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Palmitate induces apoptosis in mouse aortic endothelial cells and endothelialdysfunction in mice fed high-calorie and high-cholesterol diets

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ABSTRACT

Aims: Obesity is associated with hypertriglyceridemia and elevated circulating free fatty acids (FFA), resulting in endothelial dysfunction. Endoplasmic reticulum (ER) stress has been implicated in many of these processes. To determine if ER stress participates in palmitate-induced apoptosis, we investigated the effects of diet-induced obesity and palmitate on mouse aortic endothelial cells (MAEC) in vivo and in vitro.
Main methods: Male C57BL/6 mice were fed standard chow diets (SCD) or high-calorie and high-cholesterol diets (HCD) for 3 months. Insulin resistance was detected, and the serum, including proinflammatory indices and markers of endothelial function, was also analyzed. The ultrastructure and apoptosis of the endothelial cells in the thoracic aorta were observed. The primary MAEC were separated and treated with palmitate at different concentrations or different times respectively to observe any changes in cellular proliferation, intracellular reactive oxygen species (ROS) levels and apoptosis. Finally, the ER stress markers C/EBP homologous protein (CHOP) and glucose-regulated protein 78 (GRP78) were analyzed.
Key findings: HCD-fed obese mice became inflammation-activated and insulin-resistant. Swollen mitochondria, expanded ER and apoptosis in the endothelial cells of the thoracic aorta were observed in HCD-fed mice. Palmitate inhibited cell proliferation, increased production of ROS and induced apoptosis in MAEC. CHOP was overexpressed and shifted into the nucleus (mainly), while the expression of GRP78 was upregulated in the palmitate-treated MAEC.
Significance: Our results indicate that diet-induced obesity results in endothelial dysfunction in vivo, and that oxidative and ER stress may be involved in apoptosis induced by the palmitate in vitro.

Introduction

Due to the excessive consumption of high-calorie and high-cholesterol diets (HCD) and lack of exercise in the global human population, obesity is increasingly prevalent worldwide, in both developing and developed countries. As a result, the metabolic syndrome (MS) associated with cardiovascular diseases and diabetes is becoming increasingly widespread. Although the mechanisms of MS are not fully understood, many studies have revealed that the generation and development of MS are closely correlated with insulin resistance (IR). Recently, studies have found that endothelial dysfunction is involved in the pathogenesis of MS. Since vascular endothelial cells constitute the innermost layer of blood vessels, damage to vascular endothelial cells is usually considered as the initial step of endothelial dysfunction (Nyenwe and Dagogo, 2011).

Excessive triglyceride (TG) accumulation in adipose tissue often activates inflammatory pathways and induces the expression of proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and chemokine monocyte chemoattractant protein-1 (MCP-1) (Guenther, 2011), which can play a central role in the development of obesity and IR (Antuna-Puente et al., 2008).

It is well known that obesity is associated with elevated circulating free fatty acid (FFA) levels which cause IR, endothelial dysfunction and inflammation (Caballero, 2003; Tripathy et al., 2003). Recent studies report that FFAs can increase the expression of lipid metabolites, proinflammatory cytokines and cellular stress (including oxidative and endoplasmic reticulum (ER) stress). The processes above are collectively termed as "lipotoxicity" which occur in many peripheral tissues such as the liver, heart, skeletal muscle and pancreas (Guenther, 2011). There have been several reports describing the direct effect of a high fat diet and its related metabolite such as FFAs and ceramides, on endothelial dysfunction (Symons et al., 2009; Zhang et al., 2012a, b),

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but few reports about the effects of FFAs on ER stress in macrovascular endothelial cells.

Vascular endothelial cells with a well-developed endoplasmic reticulum have a powerful ability for protein synthesis. The glucose-regulated protein-78 (GRP78) and C/EBP homologous protein (CHOP) (also called the growth arrest and DNA damage-inducible gene 153 'GADD153') are key proteins participating in ER stress, which is known to happen in the vascular endothelial cells of patients with hyperhomocysteinemia (Austin et al., 2004). These results suggest that ER stress probably participates in endothelial dysfunction.

ER stress-induced apoptosis is increasingly considered as an important pathogenic factor in a number of widespread devastating diseases, including diabetes, atherosclerosis and renal diseases (Toru and Ozawa, 2010). Elevated plasma saturated fatty acids (rather than unsaturated fatty acids) induce the apoptosis of human coronary artery endothelial cells via the activation of the nuclear factor NF-κB (Staiger et al., 2006), however whether or not FFA-induced apoptosis is mediated by excessive ER stress in vascular endothelial cells is unknown (Zhang et al., 2012a,b). We propose the hypothesis that diet-induced obesity is related to highly circulating FFAs, which may result in the direct damage of endothelial cells and induce apoptosis through excessive oxidative and ER stress.

Wistar and Sprague Dawley rats and C57BL/6 mice are the most frequently used rodent models for mimicking human obesity and IR. Studies report that C57BL/6 mice can develop obesity, IR, diabetes mellitus and hypertriglyceridemia when fed a high-fat diet (Howard et al., 2004; Fraulob et al., 2010). Moreover, high-calorie and high-cholesterol diets have been reported to play roles in development of obesity, IR, diabetes mellitus, hypertriglyceridemia.

