Original Article

MOLECULAR METABOLISM

Inhibition of central *de novo* ceramide synthesis restores insulin signaling in hypothalamus and enhances β-cell function of obese Zucker rats

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ABSTRACT

Objectives: Hypothalamic lipotoxicity has been shown to induce central insulin resistance and dysregulation of glucose homeostasis; nevertheless, elucidation of the regulatory mechanisms remains incomplete. Here, we aimed to determine the role of *de novo* ceramide synthesis in hypothalamus on the onset of central insulin resistance and the dysregulation of glucose homeostasis induced by obesity.

Methods: Hypothalamic GT1-7 neuronal cells were treated with palmitate. *De novo* ceramide synthesis was inhibited either by pharmacological (myriocin) or molecular (si-Serine Palmitoyl Transferase 2, siSPT2) approaches. Obese Zucker rats (OZR) were intracerebroventricularly infused with myriocin to inhibit *de novo* ceramide synthesis. Insulin resistance was determined by quantification of Akt phosphorylation. Ceramide levels were quantified either by a radioactive kinase assay or by mass spectrometry analysis. Glucose homeostasis were evaluated in myriocin-treated OZR. Basal and glucose-stimulated parasympathetic tonus was recorded in OZR. Insulin secretion from islets and β-cell mass was also determined.

Results: We show that palmitate impaired insulin signaling and increased ceramide levels in hypothalamic neuronal GT1-7 cells. In addition, the use of deuterated palmitic acid demonstrated that palmitate activated several enzymes of the *de novo* ceramide synthesis pathway in hypothalamic cells. Importantly, myriocin and siSPT2 treatment restored insulin signaling in palmitate-treated GT1-7 cells. Protein kinase C (PKC) inhibitor or a dominant-negative PKCζ also counteracted palmitate-induced insulin resistance. Interestingly, attenuating the increase in levels of hypothalamic ceramides with intracerebroventricular infusion of myriocin in OZR improved their hypothalamic insulin-sensitivity. Importantly, central myriocin treatment partially restored glucose tolerance in OZR. This latter effect is related to the restoration of glucose-stimulated insulin secretion and an increase in β-cell mass of OZR. Electrophysiological recordings also showed an improvement of glucose-stimulated parasympathetic nerve activity in OZR centrally treated with myriocin.

Conclusion: Our results highlight a key role of hypothalamic *de novo* ceramide synthesis in central insulin resistance installation and glucose homeostasis dysregulation associated with obesity.

Keywords Ceramide; Lipotoxicity; Hypotalamus; Insulin resistance; Insulin secretion

1. INTRODUCTION

An increasing body of evidence shows that lipids control energy balance through their action on the hypothalamus [1,2]. Obesity following a high fat diet (HFD) but also intra-hypothalamic injections of saturated fatty acids (FFAs) have been shown to induce central lipotoxicity, evidenced by the deregulation of energy homeostasis [1,3]. Central lipotoxicity has also been shown to be deleterious for the hypothalamic control of glucose homeostasis [4,5]. Indeed, it is well established that in rodents fed with HFD, excessive lipid intake induces hypothalamic insulin resistance (IR) and decreases liver insulin sensitivity, resulting in increased liver glucose production [4,5].

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Abbreviations: IR, insulin resistance; HFD, high fat diet; T2D, Type 2 diabetes mellitus; PKCs, proteins kinase C; ER, endoplasmic reticulum; CerS, ceramide Synthase; Thr-308, Threonine 308; Ser-473, Serine 473; DH-C_2-cer, dihydroceramide; d4-palmitate, Deuterium-labeled palmitate; SPT2, Serine-palmitoyl transferase 2; C_2-Cer, C_2-Ceramide; LZR, Lean Zucker rat; OZR, Obese Zucker rat; ICV, intracerebroventricular; M, myriocin

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Mechanisms of hypothalamic insulin resistance induced by central lipotoxicity are still not completely understood, although it has been demonstrated that β-oxidation of FFAs may be involved [5,6]. Indeed, inhibition of the hypothalamic carnitine palmitoyl transferase 1, which regulates FFA β-oxidation [7], improves hepatic insulin action [5,6]. We and others showed previously that HFD or palmitate activation of PKCθ in hypothalamus, leading to impairment of central insulin signaling [4,8] is associated with hepatic IR [4]. Studies have shown an increase in lipids such as ceramides and diacylglycerols in the hypothalami of mice fed with HFD, centrally infused with palmitate, and in obese Zucker rats (OZR) [4,9]. Interestingly, infusion of ceramide analogs in the hypothalamus has been shown to deregulate energy homeostasis by regulating ER stress [10,11].

Since ceramides have been linked to peripheral lipotoxicity, especially in the onset of IR [12,13], the aim of the present study was to decipher the role of endogenous ceramide synthesis on the deregulation of hypothalamic insulin signaling and its consequences on glucose homeostasis. We found that *de novo* ceramide synthesis induced a hypothalamic IR through the activation of PKCζ. Interestingly, we also found that inhibition of *de novo* ceramide synthesis in the hypothalami of OZR improved glucose tolerance. The inhibition of hypothalamic ceramide synthesis slightly improved peripheral insulin sensitivity in OZR. In contrast, central inhibition of *de novo* ceramide synthesis in OZR was correlated to an increase of pancreatic β-cell area and insulin secretion by activation of the parasympathetic nervous system.

