MATERIALS AND METHODS

Reagents

Palmitic acid (HA) was purchased from NuChek Prep. 2-ClHA, 2-chloro-[d_4-7,7,8,8]-hexadecanoic acid (2-Cl-[d_4]HA), and 2-chloro-[d_4-7,7,8,8]-hexadecanol (2-Cl-[d_4]HOH) were synthesized as previously described [1]. Other reagents, unless specified, were purchased from Sigma-Aldrich (Saint Louis, MO).

Primary human monocytes isolation and activation

Human monocytes were isolated from peripheral blood as described previously [2]. In brief, anticoagulated blood was diluted with Ca^{2+}/Mg^{2+}-free HBSS and subjected to Ficoll density gradient centrifugation (density, 1.077 g/ml; 400 ×g for 45 min). The middle layer of cells containing monocytes were collected and diluted 1:1 with Ca^{2+}/Mg^{2+}-free HBSS, which was then subjected to further Ficoll density gradient centrifugation (density, 1.070 g/ml; 400 ×g for 15 min). The mid-floating layer was plated on 60-mm culture dishes in Medium 199 and cultured at 37 °C for 1 h in the presence of 5% CO2. Before the experiments, monocytes were washed three times in fresh HBSS. These cultured human monocytes were found to be void of granulocytes as determined by differential staining. Monocytes were then incubated in 2 ml of HBSS containing Mg^{2+} and Ca^{2+} at 37°C for 30 min or 1h in the presence and absence of phorbol myristate acetate (PMA) (300 nM). At indicated time points, cells were scraped into 1.6 ml of ice-cold saline on ice and stocked at -80°C until lipid extraction.

Lipid extraction and analysis

Cellular lipids were extracted by conventional liquid-liquid extraction [3, 4]. For each sample, 20 pmol of 2-Cl-[d_4]HOH and 2-Cl-[d_4]HA were added as internal standards for 2-ClHOH and 2-ClHA quantification, respectively. The final lipid extract was resuspended in 1 ml of chloroform, and then 0.1 ml and 0.4 ml sample were taken for α-ClFA and α-ClFOH analysis, respectively.

For free α-ClFA analysis, the samples in chloroform were dried under nitrogen and resuspended in 200µl of methanol/water (85/15, v/v) containing 0.1% formic acid. After vigorous vortexing, the samples were transferred to an autosampler tube with an insert for analysis by liquid chromatography-mass spectrometry (MS) following previously described methods [3, 5]. Both cellular α-ClFA and α-ClFOH were expressed in µM based on the conversion of 0.334µl cell volume in 10^6 monocytes [6].

α-ClFOH was first derivatized to its pentafluorobenzoyl ester before quantitation by gas chromatography-MS. The samples in chloroform were dried under nitrogen and 50µl of pentafluorobenzyl chloride was added. Then, the samples were capped under nitrogen and incubated at 60°C for 45 min. After incubation, the reaction was cooled down to room temperature for 10 min, centrifuged at 400 ×g for 2 min, and dried under nitrogen. The remaining derivatized products were washed twice with 2ml of hexane, resuspended in 300µl of hexane and transferred to the autosampler vial insert. After solvent was evaporated under nitrogen, the samples were resupended in 100µl of ethyl acetate before being subjected to gas chromatography-MS analyses as previously described [3].

Cell culture

Isolated primary human monocytes were washed three times in fresh HBSS and cultured in RPMI 1640 containing 10% fetal bovine serum (FBS) overnight for apoptosis assay. Before the experiments, cells were washed with the medium once and then treated with indicated concentrations of hexadecanoic acid (HA) or 2-ClHA for 3h in RPMI 1640 containing 2% FBS. Human monocytic THP-1 cells were grown in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% FBS. For experiments, THP-1 cells were seeded in 35-mm dishes with a density of 7.5×10^5 cells/mL and cultured in 2% FBS-containing fresh medium. Mouse macrophage RAW 264.7 cells were cultured in DMEM medium (Sigma-Aldrich) containing 10% FBS. Before experiments, fresh medium containing 2% FBS was added.

Cell apoptosis analysis

For THP-1 monocytes, cell apoptosis was measured by the Alexa Fluor® 488 annexin V/Dead Cell Apoptosis Kit (V13245, Invitrogen Inc.). Cells were harvested and washed twice with cold PBS. Cell pellets were resuspended in 1x Annexin V-binding buffer containing Annexin V and propidium iodide (PI). After incubation at room temperature in the dark for 15 min, the samples were diluted to 500 µL with 1 x annexin-binding buffer, and analyzed by flow cytometry measuring the fluorescence emission at 530 nm and 575 nm using 488 nm excitation.

