Original Article

MOLECULAR METABOLISM

Female sex hormones are necessary for the metabolic effects mediated by loss of Interleukin 18 signaling

Birgitte Lindegaard [1,2,,4], Julie Abildgaard [1,4], Sarah E. Heywood [1,3], Bente K. Pedersen [1], Mark A. Febbraio [3,*]**

ABSTRACT

Objective: Interleukin (IL)-18 plays a crucial role in maintaining metabolic homeostasis and levels of this cytokine are influenced by gender, age, and sex hormones. The role of gender on IL-18 signaling, however, is unclear. We hypothesized that the presence of female sex hormone could preserve the metabolic phenotype of the *IL-18R*$^{-/-}$ animals.

Methods: We studied female mice with a global deletion of the α isoform of the IL-18 receptor (IL-18R$^{-/-}$) and littermates control. Three studies were done: 1) animals fed a high fat diet (HFD) for 16 weeks; 2) animals fed chow diet for 72 weeks and 3) animals (3 weeks-old) randomized to either bilateral ovariectomy (OVX) or control surgery (SHAM) and followed for 16 weeks.

Results: Female *IL-18R*$^{-/-}$ mice gained less weight and maintained glucose homeostasis on a chow diet compared with HFD, but no differences between genotypes were observed. The maintenance of body weight and glucose homeostasis in *IL-18R*$^{-/-}$ mice was lost with aging. By 72 weeks of age, IL-*18R*$^{-/-}$ mice became heavier compared with WT mice due to an increase in both visceral and subcutaneous adiposity and displayed glucose intolerance. OVX did not affect body weight in IL-*18R*$^{-/-}$ mice but exacerbated glucose intolerance and impaired liver insulin signaling when compared with SHAM mice.

Conclusions: Female mice harboring a global deletion of the IL-18R, only present the same phenotype as reported in male *IL-18R*$^{-/-}$ mice if they are aged or have undergone OVX, in which circulating estrogen is likely to be blunted. The role of estrogen signaling in the protection against altered metabolic homeostasis in *IL-18R*$^{-/-}$ mice appears to be mediated by liver insulin signaling. We therefore suggest that the metabolic effects mediated by loss of IL-18 signaling are only present in a female sex hormone free environment.

Keywords IL-18; Obesity; Insulin resistance; Gender

1. INTRODUCTION

Interleukin (IL)-18 is a cytokine belonging to the cytokine family of IL-1. It is known to signal through stimulation of IFN-γ [1] and is activated by pro-inflammatory stimuli such as LPS, Fas ligand, and TNF-α leading to caspase-1-mediated cleavage of pro-IL-18 into mature IL-18. IL-18 signals via a heterodimer of the transmembrane IL-18 receptors (α and β) and ultimately activates NFκB and subsequently regulates gene transcription. Besides this pathway, IL-18 is also involved in mitogen-activated protein kinase PIPs kinase and Stat 3 signaling.

Over the past decade, it has become apparent that IL-18 plays a crucial role in maintaining metabolic homeostasis. While IL-18 levels are increased in both type 2 diabetes [2] and obesity [3], work from us [4,5] and others [6,7] that employed both loss and gain of function in rodent models, demonstrated that IL-18 was both necessary and sufficient for the maintenance of metabolic homeostasis. In our recent study where we showed that mice that harbor a global deletion of the functional IL-18R are prone to gain weight, increased ectopic skeletal muscle lipid expression, inflammation and, ultimately insulin resistance, all experiments were performed in male mice [4]. It is important to note that IL-18 levels are influenced by gender [8], age [9], and sex hormones [10]. Moreover, lack of female sex hormones is associated with increased adiposity and insulin resistance in both rodents [11,12] and humans, [13,14]. The

[1]Centre of Inflammation and Metabolism and the Centre for Physical Activity Research, Rigshospitalet, University of Copenhagen, Denmark [2]Department of Pulmonary and Infectious Diseases, Nordsjællands Hospital, Hillerød, Denmark [3]Cellular and Molecular Metabolism Laboratory, Division of Diabetes & Metabolism, Garvan Institute for Medical Research, Sydney, Australia

[4] These authors contributed equally.

