Brief communication

MOLECULAR METABOLISM

Notch intracellular domain overexpression in adipocytes confers lipodystrophy in mice

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ABSTRACT

Objective: The Notch family of intermembrane receptors is highly conserved across species and is involved in cell fate and lineage control. Previous *in vitro* studies have shown that Notch may inhibit adipogenesis. Here we describe the role of Notch in adipose tissue by employing an *in vivo* murine model which overexpresses Notch in adipose tissue.

Methods: Albino C57BL/6J $Rosa^{NICD/NICD}$::Adipoq-Cre (Ad-NICD) male mice were generated to overexpress the Notch intracellular domain (NICD) specifically in adipocytes. Male $Rosa^{NICD/NICD}$ mice were used as controls. Mice were evaluated metabolically at the ages of 1 and 3 months by assessing body weights, serum metabolites, body composition (EchoMRI), glucose tolerance and insulin tolerance. Histological sections of adipose tissue depots as well as of liver were examined. The mRNA expression profile of genes involved in adipogenesis was analyzed by quantitative real-time PCR.

Results: The Ad-NICD mice were heavier with significantly lower body fat mass compared to the controls. Small amounts of white adipose tissue could be seen in the 1-month old Ad-NICD mice, but was almost absent in the 3-months old mice. The Ad-NICD mice also had higher serum levels of glucose, insulin, triglyceride and non-esterified fatty acids. These differences were more prominent in the older (3-months) than in the younger (1-month) mice. The Ad-NICD mice also showed severe insulin resistance along with a steatotic liver. Gene expression analysis in the adipose tissue depots showed a significant repression of lipogenic (Fasn, Acacb) and adipogenic pathways (C/ebpα, C/ebpβ, Pparγ2, Srebf1).

Conclusions: Increased Notch signaling in adipocytes in mice results in blocked expansion of white adipose tissue which leads to ectopic accumulation of lipids and insulin resistance, thus to a lipodystrophic phenotype. These results suggest that further investigation of the role of Notch signaling in adipocytes could lead to the manipulation of this pathway for therapeutic interventions in metabolic disease.

Keywords Notch; Adipocyte; Lipodystrophy; Insulin resistance; Lipogenesis; Adipogenesis

1. INTRODUCTION

Adipose tissue is an organ that stores excess energy in the form of fat and has endocrine properties manifested by the production and secretion of hormones such as leptin and adiponectin [1]. Adipose tissue has been studied mainly in the context of obesity and type 2 diabetes where white adipose tissue (WAT) expands so as to accommodate the surplus of energy intake. Accompanying this expansion, WAT inflammation and the secretion of inflammatory cytokines have been associated with the development and exacerbation of insulin resistance [2]. Limiting the expansion of WAT is of major importance since surplus lipids cannot be stored in the form of triglycerides; excess lipids then accumulate in ectopic sites such as liver and muscle, worsening insulin resistance [3].

The importance of the capacity and expandability of WAT becomes much more evident in the case of lipodystrophy. Lipodystrophy is a disorder characterized by selective loss of body fat and is accompanied by severe insulin resistance. A series of genetic lipodystrophies has been reported in humans and reflect mutations in genes such as *AGPAT2*, *BSCL2*, *CAV1*, *PTRF*, *CIDEC*, *PPARG* [4]. These genes are implicated in triglyceride synthesis, fusion of lipid droplets and adipogenesis. Although lipodystrophies are relatively rare diseases, there is an increased interest in the development of mouse models so as to better understand the pathophysiological mechanisms of lipodystrophy and the role of specific genes in adipose tissue functionality. Current mouse models are genetic knock-outs of *C/ebpa*, *Pparg*, *Agpat2*, *Cav1* or transgenic mice such as AZIP/F1 and ap2-*Srebp-1c* [5]. The usefulness of these mouse models lies not only in the fact that they can be used to understand the functions of specific genes but also can expand our understanding of the pathophysiology of insulin resistance [6].

