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Influence of the adipose derived hormone resistin on signal transducer and activator of transcription factors, steroidogenesis and proliferation of Leydig cells

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ABSTRACT

Objective: To explore whether resistin, an adipose derived hormone linked to insulin resistance, influence steroidogenic genes expressions and Leydig cells function or not. **Methods:** Various Leydig cell lines were exposed to increasing doses of resistin with or without cAMP. Changes were monitored at the protein level for signal transducer and activator of transcription (STAT) factors and steroidogenic components, steroidogenic acute regulatory protein (STAR) and cholesterol side-chain cleavage enzyme (CYP11A1), for progesterone production and cell viability. **Results:** Resistin had no effect on progesterone production, despite an increase in nuclear translocation of STAT1, STAT3 and STAT5 and unexpected synergy with cAMP in the synthesis of STAR and CYP11A1. In addition, exposure to normal levels of resistin (10 ng/mL) seemed to have beneficial effects on Leydig cell function, as it increased cells viability and proliferation. **Conclusions:** Our results suggest that resistin may function as an endocrine mediator linking metabolism and male reproduction.

1. Introduction

The adipose tissue is an important endocrine organ that secretes several protein hormones, including leptin, adiponectin, and resistin. These hormones generally influence energy metabolism, closely associated to type 2 diabetes mellitus and obesity. Their roles in appetite, insulin resistance and atherosclerosis have been extensively studied, making a strong relation between obesity and increased morbidity. Lately, a direct relationship between obesity and lower steroids production and infertility has been made.

Resistin is primarily involved in the modulation of insulin sensitivity and adipocyte differentiation. Plasma resistin is correlated with insulin resistance in lean and obese human subjects[1]. However, although serum resistin levels were found to be elevated in rodent models for obesity such as *ob/ob* mice, *db/db* mice, and diet-induced obesity[2], others have shown a decrease in resistin production in certain obese rodent models[3,4]. Nonetheless, with its increased production related to the severity of obesity, resistin plays a major role in linking adipose tissue accumulation to type 2 diabetes[2]. Oppositely, deficiency in resistin production decreases hepatic gluconeogenesis and serum glucose levels[5]. Additional metabolic or endocrine functions for this hormone remain largely unexplored[5]. Moreover, a cellular receptor specific for resistin has not yet been identified.

Transcription factors of the signal transduction and activation of transcription (STAT) family are central mediators of adipose derived hormones such as leptin,

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adiponectin and resistin. Highly homologous STAT5A and STAT5B proteins mediate antiapoptotic effects of cytokines in cells of hematopoietic origin[6]. The activity of STAT factors is finely regulated by tyrosine kinases. Phosphorylation of a tyrosine residue at a conserved position is necessary for dimerization, nuclear translocation, followed by binding to STAT-specific regulatory elements of gene promoters[7]. In addition, serine kinases can also regulate the activity of STAT proteins. STAT transcription factors are also known to regulate steroidogenic genes such as *HSD3B2*[8] and *TSPO*[9].

The expression of the resistin gene has been demonstrated in rat testis throughout postnatal development, with maximum mRNA levels in adults[10]. At this age, resistin is detected in interstitial Leydig cells and Sertoli cells of the seminiferous tubules, and its production is regulated by pituitary gonadotropins[10]. Thus, resistin is among other adipose derived hormones that might play a role in steroidogenic genes regulation in Leydig cells.

Because testicular Leydig cells are exposed to increasing concentrations of resistin during obesity and inflammation and that these conditions are linked to reduced testosterone production and subfertility, we hypothesized that resistin might influence steroidogenic genes expression and Leydig cells function. Here, we report that resistin has no effect on progesterone production, despite an increase in nuclear translocation of STAT factors and unexpected 3′,5′-cyclic adenosine monophosphate (cAMP) dependent increase in synthesis of steroidogenic acute regulatory protein (STAR) and cholesterol side-chain cleavage enzyme (CYP11A1). In addition, exposure to normal doses of resistin could have beneficial effects on Leydig cell function, as it contributes to increase the viability and proliferation of these cells.

