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The effects of irisin and leptin on steroidogenic enzyme gene expression in human granulosa cells: *In vitro* studies

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ABSTRACT

Reproduction and energy metabolism are closely related, and fertility can be directly affected by either obesity or malnutrition. In this study, we investigated the *in vitro* effects of irisin and leptin, two hormones primarily involved in energy metabolism, on the expression of genes encoding key steroidogenic enzymes in primary cultures of human granulosa cells. Granulosa cells were purified from follicular fluid samples obtained during *in vitro* fertilization (IVF) procedure, cultured, and treated with irisin (125-2000 ng/ml) or leptin (25–400 ng/ml) for 1–3 days. mRNA expression levels of cytochrome P450 enzymes [CYP11A1, CYP19A1, CYP21A2], hydroxy-delta-5-steroid dehydrogenase, 3 beta and steroid delta-isomerase 1 (HSD3B1), and hydroxysteroid 17-beta dehydrogenase 3 (HSD17B3) were measured using qRT-PCR analysis. Irisin significantly upregulated *CYP19A1* mRNA levels, while leptin upregulated *CYP19A1* and *HSD3B1* mRNA levels. These preliminary results show that irisin and leptin may directly affect the expression of the genes important for ovarian steroidogenesis and female reproduction.

1. Introduction

Reproductive status is closely associated with energy metabolism, but the underlying molecular mechanisms involved in this association are poorly understood. Female infertility is a growing problem since the childbearing age in women increases proportionally with educational and economic status. According to the Centers for Disease Control and Prevention (CDC), infertility affects about 1 in 5 women in the United States [1]. Irisin and leptin are two hormones whose primary function is to regulate energy metabolism. Irisin is myo- and adipokine produced by the skeletal muscles and brown adipose tissue in response to physical activity or cold exposure. It is a type I transmembrane glycoprotein, a product of fibronectin type III domain-containing protein 5 (FNDC5) precursor that regulates lipid and glucose metabolism in skeletal muscle, liver, and adipose tissue [2]. As a transcriptional activator of peroxisome proliferator-activated receptor γ co-activator-1α (PGC-1α), irisin stimulates the browning of the white adipose tissue [3]. Leptin, first described as a satiety hormone regulating energy metabolism [4], is produced predominantly in preadipocytes and adipocytes but also in the placenta, stomach, and skeletal muscle [5]. Mutations of the leptin gene in mice or humans result in morbid obesity and cause reproductive anomalies [4,6,7].

In addition to their metabolic function, irisin and leptin participate in the regulation of the reproductive system. Leptin accelerates gonadotropin-releasing hormone (GnRH) pulsatility [8] and is involved in the control of human ovarian granulosa cell hormone release [9]. Using cultured pituitary and ovarian granulosa cells, we reported that *in vitro*, irisin may interfere with the GnRH effect on luteinizing hormone (LH) in the pituitary and directly stimulates estradiol production in the ovary [10]. One recent study reported that irisin-deficient mice exhibit decreased fertility [11]. Additionally, irisin treatment improved ovarian morphology and sex steroid hormone secretion in mice [12]. In the current *in vitro* study, we aimed to investigate the effects of irisin and leptin on human ovarian granulosa cell expression of genes encoding key steroidogenic enzymes.

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2. Materials and methods

2.1. Human subjects

This study was approved by the Institutional Review Board (IRB) of The Feinstein Institutes for Medical Research, Northwell Health (IRB# 20–0449). The study subjects included 42 women undergoing *in vitro* fertilization (IVF). The study participants signed an informed consent and health insurance portability and accountability act (HIPAA) authorization form for participation after initial evaluation for eligibility. The inclusion and exclusion criteria for the study are listed in Table 1. Subjects' clinical characteristics and the reasons for undergoing IVF are listed in Table 2.

