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Oleate protects macrophages from palmitate-induced apoptosis through the downregulation of CD36 expression

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ABSTRACT

In obese patients, free fatty acids ectopically accumulated in non-adipose tissues cause cell death. Saturated fatty acids are more deleterious to non-adipose cells, and supplementation with monounsaturated fatty acids has been proposed to rescue cells from saturated fatty acid-induced cytotoxicity; however, the mechanisms are not well understood. To understand the cytoprotective role of monounsaturated fatty acids in lipotoxic cell death of macrophages, we investigated the antagonizing effect of oleate and the underlying mechanisms in palmitate-treated RAW264.7 cells. Palmitate strongly induced apoptosis in macrophages by increasing CD36 expression, which was identified to mediate both endoplasmic reticulum stress and the generation of reactive oxygen species. Co-treatment with oleate significantly reduced CD36 expression and its downstream signaling pathways of apoptosis in palmitate-treated cells. These findings provide a novel mechanism by which oleate protects macrophages from palmitate-induced lipotoxicity.

1. Introduction

Obesity is associated with many medical complications. Plasma free fatty acids (FFAs) are usually elevated in obesity, resulting in their accumulation in non-adipose tissues, which causes deleterious effects such as cellular dysfunction and cell death (lipotoxicity) [1]. Lipotoxicity is widely accepted as a key factor contributing to the pathogenesis of obesity-related complications such as type 2 diabetes mellitus, metabolic syndrome, and cardiovascular diseases [2], therefore, inhibition of the lipotoxicity-mediated cell death pathways and signaling cascades is an important strategy to prevent and treat obesity-related metabolic complications. Lipotoxic activities are strikingly different according to the type of FFA considered; saturated fatty acids (i.e., palmitate, C16:0) are almost universally toxic, whereas the unsaturated fatty acids (i.e., oleate, C18:1) are either non-toxic or even cytoprotective in many tissues [3–5]. In this regard, the fatty acid composition of dietary fats is a critical factor determining the occurrence of lipotoxic complications [6].

Macrophages are the main players regulating the local immune response. Like other immune cells, macrophages are constantly exposed to environmental challenges and must rapidly change their physiology accordingly to maintain homeostasis. FFAs or their derivatives are principal signals for macrophages in metabolic tissues, and their roles in the modulation of the functions and survival of macrophages have been demonstrated [7,8].

CD36 is a multifunctional membrane glycoprotein receptor that is present on various cell types, including macrophages, platelets, endothelial cells, cardiomyocytes, and adipocytes [9]. Upon binding to an extracellular ligand, CD36 exerts diverse cellular functions in a cell-specific manner. It is well known that CD36 mediates macrophage foam cell formation which promotes atherosclerosis. In addition, CD36 is implicated in some of the mechanisms attributed to the apoptosis of macrophages, such as endoplasmic reticulum (ER) stress, oxidative stress, and ceramide synthesis [10,11]. However, the roles of CD36 in the fatty acid-mediated regulation of apoptosis in macrophages are still not fully elucidated.

Therefore, in this study, we investigated the antagonizing effect of oleate on palmitate-induced cytotoxicity in RAW264.7 cells. We also examined the underlying mechanisms involved in the regulation of apoptosis by oleate and palmitate, focusing on the role of CD36. Our study provides new insights into lipotoxicity in the pathophysiology of obesity-related metabolic complications.

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2. Materials and methods

2.1. Cell culture

Murine macrophage RAW264.7 cells (American Type Culture Collection, Manassas, VA, USA) were maintained in Dulbecco's Modified Eagle's medium (Gibco, Green Island, NY, USA) containing 20 mM HEPES (Sigma, St Louis, MO, USA), 10% fetal bovine serum (Gibco), and gentamycin (100 μg/ml, Gibco) in a humidified 37 °C, 5% CO_2 tissue culture incubator.

2.2. Cell treatment and viability test

Sodium palmitate and sodium oleate (Sigma) were dissolved to a final concentration of 50 mM in 0.1 N NaOH/70% ethanol and in ethanol, respectively, and added directly to growth media. For dose-dependent experiments, the cells were treated for 24 h with palmitate (100–500 μM) or oleate (12.5–100 μM) as indicated, and for time-dependent experiments, 500 μM of palmitate and 100 μM of oleate were used. In some experiments, chemical inhibitors such as CD36 inhibitor sulfosuccinimidyl oleate (SSO, 100 μM) (Cayman, Ann Abor, MI, USA), ER stress inhibitor 4-phenylbutyric acid (4-PBA, 4 mM) (Sigma) or a reactive oxygen species (ROS) inhibitor *N*-acetyl-L-cysteine (NAC, 10 mM) (Sigma) were co-treated. The cell viability was determined by cell number counting using trypan blue exclusion method.