2. Materials and methods

2.1. Chemicals

Mouse recombinant resistin and 8-Br-cAMP were purchased from Sigma Aldrich Canada (Oakville, ON, Canada).

2.2. Cell culture

Mouse MA-10 Leydig cells[11], provided by Dr. Mario Ascoli (University of Iowa, Iowa City, IA, USA), were cultured as described by Martin *et al*[12]. The rat R2C (constitutively producing progesterone) and mouse TM3 (derived from normal testis) Leydig cell lines were obtained from American Type Culture Collection. The R2C cell line was cultured in F-12 Nutrient Mixture (Ham) supplemented with 2.5% (v/v) fetal bovine serum and 15% (v/v) horse serum. The TM3 cell line was cultured in Dulbecco modified Eagle medium (DMEM) supplemented with 10% (v/v) fetal bovine serum.

2.3. Protein purification and western blots

Mouse MA-10 Leydig cells were incubated in serum-free medium containing 0.5 mmol/L 8-Br-cAMP with or without resistin at 1 000 ng/mL for times ranging from 0 to 12 h. MA-10 cells were then rinsed twice with ice cold PBS and harvested for nuclear and cytoplasmic proteins extractions according to the procedure outlined by Schreiber *et al*[13]. Protein concentrations were estimated using standard Bradford assay. Ten microgram of nuclear proteins were boiled for 10 min in a denaturing loading buffer, fractionated by SDS-PAGE, and transferred onto polyvinylidene fluoride (PVDF) membrane (Amersham, Quebec, Canada). For cytoplasmic proteins, 50 μg was used.

Immunodetection was performed using horseradish peroxidase enzyme activity and the chemiluminescence substrate Luminata Forte (Millipore; Billerica, MA, USA). Detections of STAT1, STAT3 and STAT5 from nuclear extracts were performed using polyclonal anti-STAT1, anti-STAT3 and anti-STAT5 antibodies (detects endogenous levels of total STAT5A and STAT5B proteins) (1:1 000 dilutions; Cell Signaling; Danvers, MA, USA). Detections of STAR, CYP11A1 and alpha-tubulin from cytoplasmic extracts were performed using an anti-STAR polyclonal antiserum (FL-285, 1:5 000 dilution; Santa Cruz Biotechnologies; Santa Cruz, CA, USA), a polyclonal anti-CYP11A1 antibody (1:1 000 dilution; Proteintech Group; Chicago, IL, USA) and a monoclonal anti-alpha-tubulin antibody (1:1 000 dilution; Millipore; Billerica, MA, USA) respectively.

2.4. Progesterone assays

MA-10 and R2C Leydig cell lines were incubated for 6 or 12 h with increasing concentrations of resistin (10, 100 and 1 000 ng/mL) with or without 0.5 mmol/L 8-Br-cAMP. Progesterone present in the cell culture medium was analyzed using an enzyme immunoassay kit EA74 for progesterone (Oxford Biomedical Research; Oxford, MI, USA) according to the manufacture protocol.

2.5. Viability

MA-10 cells were incubated for up to 24 h with increasing concentrations of resistin (10, 100 and 1 000 ng/mL). Cell proliferation and viability were measured using the Cell Titer-Blue cell viability assay (Promega; Madison, WI, USA). Readings were done on a Varioskan microplate reader (Thermo Scientific; Waltham, MA, USA) for fluorescence with 560 nm excitation and 590 nm emission.

2.6. RNA isolation, reverse transcription and RT-PCR

Total RNA was isolated from MA-10 and TM3 Leydig cells using E. Z. N. A. extraction kit (Omega Bio-tek; Norcross, GA, USA). First-strand cDNAs were synthesized from a 0.5