2. MATERIAL AND METHODS

2.1. Materials

Tissue culture medium was from Gibco. [γ-^{32}P]ATP was purchased from PerkinElmer. Palmitate, fatty-acid-free BSA, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide], ethylenediamine-tetra-acetic acid (EDTA), disodium salt, perchloric acid, hydrogen chloride, Anti-HA (hemagglutinin), SPT2 antibodies, and insulin human solution were from Sigma–Aldrich. Myriocin, L-cycloserine, D609, C_2-ceramide, C_2-dihydroceramides, and N-butyldeoxynojirimycin were from Enzo Life Sciences. Ro31-8220 and DAG kinase were from Calbiochem. All solvents were from Merck Eurolab or Fisher Scientific. phospho-Akt (Thr 308), phospho-Akt (Ser 473), Akt, p-PKCζ (Thr 410-403), PKCζ, and β-actin antibodies were from Cell Signaling. Ultrapure water was obtained with a Milli-Q system (Millipore, Bedford, MA, USA). Adenoviruses containing the cDNA of GFP, wild-type PKCζ (WT- PKC), constitutive active PKCζ (CA-PKCζ), or kinase-dead PKCζ (KD-PKCζ) were prepared as previously described [14]. All PKCζ constructs contained an HA tag for monitoring their expression.

2.2. Cell culture

GT1-7 murine hypothalamic cells (kindly provided by the Dr. P. Mellon, UCSD, USA) were cultured in DMEM medium supplemented with 10% FBS and 100 U/mL penicillin/streptomycin [15]. Palmitate was conjugated with fatty acid-free BSA (molar ratio FFA/BSA was 5:1) and diluted in DMEM supplemented with 1% FBS to obtain a 0.5 or 1 mM final concentration. After palmitate treatment, cells were stimulated with insulin (100 nM) for 5 min.

2.3. Measurement of cell viability

Cells were treated with MTT (1 mg/ml) for 4 h. Supernatants were discarded, and DMSO was added. Absorbance was measured at 560 nm using a microplate reader (Dynex-MRX).

2.4. Implantation of intracerebroventricular (icv) cannulae and central treatments

Ten-weeks-old male obese Zucker rats (OZR; 300–350 g) and lean Zucker rats (LZR; 250–300 g) (Charles River) were used. The experimental protocol was approved by the institutional animal care and use committee of the Paris Diderot University (CEEA40). Chronic icv cannulae were implanted under isoflurane anesthesia, and animals received a 10 μg/kg i.p. administration of xylazine. They were then placed on a stereotaxic frame. A catheter tube was connected from the brain infusion cannulae to an osmotic minipump flow moderator (model 2004; Alzet, Rabalot, France). The minipump was inserted in a subcutaneous pocket on the dorsal surface of the animal, created using blunt dissection and connected through a catheter to a depth-adjustable cannula implanted on ICV (X:−0.8 mm; Y:−1.5 mm; Z:−3.5 mm). Specificity of the ICV injections was controlled as previously described [16]. Zucker rats were then chronically infused for 28 days with either (300 nM) myriocin or vehicle (NaCl 9‰). Myriocin (300 nM) was prepared in NaCl 9‰. In Wistar rats, either C2-ceramide or DH-C_2-ceramide or vehicle (DMSO 5%/NaCl 9‰) were acutely ICV injected and insulin signalling was determined after 2 h post-injection. C_2-ceramide/DH-C2-ceramide solution (25 nM) was prepared in 5% DMSO. The selection of these doses was based on previous reports [10]. Prior to sacrifice, animals received an injection of insulin (2 mUI) or saline icv using the cannulae. Animals were sacrificed after 5 min, and tissues were collected and immediately frozen.

2.5. Glucose and insulin tolerance tests

Fourteen and 21 days after stereotaxic surgery, blood glucose levels were measured after glucose administration (oral glucose tolerance test, OGTT) or insulin (insulin tolerance test, ITT), respectively, with a glucometer (Accucheck, Roche) in overnight-fasted rats. 0.75 UI/kg insulin (Actrapid, Novonordisk) via intra-peritoneal injection was used for ITT, and 2 g/Kg D-glucose (Sigma–Aldrich) was administered orally via gavage for OGTT. During OGTT, blood samples were collected from the tail, and plasma was extracted and stored at −80 °C before further analysis. Insulin concentration was evaluated using ELISA methods (Rat Insulin Elisa, ALPCO, Eurobio, Courtaboeuf, France).

2.6. Western blotting

Cells lysates were obtained and analyzed by immunoblotting, as described [17]. A list of the antibodies used is shown in Supplementary Table 1.

2.7. Measurement of ceramide levels

Ceramide levels were measured by the diacylglycerol (DAG) kinase enzymatic method as previously described [17]. Briefly, lipid extracts were incubated in the presence of *E. coli* DAG kinase and [γ-^{32}P]-ATP. Reaction was stopped, and [γ-^{32}P]-ceramide phosphate was resolved by TLC with chloroform/acetone/methanol/acetic acid/water (10:4:3:2:1, by vol.) and quantified using a FLA700 PhosphorImager (GE Healthcare). An aliquot was used to quantify total phospholipid levels as described previously [18]. Ceramide levels are expressed in pmol per nmol of phospholipids (PL) levels.

2.8. *In vitro* insulin secretion from isolated islets

Rats were anesthetized with pentobarbital. Islets of Langerhans were isolated after collagenase digestion of the pancreas as previously described [19]. *In vitro* insulin release was assayed on islet after overnight culture. Floating Islets were pre-incubated in KRBH-0.05% BSA with 2.8 mM of glucose for 30 min, followed by 60 min