For primary human monocytes and RAW 264.7 cells, apoptotic cells were determined by an *in situ* cell death detection kit from Roche. Cells were washed once with PBS and then fixed in 4% paraformaldehyde for 1h. After washing with PBS, the samples were permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 2 min and labeled with terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) reaction mixture as well as 4',6'-diamidino-2-phenylindole. Finally, the samples were washed with PBS twice and evaluated by fluorescence microcopy.

Caspase 3 activity assay

Caspase 3 activity in cell lysates was measured by a fluorometric EnzChek® Caspase 3 Assay Kit (Cat. E13183) from Invitrogen Inc. Briefly, cells were washed in cold PBS twice and then resuspended in 1x cell lysis buffer for protein extraction. The protein concentration of the samples was determined using a Bio-Rad protein assay kit. 50 µl of samples and 50 µl of 2x reaction buffer containing 200 µM of substrate N-Benzoyl-DEVD-amino-4-methylcoumarin were added in a black 96-well plate with flat bottom (FALCON Inc.) and incubated at 37°C in a plate reader (BIO TEK Inc.) for monitoring the fluorescence using 342/441 nm excitation/emission wavelengths at multiple time points up to 1h. The rate of fluorescence increase indicates the caspase 3 activity in each sample. Finally, the caspase 3 activity of the fluorescence increase was normalized to the sample protein content.

Western blots

Cells were washed once with cold PBS and lysed in RIPA buffer (Pierce, Lot. 89900) containing protease inhibitor cocktail (Roche). After incubation for 15 minutes on ice, the samples were centrifuged at 14,000g for 15 min at 4°C, and the supernatant was collected as whole cell lysate and stored at -20°C until use. Equivalent amounts of protein were separated

on SDS-PAGE and transferred to polyvinylidene fluoride membranes. The following primary antibodies were used: cleaved poly (ADP-ribose) polymerase (PARP, Cat.556362, BD Pharmingen), CHOP (sc-7351, Santa Cruz), GRP78 (sc-13968), ATF4 (sc-200), eIF2α (Cat.5324, Cell Signaling) and phospho-eIF2α (Cat.9721, Cell Signaling). The antibody of β-actin (A3854, Sigma) was used as the loading control.

RNA isolation and analysis

Total RNA was isolated using TRIZOL reagent (Invitrogen Inc). After DNase treatment, 1 μg of RNA was reverse transcribed to cDNA using Taqman First Strand Synthesis kit (Invitrogen) with oligo-dT priming. Quantitative realtime PCR (qRT-PCR) was performed in triplicate in a final volume of 10 μl using SYBR green PCR master mix (Applied Biosystems) on Roche 480 lightcycle instrument with the following amplification conditions: 95°C for 10 min, 45 cycles at 95°C for 15s and 60°C for 30s. At the end of each run, a melting curve analysis from 55°C to 90°C was performed to ensure the absence of non-specific products and primer dimers. Glyceraldehyde phosphate dehydrogenase (GAPDH) and β-actin were used as control genes for human and mouse gene expression, respectively, with the following conditions: 95°C for 5 min, 32 cycles at 94°C for 30s, 53°C for 30s and 72°C for 30s and a final extension step at 72°C for 5 min.

For detection of Xbp-1 mRNA splicing, PCR amplification of cDNA prepared as described above was performed using REDTaq ReadyMix PCR Reaction Mix (Sigma) with the following conditions: 95°C for 5 min, 32 cycles at 94°C for 30s, 53°C for 30s and 72°C for 30s and a final extension step at 72°C for 5 min. The PCR products were separated on 2% agarose (Invitrogen) gel and visualized with ethidium bromide staining. The information of all the primers is provided in Table SI.

Hydrogen peroxide determination

Hydrogen peroxide (H_2O_2) in cell culture medium was determined using Amplex Red (90101, Sigma). Cell culture media was collected and centrifuged at 5000 rpm to remove cell debris. A total reaction volume of 100 μL containing 50 μL of media, 50 μM of Amplex Red, and 0.01 mU of horseradish peroxidase (HRP, Sigma) was added in a black 96-well plate with flat bottom. Fluorescence was monitored for up to 30 min using 530/590 nm excitation/emission wavelengths. The relative H_2O_2 levels in samples were evaluated by determining the rate of fluorescence increase.

Transient transfections

Negative control siRNA (sc-36868) and human CHOP siRNA (sc-35437) were transfected to THP-1 monocytes using transfection reagent (sc-39528) from Santa Cruz Inc. according to the kit instructions. Cells were cultured in media with the siRNA present for 6 h, and the transfection efficiency was subsequently estimated by observing the fluorescence under microscopy since the control siRNA is fluorescein isothiocyanate-conjugated. Next, the siRNA was removed by replacing with fresh medium containing dimethylsulfoxide or 2-ClHA for another 18 h.

Statistical analysis

All data are presented as means ± std. dev. One-way ANOVA followed by Dunnett post-hoc test was used to determine statistical differences between control and experimental groups. $P<0.05$ was considered statistically significant.

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