2.2. Human ovarian granulosa cells

Ultrasound examination and circulating hormone concentration testing were part of the monitoring for the IVF procedure. At the end of the stimulation period, follicular fluid was aspirated and collected from ovarian follicles, and the oocytes were retrieved by an embryologist. The remaining follicular fluid was collected, and the samples were transported to the Endocrine Research Laboratory at the Division of Endocrinology, Lenox Hill Hospital, Northwell Health.

Follicular fluid samples were centrifuged at 1000 g for 5 min. The pellet was resuspended in 20 ml of PBS, and granulosa cells were purified by gradient centrifugation (400 g for 30 min) using Percoll PLUS reagent. After being washed twice with PBS, cells were plated in cell culture dishes with DMEM/F12(50:50) medium with L-glutamine and 15 mM HEPES, supplemented with 10% FBS and antibiotic/antimycotic mixture. For experiments, cells were plated in 6-well plates (0.3×10^6 cells per well), initially in complete cell culture medium for 24 h, then in serum-free medium supplemented with irisin (125, 250, 500, 1,000, and 2000 ng/ml) or leptin (25, 50, 100, 200, and 400 ng/ml) for 24, 48, and 72 h.

2.3. RNA extraction and qRT-PCR analyses

Total RNA from cultured granulosa cells was extracted using TRIzol reagent and quantified using NanoDrop One spectrophotometer. All samples were normalized to 1 μg total RNA and converted to cDNA using qScript cDNA SuperMix. Quantitative RT-PCR analyses to measure mRNA expression of UCP1, CYP11A1, CYP19A1, CYP21A2, HSD3B1, HSD17B3, and GAPDH (house-keeping gene) were performed using PerfeCTa SYBR Green Fast Mix and QuantStudio 3 Real-Time PCR System.

Table 1
Inclusion and exclusion criteria for the study.

Inclusion criteria
• Women scheduled to undergo IVF procedure.
• Basic knowledge of the English language.
• Any race.
• 21–43 years of age.
• Signed informed consent and HIPAA authorization forms.
• Women with more than three oocytes retrieved during the IVF procedure.
• Any body mass index (BMI).
Exclusion criteria:
• Acute inflammatory disease (except endometriosis).
• Prescribed medications that may affect inflammation (Doxycycline and anti-inflammatory medications or monoclonal antibodies).
• Diagnosed malignancies.
• Cognitive impairment.
• Women who gained/lost more than 10% of body weight during the last three months before index admission.

Table 2
Subjects' clinical characteristics and reasons for undergoing IVF treatment.

	(N = 42) Mean ± SD
Age (y)	36.5 ± 3.4
Body Mass Index (kg/m^2)	25.8 ± 5.9
Hemoglobin A1c (%)	5.2 ± 0.3
Reasons for undergoing IVF:	
• Diminished ovarian reserve	
• Recurrent miscarriages	
• Endometriosis	
• Genetic carrier states	
• Polycystic ovary syndrome	
• Mullerian anomalies	
• Male factor infertility	
• Tubal factor infertility	
• Uterine factor infertility	

2.4. Statistical analyses

Data were presented as fold-change ($2^{-\Delta\Delta Ct}$) of normalized gene expression ($2^{-\Delta Ct}$) in irisin- or leptin-treated samples *vs.* vehicle-treated controls and analyzed using One-way analysis of variance (ANOVA). Tukey's *post-hoc* test was performed to evaluate further differences between the group pairs. Statistical significance was accepted when $p \leq 0.05$.

3. Results

Irisin significantly upregulated *CYP19A1* expression in all tested concentrations by up to 1.80-fold. The mRNA levels of *CYP11A1*, *CYP21A2*, *HSD3B1*, and *HSB17B3* were not significantly affected (Fig. 1a). Leptin upregulated *CYP19A1* mRNA expression at two of the tested concentrations (100 and 200 ng/ml). Additionally, when administered at concentrations between 100 and 400 ng/ml, leptin upregulated granulosa cell *HSD3B1* mRNA expression. Leptin did not significantly affect the expression of *CYP11A1*, *CYP21A2*, and *HSB17B3* mRNA (Fig. 1b).