mg aliquot of the various RNAs using the high capacity cDNA Reverse Transcription System (Applied Biosystems; Carsbad, CA, USA). MA-10 and TM3 Leydig cells were cultured in serum-free medium containing either vehicle or 0.5 mmol/L 8-Br-cAMP for the indicated times prior to RNA isolation. PCR reactions were performed using *Taq* DNA polymerase (New England Biolabs; Mississauga, ON, Canada) and oligonucleotide primers specific for leptin (*Lep*) (forward, 5′-CCC TGC TCC AGC AGC TGC AAG-3′ and reverse, 5′-GGG AAG GCA GGC TGG TGA GG-3′), adiponectin (*Adipoq*) (forward, 5′-GTG CAG GTT GGA TGG CAG GCA-3′ and reverse, 5′-CGG GTC TCC AGC CCC ACA CT-3′), resistin (*Retn*) (forward, 5′-TCT GCC ACG TAC CCA CGG GAT GA-3′ and reverse, 5′-AGC GGG CTG CTG TCC AGT CTA-3′), and ribosomal protein L19 (*Rpl*19) (forward, 5′-AGT GTC CTC CGC TGC GGG AA-3′ and reverse, 5′-AGC CTC AGC CTG GTC AGC CA-3′) as an internal control. The various PCRs were done on a Mastercycler Pro (Eppendorf, Mississauga, ON, Canada) using the following conditions: 30 s at 95 °C followed by 35 cycles of denaturation (20 s at 95 °C), annealing (20 s at 60 °C), extension (30 s at 68 °C), and a final extension of 5 min at 68 °C. After PCRs, reaction products were analyzed using 1.2% agarose gel electrophoresis and visualized using ethidium bromide staining. The specificity of PCR products was confirmed by DNA sequencing.

2.7. Statistical analyses

To identify significant differences between groups, statistical analyses were done using two-way analysis of variance to compare hormone concentrations to times of incubation. One-way analysis of variance followed by Dunnett's multiple comparison tests were used to compare different treatments to control. For all statistical analyses, $P<0.05$ was considered significant. All statistical analyses were done using the GraphPad Prism 5 software package (GraphPad Software Inc.; La Jolla, CA, USA).

3. Results

3.1. Resistin synergizes with cAMP in STAR and CYP11A1 protein expressions

Resistin is known to influence STAT3 activation and nuclear translocation in other cell types[14]. In addition, STAT3 and STAT5B are important regulators of Leydig cells function[9,15]. Thus, we thought that the nuclear translocation of STAT family members in Leydig cells could be influenced by exposure to resistin. Indeed, resistin slightly increased the accumulations of STAT1, STAT3 and STAT5 in the nuclear compartment of MA-10 Leydig cell line (Figure 1). Interestingly, cAMP, an important second messenger involved in the increase of steroidogenesis in Leydig cells, further increased STAT1 and STAT3 nuclear translocations, whereas STAT5 was downregulated by combination of cAMP and resistin.

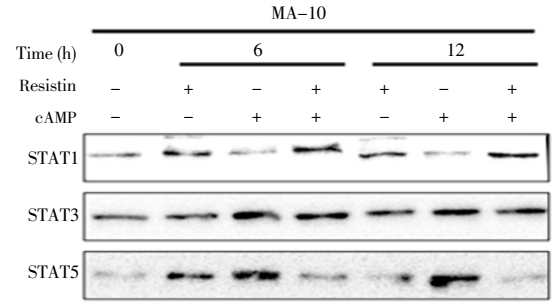

Figure 1. Resistin increases STAT members nuclear translocation.
MA-10 Leydig cells were incubated with combinations of 1 000 ng/mL resistin and 0.5 mmol/L 8-Br-cAMP for 6 and 12 h. Nuclear extracts and Western blots were done as described in Materials and methods. Experiments were repeated three times and produced identical results.

Since steroidogenesis is known to be regulated by STAT transcription factors in granulosa cells[16], we looked at the influence of resistin on the protein expression of steroidogenic genes. Surprisingly, a synergistic effect of resistin and cAMP resulted in a 2-fold increase in STAR and CYP11A1 protein expressions in MA-10 Leydig cells after 6 h of stimulation (Figure 2). In addition, this synergistic effect was maintained for the STAR protein after 12 h of incubation.

