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RESEARCH LETTER

Resistin expression in human granulosa cells

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Introduction

Resistin, also known as found in inflammatory zone 3 (FIZZ 3), is a cysteine-rich low molecular weight member of the FIZZ family of proteins. Resistin levels are elevated in obese mice, and those treated with the recombinant protein exhibit increased insulin resistance, whereas treatment with an antibody against resistin attenuated this condition [1]. Moreover, resistin expression is inhibited by thiazolidinediones which exert insulin-sensitizing effects via the peroxisome proliferator-activated receptor γ (PPARγ). On the basis of these findings, it was suggested that resistin may be a link between obesity and type-2 diabetes [1]. However, subsequent studies aimed at establishing a similar link between obesity, resistin levels, and type-2 diabetes in humans have been inconclusive [2]. Recent studies show that resistin is produced in the human placenta and its expression in the term placenta is significantly higher than that observed in the first trimester [3]. We have examined for the first time, the mRNA and protein expression of resistin in human granulosa cells obtained from patients undergoing fertility treatment. The mRNA expression of PPARγ, which is involved in the regulation of resistin gene transcription [4], was also examined.

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Materials and methods

Sample collection

Human granulosa cells were obtained from preovulatory follicles of women undergoing oocyte retrieval in the in vitro fertilization program at McMaster University Health Sciences, Hamilton, ON, Canada. Ovarian stimulation was accomplished using a long luteal protocol of gonadotropin-releasing hormone agonist Lupron (Abbott Laboratories, Montreal, Que.) at 0.5 mg per day for 10–14 days and recombinant follicle-stimulating hormone, Gonal F (Serono Canada, Oakville, ON), followed by human chorionic gonadotropin (hCG), Profasi (Serono Canada). Follicles were aspirated under transvaginal ultrasound guidance 36 h after hCG administration. Granulosa cells were recovered from follicular aspirates by centrifugation at $300 \times g$ for 5 min. Red blood cells were removed by hypo-osmotic lysis, as described previously [5]. Samples were frozen at −80 °C until used for mRNA or protein analysis.

RT-PCR

RNA was extracted using TRIzol as described by the supplier (Invitrogen Canada Inc., Burlington, ON, Canada). cDNA was synthesized from 2 μg of DNAse-treated RNA, using an Omniscript Reverse Transcriptase kit (Qiagen) and oligo(dT) primers. RT product (4 μl) was amplified using the HotStarTaq Master Mix Kit (Qiagen) for 38 cycles as follows: 94 °C for 30 s, 55 °C for 1 min, 72 °C for 1 min, and a final incubation at 72 °C for 10 min. PCR forward (F) and reverse (R) primers ($5' \rightarrow 3'$) were as follows: Resistin (F): ctagcaagaccctgtgctcca; (R): cactggcagtgacatg tggtc (229 bp); and PPARγ (F): tgatatcgaccagctgaatcc; (R):

atcaaggaggccagcattgtg (405 bp). Amplified cDNA bands were separated on a 2 % agarose gel, stained with ethidium bromide and digitally scanned using an AlphaImager™ 2200 program (Kodak), as reported previously [6].

Western blotting

Proteins were extracted in lysis buffer (20 mM Tris–HCl, pH 8.0,150 mM NaCl, 1 mM EDTA, 1 % Triton X-100) supplemented with a complete protease inhibitor cocktail (Roche Molecular Biochemicals, Laval, Quebec). Extracted proteins (50–100 µg) or a positive control of human recombinant resistin protein (~5 µg; Alpha Diagnostic International, San Antonio, TX) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transblotted overnight at 4 °C. Blots were blocked with 3 % non-fat dry milk in TBS-T buffer (50 mM Tris–HCl, 150 mM NaCl, 0.1 % Tween 20) for 1 h at room temperature, and then incubated with a 1:200 dilution of rabbit anti-human resistin antibody (Alpha Diagnostic International, San Antonio, TX) for 24 h at 4 °C. Membranes were washed with TBS-T and incubated with a horseradish peroxidase-conjugated anti-rabbit IgG (1:3,000; Santa Cruz Biotechnology, Santa Cruz, CA) in 3 % skim milk-TBS-T for 2 h. After washing, proteins were detected by enhanced chemiluminescent-autoradiography, as reported previously [6].

