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Fabrication of size-controlled linoleic acid particles and evaluation of their in-vitro lipotoxicity

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ABSTRACT

The biological activities of fatty acids (FAs) can differ with size even for lipids of similar compositions. The aim of this study was to develop size-controlled FA particles and to evaluate their toxicity as a function of size. Well-stabilized nano- and microscale linoleic acid (LA) were fabricated based on specific physical factors. Then, resulting LAs were characterized by size distribution, surface charge, assembly structure, composition, and serum effects. The sizes of the nano- (LA_{nano}) and microscale (LA_{micro}) LAs, determined by electron microscopy, were 109 nm and 12 μm, respectively. LA_{nano}, a multilamellar structure as determined by cryo-electron microscopy, was rapidly internalized into cells via free fatty acid receptor 3. After internalization, LA_{nano}, but not LA_{micro}, induced nuclear translocation of fatty acid binding protein 4 (FABP4). Translocation of FABP4 into the nucleus then induced expression of the FA metabolism-related genes InsR and AdipoR1. Their expression was significantly increased in the presence of only LA_{nano}. Cytotoxicity was also significantly increased in cells treated with LA_{nano}, but not LA_{micro}, as indicated by the endoplasmic reticulum stress markers CHOP and GRP78. Therefore, our results demonstrated that FAs with the same composition but varying in size can cause different cellular responses.

1. Introduction

Fatty acids (FAs), important sources of energy, are primarily stored as triacylglycerols in adipose tissue. When required by other tissues, certain FAs are released into the bloodstream and their transport is hindered in the aqueous environment because of their extremely low solubility. Thus, transport of FAs is facilitated by their high-affinity binding to albumin (Pavicevic et al., 2016). FAs can then be circulated as FA/albumin complexes, with ratios of FA to albumin determined by environmental metabolic conditions, dependent on the accessibility of albumin. Under certain pathological conditions, the FA/albumin ratios become imbalanced, altering the levels of FAs and potentially leading to atherosclerosis (Michaud and Renier, 2001), diabetes mellitus (Anguizola et al., 2016), or liver disease (Scorletti and Byrne, 2013).

However, the cellular mechanisms of the effects of FAs still remain controversial. Linoleic acid (LAs, C18:2) is an essential polyunsaturated acid (omega 6) and generally known to suppress proinflammatory responses in macrophages (Ishiyama et al., 2011; Zhao et al., 2005) and liver cells (Wei et al., 2009). However, Zhang et al. (Zhang et al., 2012). demonstrated that LAs induced significantly more apoptosis than saturated FAs, such as palmitic acid, by increasing endoplasmic reticulum (ER) stress. Additionally, Wei et al. (Wei et al., 2007). reported that LAs increased ER stress in liver cells. In smooth muscle cells, it was reported that LAs induced apoptosis by activation of caspases (Artwohl et al., 2009). Indeed, it is difficult to control the size of FA/albumin complexes because of self-assembly of hydrophobic chains in cell culture medium that includes serum. In *in vivo* environments, a broad spectrum of FA/albumin ratios can give rise to various sized FA/albumin complexes, leading to physiological responses through their ensemble effects. However, little is known about the dependence of cellular responses on FA size because of practical difficulties in synthesizing size-controlled free FAs.

The aim of this work was to develop a method to fabricate size-

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controlled linoleic acid (LA) particles and to systemically investigate the differential size effects of LAs on cellular uptake, intracellular trafficking and toxicity as well as the underlying molecular mechanisms associated with these LA effects.

2. Material and methods

2.1. Synthesis of size-controlled LA particles

The LA particles were fabricated based on the principle of phase transition. The details of the synthesis method are written in Supplementary Information and crucial experimental parameters are explained in Results. Briefly, linoleic acid (C18:2, LA) was purchased from Sigma-Aldrich (St. Louis, MO, USA, #L1012). The initial concentrations of LAs were 0.25 M, 0.175 M, and 0.1 M in ethanol for micro-, intermediate-, and nanoscale LA particles, respectively. These three initial solutions were transferred to aqueous environments, distilled (DI) water, cell culture medium (Dulbecco's Modified Eagle's Medium, DMEM, GibcoBRL, Gaithersburg, MD, USA), or DMEM supplemented with 10% fetal bovine serum (FBS, GibcoBRL, Gaithersburg, MD, USA), with exposure to controlled dispersion pressures. The dispersion pressures were determined by the inner diameters of the syringe needles. The concentrations of LA solutions were quantified by a commercially available fatty acid assay (#ab65341; Abcam, Cambridge, Massachusetts).

2.2. Characterization of size-controlled LA particles

2.2.1. Size of LA particles

The hydrodynamic sizes of LA particles were measured by dynamic light scattering (ELS-Z; Photal Otsuka Electronics, Japan). To image the particles using a field-emission scanning electron microscope (Hitachi S4800), the LA solutions (in DMEM with 10% FBS) were dialyzed with DI water for 24 h using dialysis tubing (Pierce Biotechnology, Rockford, IL, USA) with a 3500-kDa MW cutoff. The dialyzed solutions were dropped onto a Si wafer, dried, and then coated with Pt.