4. Discussion

In the present study, we investigated the *in vitro* effects of irisin and leptin on the expression of genes involved in steroidogenesis in cultured human ovarian granulosa cells. We demonstrated that both irisin and leptin upregulated *CYP19A1* mRNA levels. In addition, leptin upregulated mRNA expression of *HSD3B1*.

The granulosa cells used for this study were obtained from follicular fluid samples of women undergoing IVF. These cells, therefore, were hyperstimulated with gonadotropins as part of the IVF procedure to induce ovulation. However, in our study, the purified cells were grown in culture medium for two to three weeks prior to the experiments. Therefore, the effect of gonadotropin stimulation during the IVF cycle was minimized.

Our results demonstrated that both irisin and leptin upregulated the expression of *CYP19A1*. *CYP19A1* is a member of the cytochrome P450 aromatase superfamily responsible for the catalysis of the last step of estrogen biosynthesis – the conversion of androgens to estrogens. Additionally, leptin upregulated the mRNA expression of *HSD3B1*. *HSD3B1* encodes the 3β-hydroxysteroid dehydrogenase (3β-HSDH) enzyme responsible for the conversion of pregnenolone to progesterone, 17α-pregnenolone to 17α-hydroxyprogesterone, dehydroepiandrosterone (DHEA) to 4-androstenedione, and androstenediol to testosterone [13]. Our dose-response experiments using irisin demonstrated a gradual upregulation of CYP19A1 mRNA levels at doses of up to 500 ng/ml, but higher irisin concentrations were less potent (Fig. 1a). Leptin caused a similar inverted U or U-shaped dose-response effect on