2.3. Detection of apoptosis

Immunoblot analysis of cleaved form of PARP-1 (c-PARP) and TUNEL assay were used for detection of apoptosis. For immunoblot analysis, total protein was extracted and electrophoresed on SDS-PAGE gels and then transferred to a PVDF (Merck Milipore, Billerica, MA, USA). After blocking for 1 h in 5% non-fat skim milk, the membrane was incubated overnight at 4 °C with rabbit monoclonal antibodies against c-PARP (1:5,000, Abcam, Cambridge, UK) and β-actin (1:10,000, Sigma), and further incubated for 1 h with horseradish peroxidase-conjugated anti-IgG (Sigma). The immunoreactive bands were visualized by the ECL method (Amersham Pharmacia, Buckinghamshire, UK), and band intensities were quantitated with Quantity One (Bio-Rad Laboratories, Hercules, CA, USA).

TUNEL assay was performed using In Situ Cell Death Kit (Roche, Manheim, Germany). Briefly, the cells were cultured on 12 mm coverslip, fixed for 10 min with 10% formalin, and incubated 20 min with TdT labeling buffer and further for 1 h with biotin-labeled dUTP. After washing three times with terminating buffer, they were incubated for 2 h with Cy3-conjugated streptavidin (Jackson immune research, West Grove, PA, USA). DAPI (Abcam) was used to counterstain the nuclei. Apoptotic cells were identified using a confocal microscope (LSM800, Carl Zeiss, Oberkochen, Germany) at 1,000× magnification. Cy3 fluorescence was measured (excitation, 550 nm; emission, 570 nm) and analyzed using ZEN 2012 software (Carl Zeiss).

2.4. Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from the cultured macrophages using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) and cDNA was synthesized with PrimeScript™ RT kit (Takara, Shiga, Japan). qRT-PCR was performed on a ABI 7300 thermal cycler (Applied Biosystems, Foster City, CA, USA) with SYBR Green Q-PCR Master Mix (Takara), and the relative amount of mRNAs was calculated using the ddCt method with the *36B4* mRNA as an internal control. The primers used were listed in Suppl. Table 1.

2.5. Measurement of cytosolic ROS generation

Cells were cultured on 12 mm coverslip and loaded with 10 μM 2′,7′-dichlorofluorescin diacetate (Sigma) for 30 min at 37 °C. After washing excess dye, fluorescence intensity was measured using a fluorescence microscope (Axioimager M1, Carl Zeiss) at 200× magnification (excitation, 490 nm; emission, 535 nm), and analyzed using ZEN 2009 software (Carl Zeiss) and ImageJ (NIH, Bethesda, MD, USA).

2.6. Statistical analysis

All values are expressed as the mean ± s.e.m. from at least three independent experiments. The data were analyzed using Student's *t*-test or one-way analysis of variance followed by Tukey's multiple comparison test. Statistical analyses were performed using GraphPad Prism 5 for Windows (GraphPad Software, La Jolla, CA, USA). P value less then 0.05 was considered significant.

3. Results and discussion

3.1. Reciprocal effects of palmitate and oleate on the apoptosis of RAW264.7 cells

When we treated the cells with an increasing concentration of palmitate for 24 h, cell viability was significantly decreased as of 200 μM, and approximately 50% cell death was observed at 500 μM (Fig. 1A, *left*). In the time-dependent study, palmitate (500 μM) significantly suppressed cell proliferation as of 6 h after the treatment (Fig. 1A, *right*). Although necrosis is known as the main mechanism for palmitate-induced cytotoxicity in macrophages [12], many reports have indicated that palmitate also induces apoptosis in many cell types [5,13,14]. In this regard, we evaluated the involvement of apoptosis in palmitate-induced cytotoxicity by determining the expression of c-PARP, a hallmark of early-stage apoptosis [15]. As shown in Fig. 1B, the c-PARP level was dose-dependently increased as of a palmitate concentration of 200 μM, the same minimum concentration at which palmitate-induced cytotoxicity was observed (Fig. 1A, *left*), and began to increase from 6 h and reached a peak at 12 h after palmitate treatment (500 μM).

Accumulating evidence suggests a protective function of oleate by counteracting the cytotoxic effect of palmitate [13]. However, the effects of palmitate and oleate on the cell survival of macrophages have been controversial. For example, oleate, but not palmitate, was shown to increase the apoptosis of macrophages [14], and palmitate prevented the survival of osteoclasts, a macrophage-related cell type [16]. In our experiment, oleate itself only slightly increased the viability of RAW264.7 cells even at 500 μM (Suppl. Fig. 1). However, co-treatment of oleate with palmitate (500 μM) significantly increased cell survival rates in a dose-dependent manner (Fig. 1C, *left*); cell viability was increased by 15% and 53% with treatment of 12.5 μM and 100 μM of oleate, respectively. Oleate treatment could not fully recover the viability even at 100 μM (82% of the control), indicating that the signaling pathways leading to cytotoxicity may be partially shared between the two fatty acids. When the cells were treated with oleate at 100 μM, the cytotoxicity induced by palmitate (500 μM) was significantly reversed as of 12 h (Fig. 1C, *right*). From these findings, we chose a concentration of 100 μM of oleate for the subsequent experiments. Oleate also reduced c-PARP expression dose-dependently; suppressive effect was obvious from 12.5 μM and c-PARP was completely disappeared as of 50 μM (Fig. 1D, *left*). In the time-dependent experiment, this reduction was observed from 6 h after the treatment with 100 μM oleate (Fig. 1D, *right*). Taken

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