In the present report, we describe a novel mouse model of lipodystrophy that arises from adipose tissue specific overexpression of the Notch intracellular domain (NICD). The Notch signaling pathway is highly conserved across species, playing roles in cellular differentiation, proliferation and apoptosis [7]. It consists of intermembrane receptors that can be bound by Notch ligands expressed on the surface of adjacent effector cells. This binding initiates the proteolytic cleavage of Notch by which NICD is released from the membrane, enters the

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nucleus to drive downstream signaling. Notch targets a number of genes, such as hairy and enhancer of split-1 (*Hes-1*), a prototypical target gene that encodes a transcriptional repressor [8].

Research on the role of the Notch pathway in the field of metabolism is relatively limited, focusing principally on the role of Notch in adipocyte differentiation using *in vitro* adipogenesis models such as 3T3-L1 cells [9,10]. Given that these studies were performed *in vitro* and taking into account recent studies on the protective effect of Notch inhibition in hepatocytes against fatty liver and insulin resistance [11,12], we investigated the role of Notch in the adipocyte by using a $Rosa^{NICD/NICD}$::Adipoq-Cre (Ad-NICD) mouse that overexpresses NICD specifically in adipose tissue as driven by the adiponectin promoter. We report herein that adipose-tissue specific overexpression of NICD results in a profound lipodystrophic, insulin resistant phenotype.

2. MATERIALS AND METHODS

2.1. Mice

All mouse experiments were performed at the University of Pittsburgh and were approved by the Institutional Animal Care and Use Committee. Gt(Rosa)26Sortm1(Notch1)Dam/J ($Rosa^{NICD/NICD}$) [13] and B6; FVB-Tg(Adipoq-cre)1Evdr/J [14] mice were obtained from Jackson Laboratories (Bar Harbor, ME). $Rosa^{NICD/NICD}$ mice were crossed with Adipoq-Cre mice and the male offspring $Rosa^{NICD/-}$::AdipoqCre were crossed with female $Rosa^{NICD/NICD}$ to get the desired phenotype of $Rosa^{NICD/NICD}$::AdipoqCre (Ad-NICD mice). Male Ad-NICD mice were mated with female $Rosa^{NICD/NICD}$ to generate the male mice used in the experiments.

$Rosa^{NICD/NICD}$, used as controls, and $Rosa^{NICD/NICD}$::AdipoqCre (Ad-NICD) were both in the albino C57BL/6J background. An experimental cohort of male mice was sacrificed at 1 month of age and another experimental cohort was sacrificed at 3 months of age. The 1-month old cohort included 8 control and 9 Ad-NICD male mice. The 3-month old cohort included 7 control and 6 Ad-NICD male mice. Another cohort (5 control and 8 Ad-NICD mice) was used to evaluate the growth curves of the mice starting from 22 days old until the age of 90 days. The genotyping primers as well as a more detailed description of the $Rosa^{NICD/NICD}$::AdipoqCre model can be found in the supplementary material. The mice were housed at 22 °C, 50% humidity with a 12 h light/dark cycle with *ad libitum* access to water and food (Prolab Isopro RMH 3000 5P76 irradiated diet, LabDiet, St Louis, MO).

2.2. Body fat composition and metabolic tests in mice

Body fat composition was assessed using NMR-MRI-based technology (EchoMRI, Houston, TX). Blood glucose was measured using a Precision Xtra glucose meter (Abbott, Chicago, IL) in blood collected from the tails of mice. Glucose tolerance was assessed by injecting intraperitoneally a single dose of D-glucose (1 g/kg) after a 16 h overnight fasting with free access to water. Insulin tolerance was assessed by injecting mice intraperitoneally with 0.75 units/kg insulin (Humalog, Eli Lilly, Indianapolis, IN) after a 4 h fasting with free access to water.

