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Exendin-4, a glucagon-like peptide-1 receptor agonist, reduces hepatic steatosis and endoplasmic reticulum stress by inducing nuclear factor erythroid-derived 2-related factor 2 nuclear translocation

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ABSTRACT

Activation of endoplasmic reticulum (ER) stress is involved in the development of nonalcoholic fatty liver disease. Glucagon-like peptide-1 (GLP-1) has been reported to reduce hepatic steatosis, but the underlying mechanism has not been fully elucidated. Here, we investigated whether exendin-4 (EX-4), a GLP-1 receptor analogue, improves hepatic steatosis through ER stress reduction. Furthermore, we explored which ER stress pathway is involved in this process, with a focus on the protein kinase RNA-like ER kinase (PERK)-nuclear factor erythroid-derived 2-related factor 2 (Nrf2) pathway. EX-4 treatment reduced hepatic lipid accumulation by suppressing the expression of lipogenic genes and restoring the expression of β-oxidation genes in palmitate-treated HepG2 cells and high fat diet (HFD)-fed mice. In addition, EX-4 treatment suppressed hepatic ER stress activation in HFD-fed mice and tunicamycin-treated mice. In particular, EX-4 treatment restored HFD- and tunicamycin-induced Nrf2 nuclear translocation to control levels. Inhibition of Nrf2 by siRNA enhanced phosphorylation of PERK and eukaryotic translation initiation factor 2α (eIF2α), as well as other substrates of the PERK pathway. Nrf2 knockdown also inhibited the protective effects of EX-4 against lipid accumulation, ER stress activation, and cell death in palmitate-treated HepG2 cells. EX-4 treatment prevents hepatic steatosis and improves cell survival by regulating hepatic lipid metabolism and reducing ER stress activation, and Nrf2 plays an essential role in the protective effect of GLP-1 on hepatic steatosis.

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is the most common cause of liver disease, and its prevalence is gradually increasing worldwide (Younossi et al., 2016). NAFLD ranges in presentation from simple steatosis with benign prognosis to a progressive form of nonalcoholic steatohepatitis (NASH). NASH is a predisposing condition for development of cirrhosis and hepatocellular carcinoma (Calzadilla Bertot and Adams, 2016). Development of NAFLD is related to obesity, insulin resistance, and type 2 diabetes (Paschos and Paletas, 2009) and is associated with cardiovascular disease and liver-related morbidity and mortality (Mikolasevic et al., 2016; Stepanova et al., 2013).

The development and progression of NAFLD are complex processes that can be explained by the 'multiple-hit' hypothesis (Buzzetti et al., 2016; Yilmaz, 2012; Yu et al., 2016). Insulin resistance is key factor in the development of NAFLD as it leads to increased hepatic de novo

Abbreviations: ATF6, Activation transcription factor-6; Bip, Binding immunoglobulin protein; CHOP, C/EBP homologous protein; CPT1α, Carnitine palmitoyltransferase-1α; DGAT1, Acyl-CoA:diacylglycerol acyltransferase-1; eIF2α, Eukaryotic translation initiation factor 2α; ER, Endoplasmic reticulum; EX-4, Exendin-4; EX 39–9, Exendin 9–39; FABP1, Fatty acid binding protein-1; FAS, Fatty acid synthase; GLP-1, Glucagon-like peptide-1; GPAT1, glycerol-3-phosphate acyltransferase-1; HFD, High-fat diet; HO-1, Heme oxygenase-1; IRE1, Inositol-requiring enzyme-1; MEM, Minimum essential medium; NAFLD, Nonalcoholic fatty liver disease; NASH, Nonalcoholic steatohepatitis; ND, Normal chow diet; Nrf2, Nuclear factor erythroid-derived 2-related factor 2; NQO-1, NAD(P)H quinine oxidoreductase-1; PERK, Protein kinase RNA-like ER kinase; PPAR, Peroxisome proliferator-activated receptor; SCD1, Stearoyl-CoA desaturase-1; siRNA, Small interfering RNAs; SREBP1c, Sterol regulatory element binding protein-1c; UPR, Unfolded protein response; XBP-1, X-box binding protein 1.

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lipogenesis and impaired inhibition of lipolysis in adipose tissue, which in turn results in increased flux of fatty acids to the liver (Bugianesi et al., 2010). After accumulation of fat in the liver, multiple pathogenic factors, such as oxidative stress, mitochondrial dysfunction, proinflammatory cytokines, and dysbiosis contribute to the progression of NAFLD. Recent studies have suggested that endoplasmic reticulum (ER) stress plays a crucial role in the development and progression of NAFLD (Puri et al., 2008; Kapoor and Sanyal, 2009; Zhang et al., 2014).

ER is a cytosolic organelle that is responsible for the synthesis and proper folding of proteins. Under stressful conditions, unfolded or misfolded proteins accumulate in the ER membranes, leading to an adaptive response, the so-called unfolded protein response (UPR), to resolve ER stress (Hotamisligil, 2010). UPR is mediated by three stress-sensing proteins: protein kinase RNA-like ER kinase (PERK), inositol-requiring enzyme-1 (IRE1), and activation transcription factor-6 (ATF6). Under normal conditions, binding immunoglobulin protein (Bip), a chaperone protein, binds to these stress-sensing proteins to suppress their activation. However, when ER homeostasis is disrupted, Bip is displaced from these sensing proteins, which then activate downstream signaling pathways. Among three UPR signaling pathways, activation of the PERK signaling pathway directly induces phosphorylation of eukaryotic translation initiation factor 2α (eIF2α) and nuclear translocation of nuclear factor erythroid-derived 2-related factor 2 (Nrf2) to promote the clearance of unfolded proteins (Hou et al., 2016). Nrf2 is a transcription factor that acts as a major regulator of the antioxidant response, which is important in cellular defense and cell survival (Cullinan and Diehl, 2004; Cullinan et al., 2003). One study showed that Nrf2 activation reduced the progression of NASH in a rat model (Shimozono et al., 2013), while another reported that Nrf2 deletion accelerated the development of NASH in a diet-induced animal model (Chowdhry et al., 2010). Thus, Nrf2 has a crucial role in the pathogenesis of NAFLD and could be a target for treatment.