*Corresponding author. Division of Diabetes & Metabolism, Garvan Institute of Medical Research, 384 Victoria St, Darlinghurst, NSW 2010, Australia. E-mail: m.febbraio@garvan.org.au (M.A. Febbraio).

**Corresponding author. Rigshospitalet, Center for Inflammation and Metabolism (CIM), The Centre for Physical Activity Research (CFAS), Blegdamsvej 9, DK-2100 Copenhagen, Denmark. E-mail: birgitte.lindegaard.madsen@regionh.dk (B. Lindegaard).

Received October 18, 2017 • Revision received April 8, 2018 • Accepted April 11, 2018 • Available online xxx

https://doi.org/10.1016/j.molmet.2018.04.005

role of IL-18 signaling on metabolic homeostasis in female mice and, importantly, the role that the sex hormones may play in this experimental model is unclear. We, therefore, hypothesized that the presence of female sex hormone could preserve the metabolic phenotype of the *IL-18R*$^{-/-}$ animals. We tested the hypothesis in a diet-induced obesity model in both aged female mice and ovariectomized *IL-18R*$^{-/-}$ animals. We show that the lack of IL-18 signaling and female sex hormones combined, but not IL-18 or female sex hormone *per se*, leads to systemic insulin resistance and impaired insulin signaling in the liver.

2. MATERIALS AND METHODS

2.1. Animal experimental protocol

All experiments were approved by The Animal Experiments Inspectorate in Denmark. For the aging study, twelve-week-old *IL18R*$^{-/-}$ (mice deficient in IL-18 receptor 1, backcrossed 11 generations to C57BL/6J) and wild-type C57BL/6J mice were obtained from Charles River Laboratories (L'Arbresle, France). For the ovariohysterectomy (OVX) and diet-induced obesity (DIO) study, IL18R$^{-/-}$ and littermate control (WT) mice were obtained from heterozygous breeding. Mice were maintained on a 12-h light, 12-h dark cycle on a standard rodent chow diet (27%, 13%, and 60% kcal from protein, fat, and carbohydrate, respectively) for the aging and OVX study. For the DIO study, a high fat diet composed of 60% calories from fat (Research Diet 12492) was administered when the mice were 16 weeks old of age and continued for 16 weeks (DIO study). For the OVX study, 3 weeks old female, *IL18R*$^{-/-}$, and WT mice were randomly divided into 2 groups, sham (SHAM) operated and OVX operated, and anesthetized with a Hypnorm (10 mg/ml) (vetPharma Ltd, Leeds, UK) and Midazolam (5 mg/ml) (Hameln pharmaceuticals, Hameln, Germany) (1:1:2) anesthetic mixture with sterile water as the last component, administered subcutaneously (s.c) (0.1 ml per 10 g body weight) and were OVX or SHAM operated. In the OVX animals, the ovary was isolated and allocated to the incised muscles; the oviduct was ligated with 6-0 absorbable Vicryl ligature (Ethicon, Norderstedt, Germany), and the ovary removed. This procedure was performed on both ovaries and the muscle incisions and the skin incision were closed with 5-0 absorbable Vicryl (Ethicon) with interrupted sutures. The SHAM operated females were incised as described above, through the skin and abdominal muscles, and the incisions were closed as described above.

2.2. Body composition measurements

Body weights were monitored weekly throughout the DIO and OVX study. At the cessation of the studies, we analyzed body composition by quantitative MRI using EchoMRI (Echo Medical Systems, Houston, TX, USA). Fat and lean mass percentages were calculated in relation to total body weight, which was measured prior to the MRI scan. To confirm the MRI findings, mice were killed and the gonadal, retroperitoneal, and inguinal fat pads were dissected and weighed.

2.3. Insulin tolerance tests

In the DIO-study, we performed an insulin tolerance test after 16 weeks of HFD, in the aging study in 16 months-old female mice, and in the OVX study 16 weeks after OVX/SHAM operations, 4 h after removal of food. Blood samples were obtained by tail cut and analyzed for glucose using a glucometer (Accu-chek Compact plus) immediately before and at 15, 30, 45, 60, 90, and 120 min after an intraperitoneal injection of insulin (0.75 U/kg lean body weight Actrapid, Novo Nordisk, DK).

