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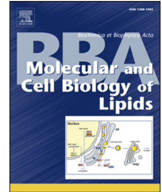

Characterization of the role of sphingomyelin synthase 2 in glucose metabolism in whole-body and peripheral tissues in mice

Masayuki Sugimoto [a,b], Yoichi Shimizu [c,d], Songji Zhao [e], Naoyuki Ukon [e], Ken-ichi Nishijima [a,c], Masato Wakabayashi [b], Takeshi Yoshioka [b], Kenichi Higashino [b], Yoshito Numata [b], Tomohiko Okuda [f], Nagara Tamaki [g], Hisatoshi Hanamatsu [h], Yasuyuki Igarashi [h], Yuji Kuge [a,c,*]

[a] Department of Integrated Molecular Imaging, Graduate School of Medicine, Hokkaido University, Sapporo, Japan
[b] Shionogi Innovation Center for Drug Discovery, Discovery Research Laboratory for Innovative Frontier Medicines, Shionogi & Co., Ltd., Sapporo, Japan
[c] Central Institute of Isotope Science, Hokkaido University, Sapporo, Japan
[d] Laboratory of Bioanalysis and Molecular Imaging, Faculty of Pharmaceutical Science, Hokkaido University, Sapporo, Japan
[e] Department of Tracer Kinetics & Bioanalysis, Graduate School of Medicine, Hokkaido University, Sapporo, Japan
[f] Drug Discovery Technologies, Discovery Research Laboratory for Core Therapeutic Areas, Shionogi & Co., Ltd., Osaka, Japan
[g] Department of Nuclear Medicine, Graduate School of Medicine, Hokkaido University, Sapporo, Japan
[h] Department of Biomembrane and Biofunctional Chemistry, Faculty of Advanced Life Science, Hokkaido University, Sapporo, Japan

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ABSTRACT

Sphingomyelin synthase 2 (SMS2) is a proposed potential therapeutic target for obesity and insulin resistance. However, the contributions of SMS2 to glucose metabolism in tissues and its possible therapeutic mechanisms remain unclear. Thus, to determine whole-body glucose utilization and the contributions of each insulin-targeted tissue to glucose uptake, we performed a glucose kinetics study, using the radiolabeled glucose analog ^{18}F-2-fluoro-2-deoxy-D-glucose (^{18}F-FDG), in wild-type (WT) and SMS2 knockout (KO) mice. Insulin signaling was enhanced in the liver, white adipose tissue and skeletal muscle of SMS2 KO mice compared with those of WT mice. In addition, compared with in WT mice, blood clearance of ^{18}F-FDG was accelerated in SMS2 KO mice when they were fed either a normal or a high fat diet. ^{18}F-FDG uptake was also increased in insulin-targeted tissues such as skeletal muscle in the SMS2 KO mice. Whereas skeletal muscle sphingolipid content was not clearly affected, plasma levels of very long-chain fatty acid (VLCFA)-containing ceramides were markedly increased in SMS2 KO mice, compared with in WT mice. We also generated liver-conditional SMS2 KO mice and performed glucose and insulin tolerance tests on mice with a high fat diet. However, no significant effect was observed. Thus, our study provided evidence that genetic inhibition of SMS2 elevated glucose clearance through activation of glucose uptake into insulin-targeted tissues such as skeletal muscle by a mechanism independent of hepatic SMS2. Our findings further indicate that this occurs, at least in part, *via* indirect mechanisms such as elevation of VLCFA-containing ceramides.

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Abbreviations: SM, sphingomyelin; SMS, sphingomyelin synthase; Cer, ceramide; CerS, ceramide synthase; S1P, sphingosine 1 phosphate; LC/ESI-MS/MS, liquid chromatography/electrospray ionization-tandem mass spectrometry; VLCFA, very long-chain fatty acid; HexCer, hexosylceramide; DhCer, dihydroceramide; ^{18}F-FDG, ^{18}F-2-fluoro-2-deoxy-D-glucose; OGTT, oral glucose tolerance test; ITT, insulin tolerance test; GSIS, glucose stimulated-insulin secretion; HOMA-R, homeostasis model assessment-insulin resistance; T2DM, type 2 diabetes mellitus; PET-CT, positron emission tomography-computed tomography; TG, triacylglycerol; DG, diacylglycerol; FFA, free fatty acid; TC, total-cholesterol; ALT, alanine aminotransferase; AST, aspartate aminotransferase; WAT, white adipose tissue (e, epididymal, m, mesenteric); iBAT, intrascapular brown adipose tissue; Qua, quadriceps femoris; Gas, gastrocnemius; Sol, soleus; Spi, spinotrapezius; Glu, gluteus maximus; Dia, diaphragm; FAO, fatty acid oxidation; PCR, polymerase chain reaction; NEFA, non-esterified fatty acid; SPLs, sphingolipids; ER, endoplasmic reticulum; GLUT, glucose transporter; IRβ, insulin receptor β subunit; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; AUC, area under the blood concentration-time curve; WT, wild-type; KO, knockout; ND, normal diet; HFD, high fat diet; Het KO, heterozygous knockout; LcKO, liver-conditional knockout; T/B ratio, tissue/blood ratio; ES, embryonic stem; H&E, hematoxylin and eosin; IL-6, interleukin-6; NMA, normalized area.