Glucagon-like peptide (GLP)-1 is an incretin hormone secreted from L-cells in the distal small intestine and colon following nutrient ingestion (Willard and Sloop, 2012). GLP-1 stimulates insulin secretion from pancreatic β cells and reduces glucagon secretion from pancreatic α cells, thereby delaying gastric emptying and suppressing appetite. Synthetic GLP-1 receptor analogues have been developed and are widely used to treat type 2 diabetes (Lindamood and Taylor, 2015). Recent studies indicate that GLP-1 receptor analogues have therapeutic effects not only on glucose metabolism, but also on the progression of NAFLD (Lee et al., 2012a; Mells et al., 2012; Klonoff et al., 2008; Cuthbertson et al., 2012). However, the precise mechanisms by which GLP-1 contributes to NAFLD and ER stress signaling have not been fully elucidated. Therefore, we investigated whether exendin-4 (EX-4), a GLP-1 receptor analogue, ameliorates hepatic steatosis in a diet-induced animal model and evaluated the mechanism by which it is involved in hepatic ER stress signaling, with a focus on the PERK-Nrf2 pathway.

2. Materials and methods

2.1. Cell culture and treatment

HepG2 cells (human hepatoblastoma cell line) were grown in minimum essential medium (MEM, Gibco, Grand Island, NY, USA) supplemented with 4.73 g/l HEPES, 3.7 g/l sodium bicarbonate, 10% FBS, and 1% penicillin/streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2. Palmitate (Sigma Aldrich, St. Louis, MO, USA) was dissolved in ethanol and diluted in MEM with 0.5% BSA. Cells were treated with 0.5 mM palmitate for 24 h. Cells were cultured in MEM with 0.5% BSA as a control. EX-4 (Sigma Aldrich) was dissolved in distilled water and added to cells 2 h before palmitate treatment.

LX-2 cells (human stellate cells) were cultured in Dulbecco's modified eagle medium (DMEM, Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA) and 1% penicillin/streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2. LX-2 cells were treated for 4 days with 10 nM EX-4 dissolved in distilled water.

2.2. Animal model

Five-week-old male C57B/6 J mice were purchased from Central Lab Animal (Central Lab Animal Inc. Seoul, South Korea). All animals were maintained on a 12 h light/dark cycle with free access to normal chow and water for 1 week. After acclimation, mice were divided into four groups and fed either a normal chow diet (ND), high-fat diet (HFD), HFD with EX-4 administration, or provided the same amount of food as the EX-4 administration group (pair-feeding group) for 9 weeks. The HFD contained 60% fat (Research Diets catalog number D12492: 60.0 kcal % fat, 20.0 kcal % protein, and 20.0 kcal % carbohydrate). Mice were administered EX-4 at 20 nM/kg twice per daily by intraperitoneal injection. For experiments involving tunicamycin, mice were injected intraperitoneally with 0.5 μg/g tunicamycin once a day for 1 week. All animal experiments were approved by the Animal Research Ethics Committee of Kyung Hee University Hospital at Gangdong.

2.3. Measurement of cell viability

Cells were seeded in 96-well plates and cultured for 48 h with different concentrations of palmitate or EX-4. After incubation, cells were treated with MTT solution (1 mg/ml) for 4 h. Dark blue formazan crystals that formed in intact cells were solubilized by DMSO. Then, the absorbance at 570 nm was measured using an E max precision microplate reader (Molecular Devices, Sunnyvale, CA, USA) and analyzed using SoftMax software (Molecular Devices).

2.4. Oil red O staining

HepG2 cells were grown to a density of 10^4 cells per well in a 96-well plate with different concentrations of the indicated chemicals. Cells were then fixed in 4% paraformaldehyde for 1 h and washed four times with cold PBS. Cells were stained with 60% Oil Red O solution (working solution, 1.8 g Oil Red O powder dissolved in isopropanol) for 1 h at room temperature. Stained cells were observed using an inverted Olympus CKX41 microscope (Olympus, Tokyo, Japan). Oil Red O dye was extracted from the stained cells in 250 μl isopropanol, and 200 μl was transferred to a 96-well plate. The absorbance at 490 nm was measured using an E max precision microplate reader (Molecular Devices, Sunnyvale, CA, USA) and analyzed using SoftMax software (Molecular Devices).

2.5. Histologic analysis

After sacrifice, liver tissues were fixed in formalin and embedded in paraffin. Specimens were cut to 5 μm thickness and stained with hematoxylin and eosin (H&E). In addition, fresh liver tissues were frozen in OCT Embedding Matrix (Cellpath Ltd., Powys, UK) and cut at 5 μm thickness. Sections were sequentially stained with Oil Red O solution and hematoxylin for counter staining. Liver histological analysis was performed by a single pathologist, who was blinded to the experimental details, using light microscopy (x400 magnification).

2.6. RNA isolation and RT-PCR analysis

Total RNA was isolated from cultured cells and liver tissue using Trizol reagent (Invitrogen, Carlsbad, CA, USA). RNA was extracted by adding 200 μl chloroform into mixture of 1 ml Trizol and cells. RNA was precipitated after mixing in an equal amount of isopropanol and washed twice on ice. Finally, total RNA was dissolved in RNase free water and stored −80 °C. cDNA was synthesized from 1 μg of total RNA. The