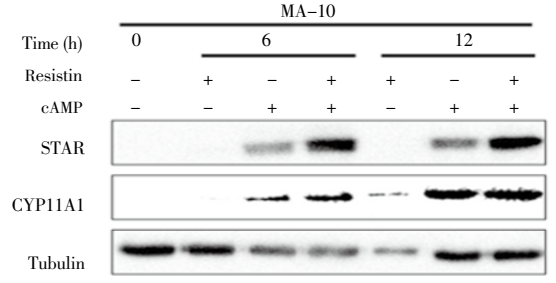

Figure 2. Resistin cooperates with cAMP to increase STAR and CYP11A1 protein expressions.
MA-10 Leydig cells were incubated with combinations of 1 000 ng/mL resistin and 0.5 mmol/L 8-Br-cAMP for 6 and 12 h. Cytoplasmic extracts and Western blots were done as described in Materials and methods. Experiments were repeated three times and produced identical results.

3.2. Leydig cells progesterone synthesis is not influenced by resistin

Given that STAR and CYP11A1 were increased in response to resistin and cAMP, we expected an increase in progesterone production in Leydig cells. However, despite an increase in the synthesis of proteins important for steroidogenesis, no effect of resistin on progesterone production was observed either in a Leydig cell line inducible by cAMP such as MA-10 (Figure 3A) or in a constitutively steroidogenic Leydig cell line such as R2C (Figure 3B). In addition, potential synergism between resistin and cAMP on progesterone synthesis could not be observed in MA-10 Leydig cells (Figure 4).

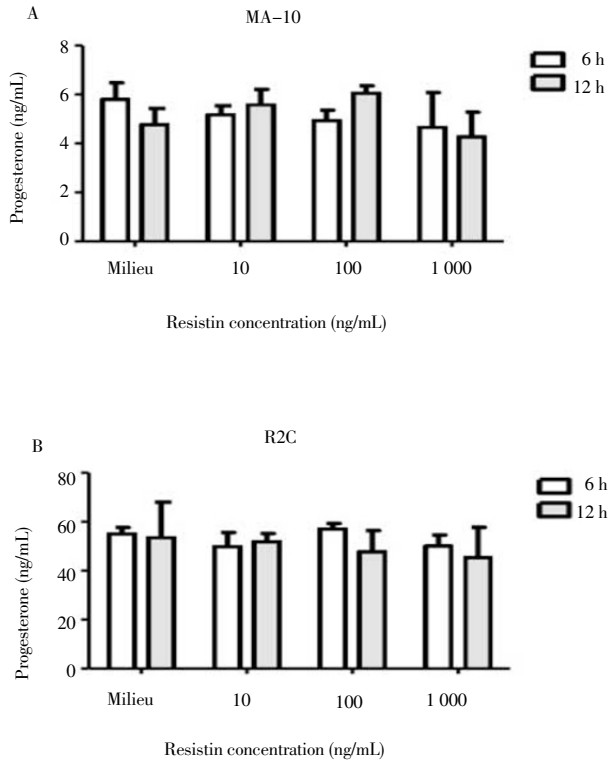

Figure 3. Regulation of progesterone production by resistin.
MA-10 (A) and R2C (B) Leydig cells were incubated with increasing concentrations of resistin (10, 100 and 1 000 ng/mL). The concentration of progesterone in the media was determined after 6 and 12 h of incubation. Data are expressed as mean±SEM and the result of four independent experiments.

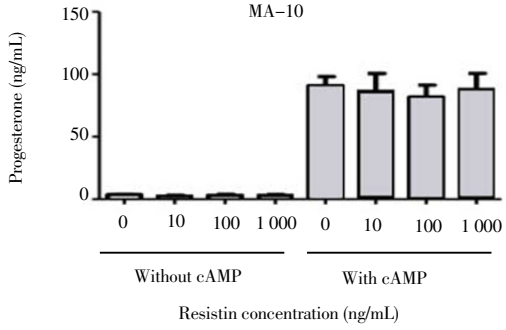

Figure 4. The increase in progesterone production in response to cAMP is not influenced by resistin.
MA-10 Leydig cells were incubated with or without 0.5 mmol/L 8-Br-cAMP and increasing concentrations of resistin (10, 100 and 1 000 ng/mL). The concentration of progesterone in the media was determined after 24 h of incubation. Data are expressed as mean±SEM and the result of nine independent experiments.

