Brief Communication

MOLECULAR METABOLISM

Inducible overexpression of adiponectin receptors highlight the roles of adiponectin-induced ceramidase signaling in lipid and glucose homeostasis

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ABSTRACT

Adiponectin and the signaling induced by its cognate receptors, AdipoR1 and AdipoR2, have garnered attention for their ability to promote insulin sensitivity and oppose steatosis. Activation of these receptors promotes the deacylation of ceramide, a lipid metabolite that appears to play a causal role in impairing insulin signaling. Here, we have developed transgenic mice which overexpress AdipoR1 or AdipoR2 under the inducible control of a tetracycline response element. These represent the first inducible genetic models that acutely manipulate adiponectin receptor signaling in adult mouse tissues, allowing us to directly assess the impact of AdipoR signaling on glucose and lipid metabolism. Overexpression of either adiponectin receptor isoform in the adipocyte or hepatocyte is sufficient to enhance ceramidase activity, whole body glucose metabolism, and hepatic insulin sensitivity, while opposing hepatic steatosis. Importantly, metabolic improvements fail to occur in an adiponectin knockout background. When challenged with a leptin-deficient genetic model of type 2 diabetes, AdipoR2 expression in adipose or liver is sufficient to reverse hyperglycemia and glucose intolerance. These observations reveal that adiponectin is critical for AdipoR-induced ceramidase activation which enhances hepatic glucose and lipid metabolism via rapidly acting "cross-talk" between liver and adipose tissue sphingolipids.

1. INTRODUCTION

The discovery of adipocyte-specific secreted molecules, termed adipokines, has dispelled the notion of adipose tissue as an inert storage depot for lipids, and highlighted its role as an active endocrine organ that monitors and alters whole-body metabolism and maintains energy homeostasis. Since its identification in the mid-90's [20], the adipokine adiponectin (also known as Acrp30, AdipoQ and GBP28) has become best known as a regulator of insulin sensitivity. The two adiponectin receptor isoforms, AdipoR1 and AdipoR2, previously have been shown to be associated with increases in activities of AMP-activated protein kinase (AMPK) and peroxisome-proliferator activated receptor (PPARα). Recently, our group has shown that adiponectin is capable of inducing ceramidase activity through its receptors, which results in the hydrolysis of ceramide to form sphingosine and free fatty acid. Sphingosine, produced in this reaction, can go on to be phosphorylated by sphingosine kinase to produce sphingosine 1-phosphate (S1P), which functions as an important signaling molecule in a number of different cellular processes. The ceramide lowering effect of the adiponectin receptors only marginally depends on the activity of AMP kinase (AMPK), but AMPK can be activated through increasing the local S1P pool. Thus, this process appears to be a proximal event in adiponectin signaling, since it is a necessary step in adiponectin's induction of AMPK activation. These data support a previous model based on data from a heterologous system that connected the family of Progesterone and AdipoQ receptors (PAQRs), which includes AdipoR1 and AdipoR2, with ceramidase activity [10,28] and suggested a revised view of adiponectin signaling with sphingolipid metabolism at its core. Sphingolipids, such as ceramides and glucosylceramides, are an important class of bioactive lipids. The levels of these lipids change as a function of adipose tissue mass and functionality, and are partially driven by cellular availability of palmitoyl-CoA. Aberrant accumulation of ceramide, glucosylceramide, and GM3 ganglioside has been implicated in a multitude of metabolic processes, including atherosclerosis, insulin resistance, lipotoxic heart failure, β-cell apoptosis, and β-cell dysfunction (reviewed in [7]). In stark opposition, the

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Brief Communication

phosphorylated sphingoid base Sphingosine 1-phosphate (S1P) is a potent inducer of proliferation and inhibitor of apoptosis [24]. The conversion of ceramide to S1P consists of deacylation of ceramide by ceramidase enzymes and a subsequent phosphorylation of sphingosine by one of two sphingosine kinase isoforms [21]. The opposing nature and simple 2-step conversion-process separating these lipids has led to speculation that the dynamic ratio of ceramide:S1P may constitute a physiological rheostat regulating in numerous cellular processes [24].

To gain further insights into the local physiological consequences of adiponectin and AdipoR-induced ceramidase activation we have embarked on two parallel approaches. Using a novel doxycycline-inducible model to allow for tissue-specific overexpression of acid ceramidase, we have determined that ceramidase activation in adipose or liver is sufficient to prevent or reverse diet-induced steatosis, insulin resistance, and glucose intolerance [29]. Here we have generated similar models allowing for AdipoR1 or AdipoR2 to be expressed under the control of a tetracycline response element (TRE-AdipoR1 or TRE-AdipoR2). We use these in order to determine which adiponectin receptor may have the most beneficial effects on glucose tolerance and whole body insulin sensitivity, and to compare and contrast adiponectin receptor action with a lysosomal ceramidase that does not promote S1P accumulation. Furthermore, we will determine whether targeting of adiponectin receptors within hepatocytes or adipocytes provides the greatest metabolic improvements and evaluate the effects of adiponectin receptor agonists for their ability to faithfully recapitulate these effects and further enhance them in transgenic mice.

