Original article

MOLECULAR METABOLISM

Adipocyte-specific blockade of gamma-secretase, but not inhibition of Notch activity, reduces adipose insulin sensitivity

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ABSTRACT

Objective: As the obesity pandemic continues to expand, novel molecular targets to reduce obesity-related insulin resistance and Type 2 Diabetes (T2D) continue to be needed. We have recently shown that obesity is associated with reactivated liver Notch signaling, which, in turn, increases hepatic insulin resistance, opening up therapeutic avenues for Notch inhibitors to be repurposed for T2D. Herein, we tested the systemic effects of γ-secretase inhibitors (GSIs), which prevent endogenous Notch activation, and confirmed these effects through creation and characterization of two different adipocyte-specific Notch loss-of-function mouse models through genetic ablation of the Notch transcriptional effector Rbp-Jk (*A-Rbpj*) and the obligate γ-secretase component Nicastrin (*A-Nicastrin*).

Methods: Glucose homeostasis and both local adipose and systemic insulin sensitivity were examined in GSI-treated, *A-Rbpj* and *A-Nicastrin* mice, as well as vehicle-treated or control littermates, with complementary *in vitro* studies in primary hepatocytes and 3T3-L1 adipocytes.

Results: GSI-treatment increases hepatic insulin sensitivity in obese mice but leads to reciprocal lowering of adipose glucose disposal. While *A-Rbpj* mice show normal body weight, adipose development and mass and unchanged adipose insulin sensitivity as control littermates, *A-Nicastrin* mice are relatively insulin-resistant, mirroring the GSI effect on adipose insulin action.

Conclusions: Notch signaling is dispensable for normal adipocyte function, but adipocyte-specific γ-secretase blockade reduces adipose insulin sensitivity, suggesting that specific Notch inhibitors would be preferable to GSIs for application in T2D.

Keywords Notch; γ-secretase complex; Insulin resistance

1. INTRODUCTION

Continued Westernization of diet and lifestyle in the setting of conducive genetics predispose to obesity, defined as excessive adipose mass [1]. Increased adiposity can then lead to insulin resistance, which predicts Type 2 Diabetes (T2D) [2]. Better understanding of the hormonal and mechanical signals underlying adipocyte-systemic crosstalk to induce insulin resistance is necessary to develop novel therapeutic targets to interrupt this burgeoning crisis.

The Notch cascade is a paracrine signaling pathway that has a well-established role in regulating normal differentiation by a complex process known as lateral inhibition [3]. Notch signaling is regulated post-translationally by ligand availability and multiple processing steps [4]. Notch receptors (Notch1-4) are activated by a transmembrane ligand of either the Jagged (Jagged1/2) or Delta-like (Dll-1/3/4) family on a neighboring cell, leading to a sequential cleavage by ADAM/TACE and the γ-secretase complex, releasing the soluble Notch intracellular domain (NICD). NICD translocates to the nucleus and activates Rbp-Jκ-dependent transcription of Notch targets, classically the *Hes* (Hairy and enhancer of split) and *Hey* (Hairy/enhancer-of-split related with YRPW motif) family of basic helix-loop-helix transcription factors, which regulate cell proliferation and embryogenesis and are indispensable for normal development [5]. More recently, Notch gain-of-function mutations have been associated with T-cell leukemia [6] and multiple solid tumors [7], leading to widespread development of Notch inhibitors as chemotherapeutic agents [8]. Of these, the most advanced are inhibitors of the γ-secretase (GSIs), a multi-protein complex consisting of catalytic (Presenilin 1 or 2), regulatory (PEN2 and Aph1a or 1b) and targeting (Nicastrin) subunits [9]. Although GSIs target numerous other Type-I transmembrane targets [10], including amyloid

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Abbreviations: 2DOG, 2-Deoxy-glucose; DBZ, Dibenzazepine; GSI, γ-secretase inhibitor; NICD, Notch intracellular domain; Rbp-Jκ, Recombination signal binding protein-Jκ; T2D, Type 2 diabetes

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precursor protein (APP) [11], knockout of multiple γ-secretase subunits phenocopy the embryonic lethality of Rbp-Jκ deletion [5,12,13], underscoring the necessity of γ-secretase function for Notch activity. We have recently shown that Notch plays a post-development role to regulate liver glucose and lipid metabolism [14,15]. Liver-specific Rbp-Jκ deletion results in increased hepatic insulin sensitivity and improved glucose tolerance; consistently, GSI-treated obese mice show marked improvements in glucose tolerance [14]. These data have since been confirmed using other GSIs and more specific Notch antagonists [15–17], leading to the hypothesis that Notch signaling may be "re-activated", and thus potentially targetable, in other tissues in the obese state. To address this question, we studied potential extra-hepatic effects of GSIs and found that while GSIs increase hepatic insulin sensitivity, they simultaneously reduce glucose uptake in white adipose tissue. To determine whether GSI-induced adipose insulin resistance was Notch-dependent, we created adipocyte-specific Rbp-Jk (henceforth, *A-Rbpj* mice) and γ-secretase (henceforth, *A-Nicastrin* mice) knockout mice, using the well-characterized Adiponectin-Cre transgenic mouse [18]. Although *A-Rbpj* and *A-Nicastrin* mice both develop normally, with unchanged body weight/adiposity as compared to Cre-littermates, *A-Rbpj* mice showed normal glucose homeostasis whereas *A-Nicastrin* mice showed a comparable reduction in adipocyte insulin sensitivity as GSI-treated mice. These data suggest that Notch activity is not required for normal adipocyte function but that γ-secretase activity regulates adipose insulin sensitivity, likely through a Notch-independent mechanism.