3.3. Leydig cells proliferation is stimulated by exposure to normal levels of resistin

Resistin induces proliferation in certain cell types such as prostate cancer cells, vascular endothelial and smooth muscle cells[17,18], whereas in others like granulosa cells, no effect is observed[19]. To investigate whether resistin could influence Leydig cells proliferation, MA-10 cells were incubated with increasing doses of resistin for up to 24 h. Interestingly, exposures to lower concentrations of resistin (10 ng/mL) resulted in a significant increase in Leydig cells proliferation from 6 to 24 h of incubation (Figure 5). Therefore, this effect of resistin on proliferation was dependent on a dose/exposure time relationship.

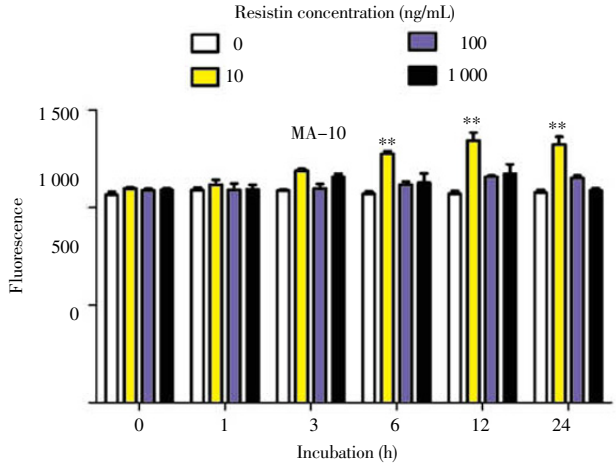

Figure 5. The proliferation of Leydig cells is increased by resistin.
MA-10 Leydig cells were incubated with increasing concentrations of resistin (10, 100 and 1 000 ng/ml). The viability/proliferation of Leydig cells was determined after 1, 3, 6, 12 and 24 h of incubation by recording fluorescence (560 nm excitation/590 nm emission). Data are expressed as mean±SEM and the result of four independent experiments. Statistically significant differences are indicated ($^{**}P<$ 0.001).

Others have shown that resistin is expressed in testicular Leydig cells[10]. To confirm these results and better define the sources of resistin that might influence Leydig cells proliferation, RT-PCRs were performed using two mouse Leydig cell lines (MA-10 and TM3). As shown in Figure 6, only resistin among adipose derived hormones investigated is expressed in both cell lines. Also, exposure to 8-Br-cAMP increased resistin mRNA expression in MA-10 Leydig cells, whereas the increase in TM3 was too weak to be conclusive.

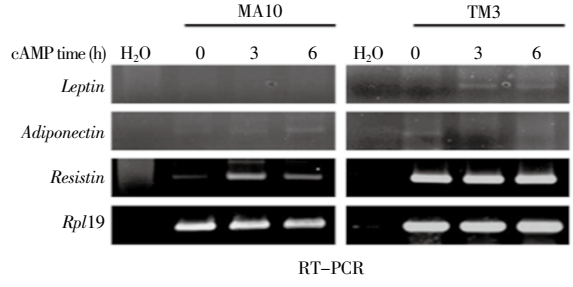

Figure 6. Resistin is expressed in Leydig cell lines.
RT-PCR analysis was used to detect *resistin* gene. Total RNA was isolated from Leydig cell lines MA-10 and TM3 incubated with 0.5 mmol/L 8-Br-cAMP for increasing times (0, 3 and 6 h). First-strand cDNAs were prepared as described in Materials and methods. Omission of cDNAs was used as negative control (H_2O). Amplification of *Rpl*19 gene was performed to validate the quantity and integrity of the cDNAs. Shown is a representative experiment of three separate amplifications using different first-strand cDNA preparations.

4. Discussion

STAT factors are involved in several regulatory pathways dependent on the actions of hormones derived from adipose tissue. We demonstrate for the first time an action of resistin on the nuclear accumulation of STAT1, STAT3 and STAT5 in testicular Leydig cells. However, the identity of genes regulated by these factors remains unknown. In human endothelial cells, STAT3 activation by resistin leads to up-regulation of the suppressor of cytokine signaling (SOCS)-3[14]. From our results, it is not clear how resistin activates STAT signaling pathways in Leydig cells and through which receptors the effects of resistin are mediated. In monocytes, resistin seems to use the toll-like receptor 4 (TLR4) for its pro-inflammatory effects[20]. Such receptor has indeed been characterized in Leydig cells and could be involved in innate immune responses[21].