2.3. Serum metabolites

Blood was drawn under isoflurane anesthesia with cardiac puncture and was allowed to clot at room temperature for 30 min. Serum was collected after centrifuging the blood at 2000 × *g* for 30 min at 4 °C. Triglycerides and cholesterol concentrations were measured using a colorimetric and a fluorometric assay kit, respectively, from Cayman Chemical (Ann Arbor, MI). Liver triglycerides were also quantified using the same kit. Concentrations of non-esterified fatty acids were measured photometrically using the NEFA-HR kit (Wako Chemicals, Japan). Leptin and insulin were measured using ELISA kits from R&D systems (Minneapolis, MN) and Mercodia (Sweden), respectively.

2.4. Histology

Tissue was immersed in 10% formalin or in O.C.T. (Sakura, Torrance, CA) for the frozen sections. Hematoxylin and Eosin (H&E) stained sections were prepared by the Department of Pathology, University of Pittsburgh. Oil-red-O (ORO) staining of frozen sections was performed by fixing them in 10% formalin, then staining with ORO and after thorough washing, the slides were counterstained with hematoxylin. Photos were taken in a Leica DM5000B microscope.

2.5. RNA preparation and quantitative real-time PCR

Total RNA was prepared using Trizol (Life Technologies) and then a RNeasy kit followed by DNAse digestion (Qiagen, Valencia, CA). RNA quality and quantity was evaluated by electrophoresis and spectrophotometry at 260/280 nm prior to reverse transcriptase reaction. cDNA was synthesized with the qScript system (Quanta Biosciences, Gaithersburg, MD). Real-time PCR was performed on an iCycler-MyIQ (Biorad, Hercules, CA) with iQ SYBR green supermix (Biorad) in tetraplicate 20 µl reactions. The PCR efficiency was determined from standard curves and the Pfaffl method [15] was used for calculations of fold changes. The evaluation of the stability of the reference genes was performed using the genorm algorithm [16]. The geometric means of the two most stable reference genes were used to normalize the gene expression data. The primer pairs used for the real-time PCR as well as the results of the genorm algorithm for each reference gene are included in the supplemental data.

2.6. Statistical analysis

The Student's t-test was used to compare the control versus the Ad-NICD mice using Prism 5 for Windows (GraphPad Software, La Jolla, CA).

3. RESULTS

3.1. Ad-NICD mice are heavier with low body fat content

Comparisons of the body weights of male Ad-NICD mice with their $Rosa^{NICD/NICD}$ (control) counterparts indicate that they weigh significantly more at weaning (22 days of age) and that this difference in weight is accentuated over time (Figure 1A). The absolute amount of food consumption was higher in the Ad-NICD mice (Figure 1B), but when normalized to body weight, no significant difference was observed (Figure 1C). However, the absolute amounts of body fat were significantly ($p < 0.05$) lower in the Ad-NICD mice at 1-month of age (1.30 g vs 2.22 g in controls) (Figure 1D) and in 3-month old mice (2.97 g vs 5.87 g in controls) (Figure 1F), as measured by EchoMRI. The fat mass to body weight ratio was significantly lower in the Ad-NICD mice both at 1 and 3 months of age (Figure 1E,G). On the other hand, the Ad-NICD mice had increased absolute lean body mass at 1 and 3 months of age (Figure 1H,J) while the lean mass as % of body weight showed little difference (Figure 1I,K).

As is explicitly shown in the necropsy images of epididymal white adipose tissue (eWAT) in Figure 1L, the 1-month old Ad-NICD mice had minimal amounts of eWAT (0.09% BW vs 0.29% BW). H&E staining of the eWAT (Figure 1M) showed a greater amount of smaller adipocytes per optical field in the Ad-NICD mice (Supplementary Fig. S1B). This difference was not so pronounced in the inguinal white adipose tissue (iWAT). On the other hand, the interscapular brown adipose tissue (iBAT) was heavier (0.47% BW vs 0.33% in controls) and paler in color (Figure 1N). As evidenced by the H&E section of iBAT, this difference

