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MOLECULAR METABOLISM

Brown adipose tissue (BAT) specific vaspin expression is increased after obesogenic diets and cold exposure and linked to acute changes in DNA-methylation

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ABSTRACT

Objective: Several studies have demonstrated anti-diabetic and anti-obesogenic properties of visceral adipose tissue-derived serine protease inhibitor (vaspin) and so evoked its potential use for treatment of obesity-related diseases. The aim of the study was to unravel physiological regulators of vaspin expression and secretion with a particular focus on its role in brown adipose tissue (BAT) biology.

Methods: We analyzed the effects of obesogenic diets and cold exposure on vaspin expression in liver and white and brown adipose tissue (AT) and plasma levels. Vaspin expression was analyzed in isolated white and brown adipocytes during adipogenesis and in response to adrenergic stimuli. DNA-methylation within the vaspin promoter was analyzed to investigate acute epigenetic changes after cold-exposure in BAT.

Results: Our results demonstrate a strong induction of vaspin mRNA and protein expression specifically in BAT of both cold-exposed and high-fat (HF) or high-sugar (HS) fed mice. While obesogenic diets also upregulated hepatic *vaspin* mRNA levels, cold exposure tended to increase *vaspin* gene expression of inguinal white adipose tissue (iWAT) depots. Concomitantly, vaspin plasma levels were decreased upon obesogenic or thermogenic triggers. Vaspin expression was increased during adipogenesis but unaffected by sympathetic activation in brown adipocytes. Analysis of vaspin promotor methylation in AT revealed lowest methylation levels in BAT, which were acutely reduced after cold exposure.

Conclusions: Our data demonstrate a novel BAT-specific regulation of *vaspin* gene expression upon physiological stimuli *in vivo* with acute epigenetic changes that may contribute to cold-induced expression in BAT. We conclude that these findings indicate functional relevance and potentially beneficial effects of vaspin in BAT function.

Keywords Brown adipose tissue; Browning; Cold exposure; DNA methylation; High-fat diet; High-sucrose diet; SerpinA12; Thermogenesis; UCP1; Vaspin

1. INTRODUCTION

Adipose tissue (AT) secretes a multitude of hormones and bioactive molecules, termed adipokines, thereby regulating a plethora of processes such as energy homeostasis or inflammation. In the obese state, the adipose gene expression profile entails a switch from a healthy, insulin-sensitizing, anti-inflammatory and anti-atherogenic secretion pattern of adipokines towards a pro-inflammatory, insulin-resistance promoting, atherogenic, and, finally, systemic pathological state [1]. The adipokine vaspin (visceral adipose tissue-derived serine protease inhibitor; SERPINA12) was initially found to be upregulated in visceral adipose tissue of diabetic Otsuka Long-Evans Tokushima fatty (OLETF) rats [2]. In murine models, vaspin overexpression as well as its exogenous administration exerts encouraging therapeutic features on metabolic parameters [3–5] while *vaspin* mRNA and serum levels correlate with obesity, BMI, and type II diabetes in humans [6,7]. Studies of central vaspin administration revealed a significant reduction of food intake in rats and mice [8,9], and, recently, a novel vaspin-mediated signaling axis between brain and liver, regulating hepatic glucose production and insulin signaling, was unraveled [10]. *In vitro* studies provide evidence of anti-inflammatory [11,12], anti-atherogenic [13–16], and anti-apoptotic [17–19] properties of vaspin in various endothelial cell lines (reviewed in [20]). Nonetheless, the underlying molecular mechanisms of vaspin action and signal transduction are not well understood. In mice, vaspin was reported to serve as a ligand for the 78-kDa glucose-regulated protein (GPR78)/MTJ-1 complex in the liver upon ER stress as well as a ligand for GPR78 and voltage-dependent anion channels in endothelial cells [3,19]. In own

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Received February 21, 2017 • Revision received March 10, 2017 • Accepted March 15, 2017 • Available online xxx

http://dx.doi.org/10.1016/j.molmet.2017.03.004

Original Article

studies, we were able to identify an insulin-degrading serine protease kallikrein 7 (KLK7) as the first target protease of vaspin [5] and provided evidence of vaspin binding to heparin sulfates in the extracellular matrix, potentially influencing protease interaction or intracellular signal transduction [21]. Among others, vaspin was also found to be strongly upregulated in brown adipose tissue (BAT) after cold exposure in microarray studies exploring intrinsic differences in cold-induced gene expression of brown and white adipose tissues in mice [22]. This feature appeared to be of interest since an accumulating number of studies indicate the involvement of adipokines in the activation and recruitment of BAT as well as browning of white adipose tissue (WAT) [23–26]. Based on the current knowledge, increasing BAT thermogenesis and inducing browning of WAT may prove to be a promising anti-obesity and anti-diabetes approaches (reviewed in [27–31]). Until now, vaspin was mainly specified in an anti-obesity and anti-inflammatory context while physiological regulation requires further clarification. This study investigates the effects of BAT activating physiological stimuli, such as high caloric diets or cold exposure, on vaspin gene expression and secretion in mice.