* Corresponding author at: Central Institute of Isotope Science, Hokkaido University, Kita 15, Nishi 7, Kita-ku, Sapporo 060-0815, Japan.
E-mail address: kuge@ric.hokudai.ac.jp (Y. Kuge).

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1. Introduction

Insulin resistance is the primary characteristic symptom in patients with type 2 diabetes mellitus (T2DM) [1]. Obesity is the major cause of insulin resistance. It is well known that excess visceral fat, primarily resulting in triacylglycerol (TG) accumulation in the liver, is tightly associated with the development of insulin resistance [2]. Whereas the causal relationship between hepatic steatosis and insulin resistance is unclear, it was reported that white adipose tissue (WAT)-derived hepatic acetyl-CoA is a primary regulator of hepatic insulin resistance [3]. Insulin resistance in skeletal muscle, the predominant target tissue for insulin-stimulated glucose uptake, is another characteristic feature of T2DM [4]. Whereas the mechanism is not yet known, it has been reported that WAT-derived excessive lipids, especially free fatty acids (FFAs) and diacylglycerol (DG), lead to insulin resistance in the skeletal muscle as well as in the liver and WAT [5–7].

Sphingolipids (SPLs), including ceramide (Cer), sphingomyelin (SM), glucosylceramide (GlcCer), GM3 and sphingosine 1 phosphate (S1P) are also important for obesity and insulin resistance [8–12]. We previously showed that high levels of serum SM species containing saturated acyl chains are closely correlated with obesity, reduced liver function and insulin resistance in obese volunteer subjects [13]. SM is synthesized from Cer by transfer of phosphocholine from phosphatidylcholine through SM synthase (SMS) 1 and 2 in the Golgi apparatus or SMS2 in the plasma membrane [14,15]. SMS1 knockout (KO) mice showed several phenotypes including impaired WAT and pancreatic functions [16,17]. In contrast, SMS2 has been proposed to be a potential therapeutic target for insulin resistance and T2DM because previous reports showed that SMS2 deficiency ameliorated diet-induced obesity, hepatic steatosis and insulin resistance without any negative effects [18–20]. SMS2 was found to be located in lipid microdomains and associated with the fatty acid (FA) transporter, CD36/FAT [18]. Thus, SMS2 modulates SM in the lipid microdomains and regulates FA uptake through CD36/FAT in hepatocytes. SMS2 KO mice showed improved glucose tolerance and elevated insulin sensitivity through activated insulin action in the liver [19]. The mechanism is not fully understood, but reduced SM and elevated Cer levels in the plasma membrane contributed to insulin signaling in SMS2 KO hepatocytes [19]. It has been demonstrated that hepatic SMS2 directly regulates hepatic steatosis [20]. However, the contributions of hepatic SMS2 to obesity and whole-body insulin resistance have not been addressed. Although skeletal muscle is an important organ for glucose metabolism, the contributions of SMS2 on this organ have not been fully addressed because research on SMS2 has focused on the liver in which the expression of SMS2 is higher than SMS1. It was previously reported that 2-deoxyglucose uptake was higher in the WAT and skeletal muscle of SMS2 KO mice, compared with WT mice [19]. However, insulin-dependent glucose uptake in different skeletal muscles and glucose kinetics has not been studied.

Fasting plasma insulin levels and homeostasis model assessment-insulin resistance (HOMA-R) are representative criteria used for screening insulin resistance [21]. The oral glucose tolerance test (OGTT) and insulin tolerance test (ITT) are also frequently used to detect abnormal glucose tolerance and insulin sensitivity [22]. However, these methods do not provide any insight as to the organ-specificity of insulin resistance and glucose metabolism. The hyperinsulinemic-euglycemic clamp is a useful technique to obtain more specific indices for insulin resistance [22]. Whereas this technique has also been referred to as the “gold standard” for measuring insulin sensitivity *in vivo* [23], it requires a great deal of skill especially when used in small animal studies. The glucose analog, ^{18}F-2-fluoro-2-deoxy-D-glucose (^{18}F-FDG) is a clinically used positron emission tomography (PET) imaging probe for evaluating glucose utilization in tissues, especially tumors [24,25]. ^{18}F-FDG, like normal glucose, is taken up by cells and phosphorylated by hexokinase [26]. However, ^{18}F-FDG cannot be further metabolized and then accumulated in cells because it lacks a 2′ hydroxyl group, which is needed for glycolysis. In several reports, tissue-specific ^{18}F-FDG distribution correlated with T2DM and insulin resistance in patients and in animal models [27–29]. Thus, ^{18}F-FDG is a convenient marker for studying glucose utilization [30].