Several publications have shown an influence of STAT factors on steroidogenesis. Since cAMP is an important mediator of the steroidogenesis regulation in Leydig cells, a different regulation of STAT1, STAT3 and STAT5 in response to resistin and cAMP could be relevant to redirect the gene expression profile of Leydig cells to a particular function. Here, we have demonstrated a synergy between resistin and cAMP in increased STAR and CYP11A1 at the protein level. This suggests that resistin may contribute to increased steroid production in Leydig cells under certain conditions. Consistent with this, resistin increased both basal and human chorionic gonadotropin-stimulated testosterone secretion in the rat testis[10]. However, in our case, no increase in progesterone production by Leydig cells could be observed even after a combined exposure of resistin and cAMP. These results suggest that the induction of STAR and CYP11A1 by resistin may be insufficient to observe a change in steroid production. In addition, STAR phosphorylation may not be increased in the presence of resistin to obtain maximal steroid production[22]. For CYP11A1, others have demonstrated an opposite action of resistin on mRNA expression in large follicles as well as an inhibition of insulin-like growth factor-1 induced progesterone synthesis by bovine small-follicle granulosa cells[23]. However, the physiological importance of resistin might be different according to gender and/or species. Indeed, adipose tissue *resistin* mRNA levels are higher in males rats compared to females[24] and its expression is enhanced by testosterone increases in male rats but not females[25]. Moreover, adipocytes Resistin expression is downregulated by estrogen[26]. In humans, higher resistin levels have been found in women compared to men[1].

In addition to the involvement of STAT signaling pathways in the action of resistin in Leydig cells, other signaling pathways may be involved. Indeed, the nuclear factor-kappa B pathway and components of the mitogen-activated protein kinases (MAPK) are involved in resistin mediated actions on inflammation[27,28]. Also, resistin inhibits AMP-activated protein kinase (AMPK) signaling pathway in liver and skeletal muscle[5,29]. Among these pathways, nuclear factor-kappa B and MAPK have been characterized in Leydig cells and are involved in apoptosis[30,31]. Therefore, these pathways could contribute to the actions of resistin in these cells.

Besides influencing steroidogenic genes expressions, resistin might also increase steroids production by Leydig cells through stimulation of cells viability and proliferation. Indeed, we show that normal levels of resistin have a proliferative action on Leydig cells. However, such concentration of resistin has no effect on progesterone production by Leydig cells. Therefore, an increased proliferation of Leydig cells in response to resistin does not lead to increased progesterone production. In humans, normal serum concentrations of resistin range between 2.5 and 21.5 ng/mL[1]. Besides the normal level of 10 ng/mL of resistin, we also tested supraphysiological doses of 100 and 1 000 ng/mL in our experiments based on previous studies on inflammation and smooth muscle cell proliferation[27,32]. Since Leydig cells produce resistin in small concentrations (our results and others[10]), they could regulate their own proliferation in an autocrine or paracrine manner. In addition, Sertoli cells may also contribute to Leydig cells proliferation by secreting resistin[10]. Evidences suggest that STAT1 promotes apoptosis in a variety of cell types, whereas STAT3 has an anti-apoptotic effect[33]. However, our results do not allow us to link the resistin-mediated expression of these STAT factors to the proliferative status of Leydig cells.

Taken together, these results provide insight into the action of resistin on Leydig cells function. Resistin synergizes with cAMP to regulate STAT factors nuclear translocation and to increase STAR and CYP11A1 protein expressions. Also, Leydig cells proliferation is increased under normal levels of resistin. Although others have shown that changes in resistin levels, as a result of genetic manipulations, had no effect on fertility[34,35], our results suggest that resistin may function as an endocrine mediator linking metabolism and male reproduction.

Acknowledgments

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Conflict of interest statement

We declare that we have no conflict of interest.

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