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Disruption of the mitochondria-associated ER membrane (MAM) plays a central role in palmitic acid–induced insulin resistance

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ABSTRACT

The mitochondria-associated ER membrane (MAM) is a specialized subdomain of ER that physically connects with mitochondria. Although disruption of inter-organellar crosstalk via the MAM impairs cellular homeostasis, its pathological significance in insulin resistance in type 2 diabetes mellitus remains unclear. Here, we reveal the importance of reduced MAM formation in the induction of fatty acid–evoked insulin resistance in hepatocytes. Palmitic acid (PA) repressed insulin-stimulated Akt phosphorylation in HepG2 cells within 12 h. Treatment with an inhibitor of the ER stress response failed to restore PA-mediated suppression of Akt activation. Mitochondrial reactive oxygen species (ROS) production did not increase in PA-treated cells. Even short-term exposure (3 h) to PA reduced the calcium flux from ER to mitochondria, followed by a significant decrease in MAM contact area, suggesting that PA suppressed the functional interaction between ER and mitochondria. Forced expression of mitofusin-2, a critical component of the MAM, partially restored MAM contact area and ameliorated the PA-elicited suppression of insulin sensitivity with Ser473 phosphorylation of Akt selectively improved. These results suggest that loss of proximity between ER and mitochondria, but not perturbation of homeostasis in the two organelles individually, plays crucial roles in PA-evoked Akt inactivation in hepatic insulin resistance.

1. Introduction

Hepatic insulin resistance, a hallmark of type 2 diabetes mellitus (T2D), is characterized by a failure of insulin to inhibit gluconeogenesis and stimulate glycogen synthesis, resulting in elevated blood glucose levels [1]. During obesity, which is a major risk factor for T2D, free fatty acid (FFA) released from visceral adipose tissue is directly transported to the liver via the portal vein, leading to hepatic insulin resistance [2–4]. Palmitic acid (PA), one of the most well-characterized FFAs associated with insulin resistance [5], alone can induce insulin resistance in cultured hepatic cell lines [6,7]. A growing body of evidence has revealed the significance of increased diacylglycerol content and the subsequent activation of protein kinase-Cε in the development of hepatic insulin resistance [8,9]. However, several other factors, including inflammation, mitochondrial dysfunction, and the endoplasmic reticulum (ER) stress response, have also been proposed to impair insulin sensitivity in the diabetic liver [1,10]. Thus, the exact mechanisms by which the insulin signaling cascade becomes dysregulated in the liver of diabetic individuals remain unknown.

Protein kinase B (Akt/PKB), a central molecule in the insulin signaling cascade, plays crucial roles in the regulation of whole-body glucose homeostasis [11]. Upon binding of insulin to its receptor, sequential phosphorylation reactions generate a binding scaffold, phosphatidylinositol-3, 4, 5-triphosphate (PIP_3), on the plasma membrane, which in turn recruits Akt and 3-phosphoinositide-dependent kinase-1 (PDK1), resulting in the phosphorylation at Thr308 of Akt. In addition, phosphorylation of Akt at Ser473 by mammalian target of rapamycin complex 2 (mTORC2) is required for its full activation [12,13]. Activated Akt downregulates hepatic glucose production by inhibiting two crucial downstream molecules, glycogen synthase kinase-3β (GSK3β) and forkhead box protein O1 (FOXO1) [14]. Thus, in T2D, a reduction in Akt phosphorylation impairs hepatic glucose

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disposal while increasing hepatic glucose production. In mice, liver-specific inactivation of the *Akt* gene aggravates systemic glucose tolerance, confirming the importance of Akt activity in the maintenance of hepatic insulin sensitivity [15,16]. However, the detailed molecular mechanisms that impair Akt activation during the development of hepatic insulin resistance are not fully understood.

Both mitochondrial and ER functions are disrupted during hepatic insulin resistance. In subjects with insulin resistance, enhanced production of reactive oxygen species (ROS) in damaged mitochondria decreases Akt activation [17,18]. Chemical suppression of the ER stress response, a homeostatic response to excessive accumulation of misfolded proteins in the ER, restores hepatic insulin sensitivity and increases Akt phosphorylation levels in diabetic mice [19]. Thus, intact mitochondria and ER are likely to be independently required for the maintenance of normal insulin signaling cascades, and distortion of either of these organelles can disrupt insulin action. On the other hand, mTORC2-mediated phosphorylation of Akt at Ser473 occurs at the contact site between mitochondria and a subdomain of the ER known as the mitochondria-associated ER membrane (MAM) [20]. In diabetic mice, MAM formation is altered concomitant with repression of Akt phosphorylation, suggesting that interaction between mitochondria and ER via the MAM is functionally important in the regulation of insulin signaling [21,22].

In this study, we investigated to what extent the ER stress response, mitochondria dysfunction, and alterations in MAM integrity contribute to development of insulin resistance. For this purpose, we adopted a simple experimental culture system in which HepG2 cells were stimulated with PA to induce insulin resistance. Our results revealed that insulin-induced Akt phosphorylation is impaired upon PA treatment, which disrupts the functional integrity of the MAM without involvement of the ER stress response or mitochondrial ROS. Forced expression of mitofusin-2 (MFN2) partially restored MAM contact area and Akt activity. Thus, our findings strongly suggest that MAM disruption plays a crucial role in the development of hepatic insulin resistance.