Thus, the aims of this study were i) to determine the contribution of each insulin-targeted organ to SMS2-associated whole-body glucose metabolism by a kinetics study using ^{18}F-FDG, and ii) to investigate the mechanisms involved using a lipidomic approach and organ-specific SMS2 KO mice.

2. Materials and methods

2.1. Animals

2.1.1. Animals and housing

The animal research protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the Graduate School of Medicine, Hokkaido University (Sapporo, Japan) and Shionogi & Co., Ltd. (Osaka, Japan). The laboratory animal facility of Shionogi & Co., Ltd. has been accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). The protocol conformed to the *Guide for the Care and Use of Laboratory Animals* (US National Institutes of Health, Bethesda, MD). Male mice were housed under controlled temperature and humidity with a 12 h light–dark or 13 h light–11 h dark cycle. Before the experiment, the mice were maintained in the facility for 1 week to acclimatize them to the conditions. Foods and water were provided *ad libitum*.

2.1.2. Generation of SMS2 KO mice

SMS2 heterozygous KO (Het KO) and KO mice were generated as described previously [18]. Mice used were on a C57BL/6N background. The same genetic background mice were used as wild-type (WT) control mice.

2.1.3. Generation of SMS2 liver-conditional KO mice

To generate *loxP*-floxed (fl; for flanked by *loxP*) SMS2 mice, a targeting vector for homologous recombination in mouse embryonic stem (ES) cells was constructed (Fig. 6A). Exon 3 of the *Sgms2* gene flanked by *loxP* and homologous arms were sub-cloned by polymerase chain reaction (PCR). These sequences were ligated with a PGK-Neo cassette [31]. A DT-A cassette was inserted at the 3′ terminus of the vector [32]. The targeting vector was linearized and transduced into ES cells derived from C57BL/6 mice by electroporation using Nucleofector Kits for Mouse Embryonic Stem Cells (Lonza Group Ltd., Basel, Switzerland) [33]. The ES cells considered recombinant clones were identified by PCR using the following primers; forward 5′-TTCC TGACTAGGGGAGGAGTAGAAG-3′, reverse 5′-ACCATATGGTGGAGCCAA AATGG-3′. Chimera mice were generated by the aggregation method as previously described [33,34]. Highly chimeric offspring were regarded as heterozygous SMS2$^{fl/+}$ mice. Male SMS2$^{fl/+}$ mice were mated with female heterozygous CAG-FLPe transgenic mice [35] to delete the PGK-Neo cassette. Obtained SMS2$^{\Delta Neofl/+}$; FLPe$^{tg/+}$ mice were bred with WT mice to remove CAG-FLPe. Obtained SMS2$^{\Delta Neofl/+}$ mice were then inbred, resulting in homozygous SMS2$^{\Delta Neofl/\Delta Neofl}$ mice. Male heterozygous SMS2$^{fl/+}$ mice were bred with female CMV-Cre transgenic mice [36] to obtain SMS2 Het KO (SMS2$^{+/-}$; CMV-Cre$^{tg/+}$) mice. To remove CMV-Cre and introduce Albumin (Alb)-Cre, SMS2$^{+/-}$; CMV-Cre$^{tg/+}$ mice were then interbred with homozygous Alb-Cre transgenic (Alb-Cre$^{tg/tg}$) mice [37]. To obtain hepatocyte-specific SMS2 liver-conditional KO (LcKO, SMS2$^{\Delta Neofl/-}$; Alb-Cre$^{tg/+}$) mice, SMS2$^{+/-}$; Alb-Cre$^{tg/+}$ mice and SMS2$^{\Delta Neofl/\Delta Neofl}$ mice were mated. To identify the genotype of obtained mice, their genomic DNA was extracted by a conventional method and PCR was performed using the following primers; forward 5′-ATTAAAATATTGGATCCAGGCGTG-3′, reverse 5′-GAGTATGCTAGGTT GCAGCTAATTC-3′ (Fig. 6B). CAG-FLPe transgenic mouse strain (C57BL/6-Tg(CAG-flpe)36Ito/ItoRbrc) was provided by RIKEN BRC

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