2. MATERIALS AND METHODS

2.1. Experimental animals

Male 8 week old *C57/BL6* mice were purchased from Jackson Laboratories. We intercrossed Adiponectin-cre [18] with *Nicastrin*$^{flox/flox}$ [19] and *Rbpj*$^{flox/flox}$ [15] mice to generate Adiponectin(cre):*Nicastrin*$^{flox/flox}$ (*A-Nicastrin)* and Adiponectin(cre):*Rbpj*$^{flox/flox}$ (*A-Rbpj*) mice. All studies were performed using male mice. Mice were weaned to either standard chow (Purina Mills 5053) or high-fat diet (HFD) (18.4% calories/carbohydrates, 21.3% calories/protein and 60.3% calories/fat derived from lard; Harlan Laboratories, TD.06414). All animal procedures were approved by the Columbia University Institutional Animal Care and Utilization Committee.

2.2. Assays

Measurement of blood glucose (One Touch), plasma insulin (Millipore), and lipids were performed as previously described [20]. Intraperitoneal glucose tolerance tests (IP-GTT) were performed after a 16 h fast with 2 g/kg glucose. Body composition was measured by NMR (Bruker Optics).

2.3. Gamma-secretase inhibitor (GSI)

GSI was used as previously described [14]. In short, (S)-2-[2-(3,5-Difluoro-phenyl)-acetylamino]-N-((S)-5-methyl-6-oxo-6,7-dihydro-5H-dibenzo[b,d]azepin-7-yl)-propionamide, also known by the trade name dibenzazepine (DBZ), was suspended in vehicle [0.5% Methocel E4M (wt/vol, Colorcon) and 0.1% (vol/vol) Tween-80 (Sigma)] [21]. Immediately prior to injection, DBZ was sonicated for 2 min to suspension.

2.4. Glucose turnover studies

To measure glucose turnover and uptake in the fasting state, we omitted the insulin infusion during our standard glucose-insulin clamp protocol, as described previously [22]. In brief, awake mice with an indwelling catheter implanted in the right jugular vein one week before the experiment, were fasted overnight, and 3-[^{3}H]glucose (Hartmann Analytical, Germany) was then infused at 0.05 ìCi/min for 120 min to determine basal glucose turnover. 10 ìCi of 2-deoxy-d-[1-^{14}C]glucose (2DOG, Hartmann Analytical, Germany) was infused within 3 min to measure organ specific glucose uptake. Blood samples were drawn by tail vein at baseline and at 120 min after the initiation of the 2DOG infusion. At study completion, mice were anesthetized and tissues were harvested, snap frozen in liquid N_2 within 3 min of collection using liquid N_2-cooled tongs, and stored at −80 °C for subsequent analysis. Intracellular (6-phosphorylated) 2DOG uptake of epididymal white adipose tissue under basal conditions was measured as described [22].

2.5. Quantitative reverse-transcription PCR

RNA was isolated from adipose and liver with RNeasy Lipid and RNeasy mini-kits (Qiagen), respectively. cDNA was synthesized with qScript cDNA SuperMix (Quanta Biosciences), and quantitative PCR performed with a CFX96 Real-Time PCR detection system (Bio-Rad) and GoTaq SYBR Green qPCR kit (Promega) using the ΔΔC(t) method, with TATA-binding protein (TBP) and/or 18S as controls to determine relative gene expression.

2.6. Western blotting

3T3-L1 cells (ATCC) were differentiated per standard protocol. Day 8–10 adipocytes were incubated with 200 nM Compound E overnight, serum starved for 4 h, then treated with 100 nM bovine insulin (Sigma) for 15 min prior to lysis. Both 3T3-L1 lysates and whole adipose extracts were lysed in Adipose Lysis Buffer (20 mM Tris, pH 7.4 150 mM NaCl, 10% glycerol, 2% Nonidet P-40, 1 mM EDTA, pH 8.0, 0.1% SDS, 0.5% sodium deoxycholate, 20 mM NaF, 30 mM NaPPi, 1 mM NaVO4), supplemented with Complete Protease Inhibitor Cocktail Tablet, EDTA-free (Roche). Immunoblots were probed with antibodies against Nicastrin (#5665), Psen2 (#2192), phospho-Akt Thr308 (#9275), total Akt (#9272), phospho-GSK-3β Ser9 (#9322), total GSK-3β (#9315), tubulin (#2148), and actin (#8456) from Cell Signaling.

2.7. Statistical analysis

All results are reported as ± SEM unless otherwise indicated. Gene expression levels were compared using Students t-test. IP-GTT area under the curve was calculated using the trapezoidal rule. P values of <0.05 were considered significant.

3. RESULTS

3.1. GSIs increase hepatic insulin sensitivity

We have previously shown that dibenzazepine (DBZ), a well-characterized, bioavailable Notch inhibitor of the GSI class [21,23], improves glucose tolerance in diet-induced or leptin-deficient (*ob/ob*) obese mice [14] but results in dose-limiting intestinal metaplasia [23]. To determine if a therapeutic window exists for safe application of this class of drugs for metabolic disease, we performed a dose-finding study. Interestingly, "low-dose" (2 mcg per kg body weight) DBZ treatment showed comparable potency to improve glucose tolerance as the previously used "high-dose" (10 mcg per kg body weight) (Figure 1A) without apparent intestinal toxicity (Supplemental Figure 1A, B). Neither dose altered food intake, adipose, or body weight (not shown, Supplemental Figure 1C, D). These data suggest differential susceptibility across tissues to Notch inhibition, and we used low-dose DBZ (henceforth, referred to as GSI) in the remainder of our experiments to minimize potential confounding effects. Based on the improved glucose tolerance phenotype of *L-Rbpj* mice, which lack