Immunocytochemistry

Human granulosa cells (~300,000) were seeded on 12 mm circular cover slips coated with 500 µl of poly L-lysine (Sigma) in 24-well culture dishes and allowed to attach overnight in 10 % FBS/DMEM. Cells were fixed with 4 % paraformaldehyde for 15 min, and then washed twice for 10 min with distilled water. To reduce background staining, 500 µl of 3 % H_2O_2 was added for 5 min, followed by three washes with PBS for 5 min. Blocking buffer (1.5 % normal goat serum/PBS) was applied for 1 h. Rabbit anti-human resistin antibody (1:200; Alpha Diagnostic International, San Antonio, TX) in blocking buffer was added and cells were incubated for 24 or 48 h at 4 °C. After three 10-min washes with PBS, biotinylated anti-rabbit IgG (1:400, Vector Laboratories, Inc., Burlingame, CA) was added to wells for 2 h at room temperature. Wells were washed with PBS three times for 10 min and the Vectastain ABC reagent (Vector Laboratories, Inc., Burlingame, CA) was added for 30 min. Following three washes with PBS for 10 min, diaminobenzidine was added, and coverslips were washed and mounted on slides with fluorescent mounting medium (DakoCytomation Inc., Mississauga, ON, Canada). A Zeiss LSM510 confocal microscope was used to capture images.

Results

As shown in Fig. 1a, resistin mRNA was detected in granulosa cells obtained from 6 patients. Initially, cells from two patients were pooled and used to establish amplification conditions. Subsequently, this pooled sample was again amplified along with four separate samples from four patients. Resistin cDNA was isolated using a Qiagen Gel Extraction kit and the sequence was confirmed at MOBIX (Institute for Molecular Biology and Biotechnology, McMaster University). A protein band of about 11 kDa, which is consistent with the resistin protein observed previously in human adipose tissue [7], was detected by immunoblotting using granulosa cell extracts obtained from other patients, and a positive control for human resistin (Fig. 1b). PPARγ mRNA exhibited consistent expression in all patient samples examined (Fig. 1c) compared with the apparent variability in the relative levels of resistin mRNA from the same samples. In keeping with the foregoing, resistin immunoreactivity was detected by immunocytochemistry in the cytoplasm of human granulosa cells, as shown in Fig. 2.

Discussion

This report indicates for the first time that the adipokine, resistin, is expressed in human ovarian granulosa cells. In addition to its well-known presence in adipose tissue, resistin has been detected in various mammalian tissues, including the mouse brain and pituitary [8] and the rat stomach, intestinal tract, adrenal gland, and skeletal muscle [9]. However, there are species differences in the tissue sources of resistin, with rodents exhibiting a predominant

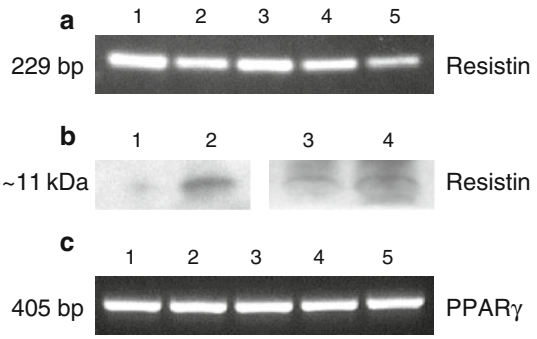

Fig. 1 Resistin mRNA and protein in human granulosa cells. **a** RT-PCR detection of resistin mRNA in pooled granulosa cell samples from two patients (*lane 1*) and in four additional separate samples (*lanes 2–5*). **b** Western blot detection of human resistin (~11 kDa) in a positive control (*lane 1*: ~ 5 µg) and in granulosa cell extracts from patients (*lanes 2–4*: 50, 75 and 100 µg protein). **c** PPARγ mRNA expression in patient samples: *lanes 1–5* as described above