2.2.2. Surface charge of LA particles

The zeta potential was measured by a Zetasizer Nano ZS instrument (Malvern Instruments, Southborough, MA, USA). Eight different results were statistically averaged and these averages are reported. LA particles in DMEM containing 10% FBS (DMEM+10% FBS) were centrifuged at 14,000 rpm for 30 min and the solutions were exchanged from DMEM+10% FBS to DI water or PBS (pH 7.4). The zeta potential was then measured.

2.2.3. Inner structure of LA particles

Cryo-electron microscopy was used to visualize the inner structure of the assembled LA_{nano} particles in DMEM+10% FBS. A total concentration of 1 mM LA_{nano} in DMEM+10% FBS was collected by centrifugation at 14,000 rpm for 20 min and resuspended in DI water. The final LA_{nano} concentration was 100 mM. The vitrified samples were observed in a bio−transmission electron microscope (Bio-TEM, Tecnai G2 Spirit, FEI, Netherlands, located at Korea Basic Science Institute, Daejeon, South Korea).

2.2.4. Surface composition of LA particles

To examine the proteins adhering to the surface of LA particles when exposed to DMEM+10% FBS, LA particles were harvested by centrifugation at 14,000 rpm for 20 min, lysed, and subjected to SDS-PAGE. The amounts of precipitated LA and protein were measured by a FA quantification kit and Bradford assay, respectively, and the ratio of LA to protein was approximately 467:1 (w:w). Coomassie brilliant blue staining showed that >95% of precipitated protein was BSA, a band running at 65 kDa.

2.3. Cell culture experiment

For incubation of LA particles with murine macrophage cells (RAW264.7, ATCC® TIB-71™), synthesized LA particles in DMEM+10% FBS were measured for FA content with a FA quantification kit (#ab65341; Abcam, Cambridge, Massachusetts). The uptake of nano-sized LA particles into RAW264.7 cells on passage 8 through 12 was visualized on the Bio-TEM. To confirm whether acute cell death was caused by endotoxins produced during LA particle synthesis, the cell culture medium ($n = 3$) was tested with an LAL Endotoxin Quantification kit (#88282, Pierce Biotechnology) according to the manufacturer's protocol. After treating RAW264.7 cells with previously synthesized LA particles (LA_{micro}, $LA_{intermediate}$, and LA_{nano}) for 12 h, the cell culture medium was harvested and analyzed in the endotoxin test.

2.4. Label-free lipid imaging using coherent anti-stokes raman scattering (CARS) microscopy imaging

LAs that had accumulated in cells were imaged by CARS microscopy. Recently, CARS has been extensively applied for biological applications (Hellerer et al., 2007; Kim, Lee, Lee, Lee, Lee, Park, et al., 2010; Pezacki et al., 2011). In one such application, lipids can be clearly imaged by CARS based on their molecular vibrations. We previously described our use of the CARS microscope (S. H. Kim et al., 2010) and more detailed information is provided in the Supplementary Information. Because CARS imaging enables label-free detection, the accumulated LAs in cells could be directly imaged from the culture dish with no staining necessary.

2.5. Immunocytochemistry

To examine localization of FABP4 after cellular uptake of LAs of various sizes, RAW264.7 cells (2×10^5 cells per well) were seeded in a 2-well Lab-tek II chambered coverglass (Nalge Nunc International, Rochester, NY, USA). After 24 h, 0.25 mM LA particles (LA_{micro}, $LA_{intermediate}$, and LA_{nano}) were incubated with cells for an additional 12 h in a 5% CO_2 equilibrated 37 °C incubator. The cells were then fixed with 4% paraformaldehyde for 10 min and permeabilized for 1 h at room temperature with PBS containing 0.1% BSA and 0.25% Triton X-100. An anti-FABP4 (1:200 of 0.1% BSA; Abcam) primary antibody was incubated with the cells at 4 °C overnight. Cells were then washed with PBS and incubated with an Alexa594 conjugated-secondary antibody (1:200; Molecular Probes, Eugene, OR, USA) for 1 h at room temperature. After washing in PBS with 0.1% BSA, cells were stained by incubating with BODIPY493/503 (1 μg/mL; Molecular Probes) and Hoechst33342 (10 μg/mL; Molecular Probes) dyes for 30 and 5 min, respectively. Images were acquired with a confocal laser scanning microscope (BX81; Olympus, Japan). In live cell imaging for late endosomes, cells were incubated with Late Endosomes-GFP, BacMam2.0 reagent (Molecular Probes, #C10588) for 16 h before treatment with LA particles.

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

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The total amount of RNA used for qRT-PCR was 1 μg. The mRNA levels of 10 genes associated with lipid metabolism (PPARα, PPARγ, PI3K, DGAT, insulin receptor (InsR), adiponectin R1 (AdipoR1), perilipin A, LCAT, ABCA1, and lipin1), six related to FA uptake (FFAR1-4, GPR132, CD36/FAT) and an internal control (GAPDH) were detected with a one-step SYBR-green qRT-PCR kit