2. METHODS

2.1. Animal models

Female C57BL/6NTac mice were purchased from Taconic Bioscience (Taconic, Lille Skensved, Denmark) at an age of 10 weeks. Mice were allowed to acclimatize for 2 weeks in pathogen-free facilities at 22 °C with a 12:12 h dark–light cycle and free access to water. After randomization for body weight, mice were assigned to different experimental groups. For dietary analyses, mice were group housed at 22 °C and fed either a high fat (D12492, 60 kJ% fat, Ssniff Spezialdiäten GmbH), high sugar (D12450B, 10 kJ% fat, 63% sucrose, Ssniff) or low glycemic reference diet (D12450J, 10 kJ% fat, high amylose starch, Ssniff) for 12 weeks (n = 8 per group). See Supplementary Table 1 for detailed nutritional composition of the matched diets.

Recent studies demonstrated that conventional housing temperatures of 22 °C already display a mild cold stress to mice [32]. Therefore, the effect of ambient temperature on *vaspin* mRNA expression and secretion was examined in mice housed at thermoneutrality (30 °C) or under cold stress (8 °C). Mice were single-housed and acclimatized for one week at thermoneutrality (30 °C) and thereafter housed at 30 °C or 8 °C for one week (n = 6 per group). Mice were fed the same low glycemic reference diet used for the diet cohort. Animals from both groups were sacrificed by CO_2 and samples from intrascapular brown (iBAT), gonadal white (gWAT), and inguinal white (iWAT) adipose tissue as well as livers were collected, snap frozen in liquid nitrogen, and stored at −80 °C until further use. All animal experiments were approved by the local authorities of the State of Saxony, Germany as recommended by the responsible local animal ethics review board (Regierungspräsidium Leipzig, TVV39/14, Germany).

2.2. Quantitative real-time-PCR (qPCR)

Total RNA was isolated from snap frozen tissue samples using RNeasy lipid tissue Mini kit (Qiagen, Hildesheim, Germany) and 1 μg RNA was reverse transcribed with QuantiTect Reverse Transcription Kit (Qiagen). RNA isolation from cultured cells was done with InviTrap Spin Tissue RNA Mini Kit (Stratec Biomedical, Birkenfeld, Germany) as specified by the manufacturer. For quantification of gene expression, qPCR was performed using the LightCycler System LC480 and LightCycler-DNA Master SYBR Green I Kit (Roche, Mannheim, Germany) as previously described [33]. Gene expression was calculated by ΔΔCT method and normalized with respect to 36B4 or NoNo levels in each sample expression [34,35]. Primer sequences are listed in Supplementary Table 2.

2.3. Western blot analyses

Organs were collected and immediately snap frozen in liquid nitrogen. Frozen tissue samples were grounded in a mortar on liquid nitrogen together with protein extraction buffer (RIPA, 150 mM NaCl, 10 mM Tris pH 7.2, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, and 5 mM EDTA) completed with protease- and phosphatase inhibitors (Roche), incubated at 6 °C for 30 min, and centrifuged (16,000 rpm, 4 °C, 15 min). Total protein concentration of lysate supernatants was determined using bicinchoninic acid (BCA) assay according to manufacturer's indications (Pierce, Thermo Fisher, Waltham, MA, USA). For immunoblotting, 20 μg of total protein were subjected to SDS polyacrylamide gel electrophoresis and subsequently transferred to a nitrocellulose membrane using tank blot method overnight. Membranes were blocked with 3% BSA for 1 h at room temperature followed by primary antibody incubation at 4 °C overnight. Specific HRP-coupled secondary antibodies were used and chemoluminescence signals were detected using a gel documentation system. Primary antibodies were the following: Vaspin (USC-PAA-706Mu01, Cloud Clone Corp., Houston, TX, USA), UCP1 (ab23841, Abcam, Cambridge, UK), β-actin (A2066, Sigma, Darmstadt, Germany), and anti-rabbit-HRP (CST#7074, Cell Signaling, Danvers, MA, USA).

2.4. ELISA analyses

For quantification of vaspin plasma levels, blood samples were taken from fasted mice by cardiac puncture at the end of experiment. To obtain clear lysates, blood-EDTA samples were centrifuged at 4 °C and 11,000 rpm for 10 min. ELISA analyses was performed according to manufactures instructions (NB-E20150; Novateinbioscience, Cambridge, MA, USA).

2.5. Cell culture experiments

Immortalized white (3T3-L1) and brown pre-adipocyte (BAT) cell lines [36] were used to analyze vaspin expression *in vitro*. Cells were cultured in Dulbecco's Modified Eagle (DMEM, Gibco) supplemented with 10% or 20% fetal bovine serum (FBS), respectively. At two days post confluence (day 2), adipogenesis was induced by addition of 0.5 mM IBMX, 1 μM insulin and 0.4 μg/ml dexamethasone to the culture medium. After 48 h, medium was changed to insulin containing DMEM for another two days and subsequently to DMEM-FBS until day 10. To determine vaspin gene expression in the course of adipogenesis, cells were either harvested when confluent (day 0) or at the end of differentiation (day 10) and subjected to RNA isolation and qPCR analyses. To evaluate the influence of the PPARγ activator rosiglitazone on vaspin expression, cells were additionally treated with 1 μM rosiglitazone starting at day 0.

2.5.1. Stimulation assay

Fully differentiated brown adipocytes were serum-starved overnight (16 h) and stimulated with Saline, 1 μM norepinephrine (NE) or 1 μM CL316,243 for 2 h and subjected to RNA isolation and qPCR analysis.

2.5.2. DNA demethylation in BAT cells

To analyze the influence of DNA methylation status on vaspin expression in BAT cells, pre-adipocytes were treated with indicated concentrations of 5′aza-2′-deoxycytidine for 48 h (medium was changed every other day) and subsequently differentiated. At day 10, cells were harvested and subjected to further analyses.