To simulate the high-calorie and high-cholesterol diets (HCD) prevalent in humans, we adopted a self-made HCD to feed C57BL/6 mice for 3 months and observed the obesity-induced pathological changes in the endothelium of the thoracic aorta. Saturated fatty acid palmitate (16:0) is the main saturated fatty acid in the HCD, so it was chosen to treat primary mouse aortic endothelial cells (MAEC) to probe the molecular mechanisms of diet-induced obesity, especially ER stress.

Materials and methods

Materials

Palmitate, tunicamycin, N-acetylcysteine and MTT (3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide) were purchased from Sigma Chemical Company (St. Louis, MO). Mouse insulin and ET-1 radio-immunity assay kits were acquired from Beijing North Institute of Biological Technology (China). FFA and NO detection kits came from Nanjing Jiancheng Bioengineering Institute (China). Mouse TNF-α, MCP-1 and IL-6 Quantikine enzyme-linked immunosorbent assay kits were purchased from R&D Systems (Billerica, MA). The In Situ Cell Death Detection Kit (POD) was purchased from Roche Molecular Biochemicals (Mannheim, Germany). The Annexin V-FITC/PI apoptosis detection kit and ROS detection kit were purchased from Nanjing KeyGEN Biotechnology Co. Ltd. (China) and Beyotime Institute of Biotechnology (China), respectively. Antibodies to factor VIII (sc-33584), β-actin (sc-47778), CHOP (L63F7) (#2895) and GRP78 (ab21685) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), Cell Signaling Technology, Inc. (Danvers, MA) and Abcam Inc. (Cambridge, UK), respectively. Goat anti-mouse and FITC-conjugated, goat anti-rabbit secondary antibodies conjugated to horseradish peroxidase were obtained from Beijing Zhong Shan Golden Bridge Biological Technology, Co., Ltd. (China). Other reagent-grade chemicals were obtained from commercial sources.

Construction of diet-induced obesity mice model

All animal procedures were performed in accordance with the principles approved by the Animal Ethics Committee of Anhui Medical University. Male C57BL/6 wide-type mice (at the age of 8 weeks and 21.43 g ± 2.08 g, n = 16) were purchased from the Experimental Animal Center of Anhui Medical University and acclimated for 1 week. The mice were housed in plastic cages (5 mice/cage) at room temperature (22–25 °C) and 35–60% humidity under a 12:12 h light and dark cycle. They were allowed free access to laboratory chow and water.

The mice were randomly divided in two groups, a standard chow diet group (SCD) and a high-calorie and a high-cholesterol group (HCD). The mice were fed either HCD (consisting of 20% lard, 20% sucrose, 10% custard powder, 1% cholesterol and 49% basic forage) or SCD for 3 months.

Glucose tolerance test (GTT) and insulin tolerance test (ITT)

Insulin sensitivity was examined using the glucose tolerance test (GTT) and insulin tolerance test (ITT). For the GTT assays, all mice were fasted overnight and given glucose (2 g/kg BW) by intraperitoneal injection. Blood samples were obtained from the tail veins at 0, 30, 60, 90 and 120 min after injection. The blood glucose was measured with a glucometer (OneTouch Ultra, Johnson& Johnson Medical), and the data were plotted as blood glucose concentration over time. ITT assays were performed by the intraperitoneal injection of neutral insulin (1 U/kg BW) after fasting for 4–6 h. The blood glucose was measured at 0, 30, 45, 60, 90 and 120 min. The data were plotted as blood glucose concentration over time.

Analysis of blood biochemical and inflammatory parameters

The mice were fasted for 12 h and anesthetized by CO_2 inhalation. Blood samples were then collected from the orbital sinus and the serum was separated for biochemical analyses, including glucose, triglyceride (TG), total cholesterol (TC), high density lipoprotein-cholesterol (HDL-C) and low density lipoprotein-cholesterol (LDL-C), by colorimetric enzymatic assays in an automatic analyzer (Olympus AU640). Serum NO and FFA levels were measured according to the kit instructions. Plasma insulin and ET-1 levels were determined by an ultrasensitive mouse insulin radio-immunity assay. Plasma TNF-α, IL-6 and MCP-1 were measured by ELISA assays.

Ultrastructural analysis of thoracic aorta

Thoracic aortas were harvested from the sacrificed mice, incised into small fragments (1 mm^3), immediately fixed in 2.5% glutaric dialdehyde for 6 h at room temperature and post-fixed in 2% osmium tetraoxide for 2 h at 4 °C. After gradual dehydration with ethanol, they were immersed for 4 h at room temperature in an acetone: epoxy resin (1:1) mixture and embedded in epoxy resin Epo812. The ultrathin sections (70 nm) were stained with 2% uranyl acetate and lead citrate, and then observed by transmission electron microscopy (JEM-1230, JEOL, Japan) to search for pathological changes in the mitochondrial and ER in aortic endothelial cells.

Detection of apoptosis of thoracic aorta in situ

Free 3′-OH strand breaks resulting from DNA degradation were detected using the TUNEL technique which was performed according to the manufacturer's instructions. Paraffin sections (5 μm) of the fixed and embedded thoracic aorta were dewaxed according to standard protocols. The tissue sections were incubated for 4 min at 37 °C with a proteinase K working solution, then the slides were rinsed 3 times with PBS, incubated with 3% H_2O_2 for 30 min at 37 °C and rinsed three additional times with PBS. Then the tissue sections were incubated with a diluted TUNEL reaction mixture for 90 min at 37 °C in a dark humidified atmosphere and rinsed three times with PBS. After the area around the sample was dried, 50 ul of Convert-POD were added and the slides were incubated in a humidified chamber for 30 min at 37 °C and