2. MATERIALS AND METHODS

2.1. Animals

All animal experimental protocols were approved by the Institutional Animal Care and Use Committee of University of Texas Southwestern Medical Center at Dallas. TRE-AdipoR1 or TRE-AdipoR2 mice were generated by subcloning the murine adiponectin receptor gene into a plasmid containing the TetO element. Following linearization, the construct was injected into C57/Bl6-derived blastocysts. Transgene-positive offspring were genotyped using PCR with the following primer sets: TRE-AdipoR1 forward, 5′-ACGTCCTGGTGGTGGGAGCAG and TRE-AdipoR1 reverse, 5′-TCATGAGGGTCCATGGTGATAC; TRE-AdipoR2 forward 5′-TGTGACATCTGGTTTCACTCTC and TRE-AdipoR2 reverse, 5′-TGGTGATACAAGGGACATCTTC.

The *Rosa26-loxP-stop-loxP-rtTA* and *Albumin-Cre* mice were lines were obtained from Jackson Laboratories. *Albumin-Cre* mice were bred with the *Rosa26-loxP-stop-loxP-rtTA* mice to achieve liver-specific expression of rtTA. This mouse was subsequently crossed to the TRE-R1 or TRE-R2 transgenic mice. The resulting triple transgenic mice expressed adiponectin receptor in liver only after exposure to doxycycline (Dox). All overexpression experiments were performed in a pure C57/Bl6 background. All experiments were conducted using littermate-controlled male mice. All Dox-chow diet (200 mg/kg Dox) or HFD-Dox (200 mg/kg Dox) experiments were initiated at approximately 6–12 weeks of age. To generate adipose-specific transgenic mice, the TRE-R1 or TRE-R2 mice were crossed with previously established Adiponectin-rtTA transgenic lines [23]. Wildtype mice for each experiment were rtTA-free or TRE-free littermates, which allowed for direct comparison between receptor transgenic and wt. For AdipoRon studies, mice were injected IP with 5 mg/kg of AdipoRon (Cayman) as previously described [17]. Serum glucose was monitored for 2 h post-injection.

2.2. Neutral ceramidase activity assay

Neutral ceramidase activity was determined by using NBD C12 ceramide as we have previously described [6].

2.3. Systemic tests

Glucose (2.5 g/kg, oral), insulin (0.75 U/kg, IP) and triglyceride (15 uL/g 20% intralipid, oral) were administered for tolerance tests as we previously described [29]. Hyperinsulinemic-euglycemic clamps were performed on conscious, unrestrained male C57 black WT and Alb-AC mice, as previously described [6,11].

2.4. Quantitative real-time PCR

Tissues were excised from mice and snap-frozen in liquid nitrogen. Total RNA was isolated following tissue homogenization in Trizol (Invitrogen, Carlsbad, CA) using a TissueLyser (MagNA Lyser, Roche) then isolated using an RNeasy RNA extraction kit (Qiagen). The quality and quantity of the RNA was determined by absorbance at 260/280 nm. cDNA was prepared by reverse transcribing 1 ug of RNA with an iScript cDNA Synthesis Kit (BioRad). Results were calculated using the threshold cycle method [14], with β-actin used for normalization.

2.5. Histology, immunohistochemistry (IHC)

The relevant adipose and liver tissues were excised and fixed in 10% PBS-buffered formalin for 24 h. Following paraffin embedding and sectioning (5 μm), tissues were stained with H&E.

2.6. Immunoblotting

Frozen tissue was homogenized in TNET buffer (50 mM Tris—HCl, pH 7.6, 150 mM NaCl, 5 mM EDTA, phosphatase inhibitors (Sigma—Aldrich) and protease inhibitors (Roche) and then centrifuged to remove any adipose layer present. After the addition of Triton X-100 (final concentration of 1%), protein concentrations were determined using a bicinchoninic acid assay (BCA) kit (Pierce). Proteins were resolved on 4–20% TGX gel (Bio-Rad) then transferred to nitrocellulose membranes (Protran). pAkt (Ser473, 4060) and total Akt (2920) (Cell Signaling Technology, Inc.) were used (1:1,000) for insulin signaling studies. Primary antibodies were detected using secondary IgG labeled with infrared dyes emitting at 700 nm (926-32220) or 800 nm (926-32211) (both at 1:5,000 dilutions) (Li-Cor Bioscience) and then visualized on a Li-Cor Odyssey infrared scanner (Li-Cor Bioscience). The scanned data were analyzed and quantitated using Odyssey Version 2.1 software (Li-Cor Bioscience).

2.7. Lipid quantifications

Sphingolipids were quantified as described previously by LC/ESI/MS/MS using a Shimadzu Nexera X2 UHPLC system coupled to a Shimadzu LCMS-8050 triple quadrupole mass spectrometer [6]. Lipid species were identified based on their molecular mass and fragmentation patterns, and verified by lipid standards. Diacylglycerol was quantified by direct-infusion mass spectrometry using an AB Sciex 5600+, as previously described [29].

2.8. AdipoRon treatment

Mice were injected IP with 5 mg/kg of AdipoRon (Cayman) as previously described [17]. Serum glucose was monitored for 2 h post-injection.

2.9. Statistics

All results are provided as means ± s.e.m. All statistical analyses were performed using GraphPad Prism. Differences between the two groups over time (indicated in the relevant figure legends) were determined by