2. Material and methods

2.1. Reagents

PA and adenosine 5′-triphosphate sodium salt n-hydrate (ATP) were purchased from Wako Pure Chemical Industries. Rhod-2AM, Fluo-4AM and MitoSOX were from Thermo Fisher Scientific. Insulin solution (Humalin-R) was from Eli Lilly. GSK2606414 and brefeldin A were from LKT Laboratories, Inc. and Merck Millipore, respectively.

Antibodies were acquired from the indicated suppliers: rabbit polyclonal anti-Akt (#9272), anti-phospho-Akt (Thr308) (#9275), and anti-phospho-Akt (Ser473) (#9271), Cell Signaling Technology; mouse monoclonal anti-α-tubulin (T9026), Sigma-Aldrich; mouse monoclonal anti-KDEL (ADI-SPA-827) for detection of GRP78, ENZO Life Sciences; mouse monoclonal anti-CHOP (MA1-250), Thermo Fisher Scientific; rabbit polyclonal anti-Lamin A/C (sc-20681) and goat polyclonal anti-VDAC1 (sc-8828), Santa Cruz Biotechnology; mouse monoclonal anti-Mitofusin 2 (ab56889) and rabbit polyclonal anti-Sigma1-receptor (S1R) (ab53852), Abcam; rabbit polyclonal anti-ACSL4 (AP2536B), Abgent. Horseradish peroxidase–conjugated anti-mouse IgG (NA9310V) and anti-rabbit IgG (NA9340V) were from GE Healthcare.

2.2. Cell culture

Human hepatocellular carcinoma cell line HepG2 was cultured in Dulbecco's Modified Eagle's Medium (DMEM, Thermo Fisher Scientific), supplemented with 10% fetal bovine serum, at 37 °C in a 5% CO_2–95% air atmosphere.

2.3. PA solution

PA solution was prepared as described previously [23].

2.4. Western blotting

Cell pellets were lysed using an extraction buffer as described previously [7]. Lysate was separated by 7–16% SDS-PAGE, and then subjected to immunoblotting. This was followed by detection using the Immobilon™ Western Chemiluminescent HRP Substrate (Millipore) and ChemiDoc™ XRS+ system (Bio-Rad). The visualized bands were quantified by Image Lab software (Bio-Rad). Tubulin was used as an internal control.

2.5. RT-PCR

Total RNA was extracted from cells and reverse transcribed with GoScript™ Reverse Transcriptase (Promega). The synthesized first-strand cDNA was subjected to PCR with Taq polymerase using primers 5′-GAAGCCAAGGGGAATGAAGTGAGG-3′ and 5′-CATGGGGAG ATGTTCTGGAGGGG-3′ to amplify the XBP1 fragment containing the splice site. The amplified products were separated by electrophoresis on an 8% polyacrylamide gel and visualized by ethidium bromide staining.

2.6. Transmission electron microscopy

Cells were fixed in 1.25% glutaraldehyde in 0.1 M sodium cacodylate (pH7.2). Post-fixation was performed in 1% OsO_4 in 0.1 M phosphate. Subsequently, samples were dehydrated and embedded in epoxy resin (Quetol-651, Nissin EM, Tokyo). Ultrathin sections (100 nm) were cut by an ultramicrotome (Ultracut N, Reichert-Nissei, Tokyo, Japan) and collected on grid meshes. The sections were stained with 1% uranyl acetate for 10 min, followed by 1% lead citrate for 5 min, and washing with distilled water. They were examined with an electron microscope (Hitachi H-600A, Hitachi, Tokyo, Japan). Mitochondria perimeter and the length of ER-mitochondria interfaces (MAM) were measured to obtain the MAM ratio using Adobe Photoshop CC 2017.

2.7. Calcium flux analysis

Cells were plated on 35 mm glass-based dishes (Asahi Techno Glass), and after two overnights treated with PA or vehicle as indicated in the figure legends. Dishes were incubated with 3 μM Rhod-2AM for staining of mitochondrial calcium or with 5 μM Fluo-4AM for staining of cytosolic calcium for 30 min in recording medium (20 mM HEPES (pH 7.5), 115 mM NaCl, 13.8 mM glucose, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$). Fluorescence images were collected every 5 s using an FV1000 confocal laser scanning microscope (Olympus). Stimulation was performed with 50 μM ATP. Signals from more than 50 cells per dish were analyzed using the ImageJ software.

2.8. ROS measurement

Cells were incubated with 5 μM MitoSOX in HBSS for 15 min at 37 °C and digested with trypsin. Data were acquired on a Cytomics FC500MPL flow cytometer (Beckman Coulter, Inc.), and the data were analyzed using FCSalyzer ver. 0.9.11-alpha (https://sourceforge.net/projects/fcsalyzer/).

2.9. Cell fractionation

Fractionation of the MAM was described elsewhere [24]. Briefly, following homogenization of cells collected from 10 dishes (ϕ 100 mm), nuclei, crude mitochondria, and cytosol fractions were prepared by