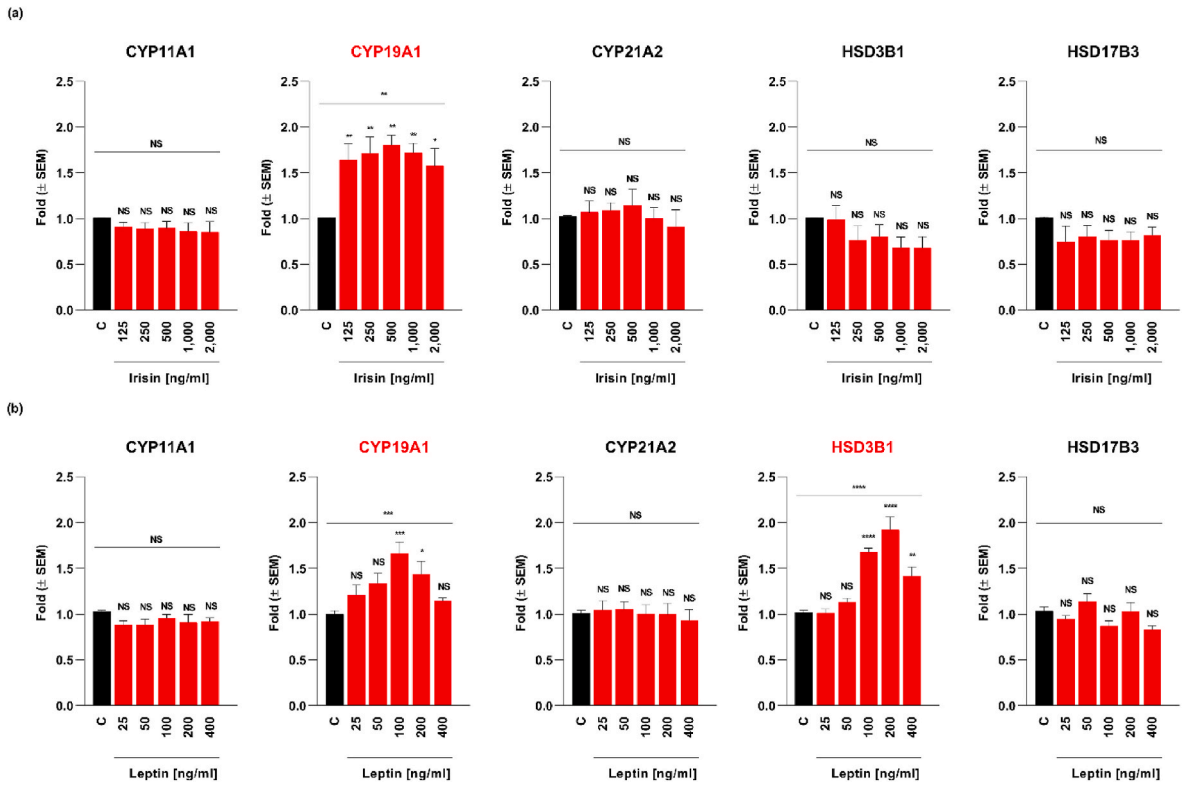

Fig. 1. ***In vitro*** **effects of irisin (a) and leptin (b) on the expression of genes involved in steroidogenesis.** Purified human granulosa cells were treated with increasing concentrations of irisin (125–2000 ng/ml) or leptin (25–400 ng/ml) for 24 h, and expression of *CYP11A1*, *CYP19A1, CYP21A2, HSD3B1,* and *HSD17B3* genes was measured by qRT-PCR analysis. Data are presented as fold-change of irisin treatment compared to vehicle-treated control cells. NS, non-significant; *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$. The names of the genes significantly modulated by the irisin or leptin treatment are marked in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

CYP19A1 and HSD3B1, with maximum upregulation at 100 ng/ml and 200 ng/ml, respectively (Fig. 1b). We hypothesize that the observed U-shaped concentration-response effects of irisin and leptin in our study may be due to cell toxicity or receptor saturation at higher treatment concentrations. Such nonmonotonic dose-responses are not uncommon in hormone/receptor interactions [14,15].

Although these results are intriguing, our study suffers from several limitations. Our data were entirely obtained during *in vitro* experiments, thus reducing the impact on translational conclusions. Literature on the levels of circulating irisin in humans remains controversial. It is commonly suggested that "normal" circulating irisin levels range between 3.6 and 4.6 ng/ml [16]. However, studies such as Hew-Butler et al. [17] or Elizondo-Montemayor et al. [16] found average levels of around 200 ng/ml. A recent review reported levels as high as 10 μg/ml [18]. These differences can be accounted for, at least in part, by the lack of reliable measurement methods. Leptin plasma concentrations directly correlate with BMI and demonstrate gender differences. In lean individuals, average serum leptin levels are 6.9 ng/ml in males and 15.2 ng/ml in females, while in obese individuals, these levels are an average of 36 ng/ml but can go as high as 100–160 ng/ml [19–22]. In our study, significant changes in steroidogenic enzyme expression levels were observed after treatment with concentrations of irisin and leptin above average circulating levels but still in the range reported for humans, as discussed above. Another limitation of our study is that the effects of irisin and leptin were examined only on gene expression level. Whether the observed mRNA modulations lead to significant changes in the circulating steroid hormone levels remains to be elucidated.

5. Conclusions

Taken together, these initial studies demonstrate that irisin and leptin may directly affect steroid hormone production in the ovary on gene expression level, thus providing a clue for possible mechanisms by which metabolic signals can modulate female fertility. Further studies are needed to elucidate the precise mechanisms of these processes and their physiological and clinical implications.

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CRediT authorship contribution statement

Leonid Poretsky: Conceptualization, Resources, Formal analysis, Investigation, Writing – review & editing. **Arielle Yeshua:** Resources. **Tal Cantor:** Resources. **Dimiter Avtanski:** Conceptualization, Investigation, Writing – original draft, Writing – review & editing. **Radoslav Stojchevski:** Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Karina Ziskovich:** Resources. **Tomer Singer:** Conceptualization, Resources.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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