2.4. Glucose tolerance tests

In the DIO study and the OVX study, we performed intraperitoneal glucose tolerance tests after 16 weeks of HFD feeding and 16 weeks after OVX, respectively, and after removal of food for 14 h. Blood samples were obtained by tail cut and analyzed for glucose using a glucometer (Accu-chek Compact plus) immediately before and at 15, 30, 45, 60, 90, and 120 min after an intraperitoneal injection of glucose (2 g/kg body weight). In the OVX and aging study, we performed an oral glucose tolerance test 16 weeks after OVX and in 9 months- (36 weeks) and in 18 (72 weeks) months-old female mice, respectively, after removal of food for 14 h. Blood samples were obtained by tail cut and analyzed for glucose content using a glucometer (Accu-chek Compact plus) immediately before and at 15, 30, 45, 60, 90, and 120 min after an oral gavage of glucose (2 g/kg body weight).

2.5. Measurement of blood samples

Blood samples were obtained from the tail vein, transferred into EDTA tubes, spun at 6000 g for 10 min at 4 C, and the plasma was removed. Plasma was stored at −80 C until analysis. Plasma insulin was determined by ELISA (Crystal Chem). Blood glucose was measured by glucometer (Accu-chek Compact Plus).

2.6. Insulin signaling tissue collection

In the OVX study, insulin signaling in liver, adipose tissue, and skeletal muscle was studied. Animals were given an intraperitoneal injection of insulin (1.5 units/kg lean body mass). After 5 min, the animals were sacrificed by cervical dislocation and the tissue removed.

2.7. Protein analyses

Tissues of interest were homogenized in an ice-cold lysis buffer (50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 5 mM NaP, 2 mM NaV, 1 mM DTT, 0.2% Ipegal-CA-630, protease inhibitor (Roche Applied Science), 1% phosphatase inhibitor cocktail, pH 7.4) for 2 min using a tissue lysate with 30 oscillations/s (TissueLyser II; QIAGEN, Germany). Lysates were generated by centrifugation (16,000 *g*) for 15 min at 4 °C. Protein concentration in lysates was measured by the Bradford reagent (BioRad) [15].

Tissue lysates were subjected to SDS-PAGE using BioRad 4−15% precast gels and I-blot semi-dry transfer machine according to the manufacturer's instructions. Samples from all groups were loaded in even numbers on the same gel. PVDF membranes were probed with primary antibodies raised against the protein of interest; p-AMPK(Thr172) (#2535, Cell Signaling Technology, Danvers, MA), p-Akt(Ser473) (#9271, Cell Signaling Technology, Danvers, MA), p-GSK3 α/β (Ser21/9) (#9331 Cell Signaling Technology, Danvers, MA, USA), p-ACC(Ser79) (#3661, Cell Signaling Technology, Danvers, MA), p-JNK(Thr183/Tyr185) (#9251, Cell Signaling Technology, Danvers, MA), p-STAT3(Tyr705) (#9131, Cell Signaling Technology, Danvers, MA), total AKT (#9272, Cell Signaling Technology, Danvers, MA). Detection of primary antibodies was performed using appropriate peroxidase-conjugated IgG and protein signals visualized using FEMTO enhanced chemiluminescence and Biorad Chemidoc XRS imager. Total protein content was quantified using reactive brown 10 (Sigma−Aldrich, St-Louis, MO). Quantification of immunoblots was done using ImageJ (NIH, Bethesda, MD, http://rsb.info.nih.gov/ij).

2.8. Statistical analyses

All analyses were performed using SAS 9.2. Analyses with two variables were tested with a two way ANOVA followed by a Tukeys post hoc test. Time-series data were analyzed with PROC MIXED. Effects of insulin stimulation on genotype and OVX were analyzed using a three-way ANOVA. $p < 0.05$ was considered significant.