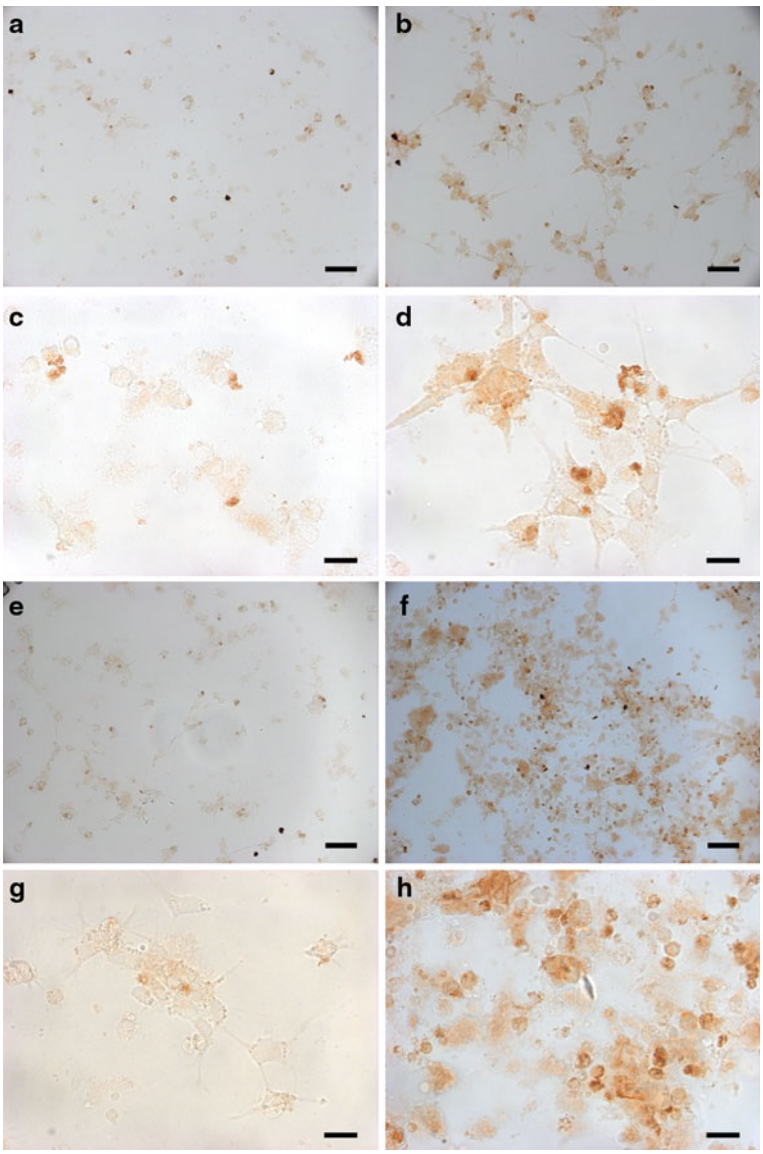

Fig. 2 Resistin immunoreactivity in human granulosa cells. Images of controls: 24-h (**a**, **c**), 48-h (**e**, **g**); and resistin: 24-h (**b**, **d**) and 48-h (**f**, **h**), are shown. *Scale bars* 50 μm (**a**, **b**, **e**, **f**) and 20 μm (**c**, **d**, **g**, **h**)

expression in adipose tissue, whereas in humans, resistin is primarily expressed in macrophages [10].

It has been suggested that resistin is the link between obesity and diabetes, since its expression is increased in mice with diet-induced or genetic obesity, while suppression of resistin levels by drug or antibody treatment ameliorates diabetic symptoms with enhanced insulin-induced glucose uptake by adipocytes [1]. Insulin resistance is a common feature of type 2 diabetes and polycystic ovarian syndrome, which predisposes to infertility in women [11].

Therefore, the expression of resistin in granulosa cells from patients undergoing ovarian stimulation raises the interesting question of its role in follicular development. Recently, resistin was shown to be produced by bovine granulosa cells and to modulate granulosa cell progesterone production in an autocrine/paracrine manner [12]. The induction of resistin by pituitary gonadotropins would suggest a significant effect of this adipokine on reproduction. Recent evidence that resistin is expressed in the human placenta, and that its levels are elevated in late pregnancy, has raised the possibility of its role in fetal development via regulation of glucose metabolism [3]. The authors suggest that the elevation in resistin levels may be involved in the decreased insulin sensitivity and associated postprandial hyperglycemia observed in the second half of pregnancy, and this may promote rapid fetal growth [3]. Therefore, it is likely that ovarian resistin is involved in follicular development and contributes to fetal survival and development.

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Ethical standards All experiments were conducted in compliance with current ethical guidelines at McMaster University, Canada.

Conflict of interest The authors declare that they have no conflict of interest.

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