RESEARCH DESIGN AND METHODS

Reagents

The PPARγ antagonists, T0070907 and bisphenol A diaglycidyl ether (BADGE), were obtained from BioMol International. Rosiglitazone was purchased from Calbiochem. Various FFAs: laurate (12:0), myristate (14:0), palmitate (16:0), stearate (18:0), oleate (18:1), linoleate (LA; 18:2), arachidonate (AA; C20:4), eicosapentaenoate (EPA; 20:5) and docosahexaenoate (DHA; 22:6) were purchased from Sigma. N-acetyl cysteine (NAC), catalase and superoxide dismutase (SOD) also were from Sigma.

Preparation of fatty acid–albumin complexes

FFAs were prepared by conjugation with albumin, as described previously (1). We have found that 3T3-L1 cells, which have high levels of TLR4 expression, are very sensitive to endotoxin. Thus, it is necessary to pass the albumin over a polymyxin B column (Sigma) to remove the small amount of endotoxin present in commercially available sources of fatty acid free albumin (FAFA, Sigma). FFAs were first dissolved in NaOH at 100 mM and conjugated with FAFA at a molar ratio of 3:1 (FFA/albumin). Control solution containing NaOH and FAFA was similarly prepared. All unsaturated FFAs were freshly prepared to prevent oxidation. All reagents used were tested for endotoxin level using the *Limulus* amebocyte lysate (LAL) test kit (Sigma).

Cell lipid content and hypertrophy

Total lipids were extracted from 3T3-L1 adipocytes exposed to 5 or 25mM glucose or/and various FFAs using the method of Folch (2) . The cellular content of triglycerides was measured using a colorimetric kit (Roche). To assess intracellular neutral lipid content and hypertrophy of adipocytes, the cells were stained with HCS LipidTOX (Invitrogen) and analyzed by measuring the lipid droplet area using the ImagePro Plus program (MediaCybernetics) and hemacytometer as a calibrator.

Multiplex real-time quantitative reverse-transcription polymerase chain reaction

Real-time reverse-transcription polymerase chain reaction (RT-PCR) was performed using the TaqMan Master kit (Applied Biosystems) in the Stratagene MX3000P system (3). Adiponectin, SAA1, SAA3, MCP-1, hyaluronan synthase 2 (HAS2), 18S rRNA, β-actin primers and a FAM probe were obtained from Applied Biosystems (Assay-on-Demand). Primers and TaqMan probes specific for GAPDH are as follows: for GAPDH, forward primer, 5'AGCCTCGTCCCGTAGACAAA3'; reverse primer, 5'ACCAGGCGCCCAATACG3'; probe, HEX-5'AAATCCGTTCACACCGACCTTCACCA3'-BHQ1. For normalization, 18S rRNA, β-actin or GAPDH were used as endogenous controls. There were no significant differences between each treatment in these endogenous controls. Therefore, GAPDH was used routinely. Each sample was analyzed in triplicate and presented in multiplex reactions using GAPDH as control.

Hyaluronan enzyme-linked immunosorbent assay

HA content was measured in cell extracts from 3T3-L1 adipocytes (4). In brief, samples were digested with pronase (500 μg/ml, Sigma) in 0.5 M Tris pH 6.5 at 37°C overnight. Following digestion, the pronase was inactivated by heating to 100°C for 20 min. We used a modification of a competitive ELISA in which the samples were first mixed with biotinylated HA binding protein isolated from bovine cartilage and then added to human umbilical cord HA coated microtiter plates, the final signal being inversely proportional to the level of HA added to the biotinlyated HA binding protein. The resulting absorbances were measured at 405/570 nm on a microplate reader (Molecular Devices) using SOFTmax PRO (version 4.3) software (Molecular Devices).

Immunohistochemistry

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3T3-L1 adipocytes grown on coverslips were incubated for 7 days in 5 or 25 mM glucose with/without 250 μM FFAs, fixed with 4% paraformaldehyde for 5 min, and permeabilized with 0.4% Triton X-100for 15 min. The specimens were blocked with 3% (w/v) BSA in PBS for 1 h and incubated with rabbit polyclonal antibody against the p65 subunit of NFκB (Santa Cruz Biotechnology) in 1% BSA at 4 ° overnight. The specimens were then washed with PBS and incubated with biotinylated goat anti-rabbit IgG (Jackson Immunoresearch Laboratory) for 1 h. After washing twice with PBS, the specimens were incubated with FITC-conjugated streptavidin (Jackson Immunoresearch Laboratory) for 1 h and then washed twice with PBS. Coverslips with the stained cells were mounted in 80% glycerol in PBS and photographed using a fluorescent microscope (Nikon Eclipse 80i).

FACS analysis for cell death

Since certain FFAs can elicit apoptosis (5) , we also measured cell death in adipocytes cultured in 5 or 25 mM glucose with or without FFAs. Characterization of cell death was carried out after propidium iodide (PI) and Alex Fluro 488-labeled annexin V staining followed by flow cytometric analysis (FASCanto, Becton Dickinson) according to the manufacturer's instruction (Vybrant apoptosis assay kit, Invitrogen). H_2O_2 was used as a positive control. Dead cells show PI and/or annexin V staining fluorescence, and live cells show a little or no fluorescence

Monocyte adhesion assay

Differentiated 3T3 L-1 adipocytes were cultured for 7 days in 5 mM or 25 mM glucose concentrations with/without 250 μM FFAs. To examine how many monocytes attached to the cultured adipocytes, U937 cells (10^6 cells /ml) were fluorescently labeled by incubation with calcein-AM (5 μg/ml, Molecular Probes) for 30-45 min, and washed twice in phenol red-free RPMI medium (6). Suspended U937 cell were then added to the 3T3-L1 adipocyte monolayers and allowed to adhere for 90 min at 4 °C. Plates were washed gently three times and visualized to ensure monolayer integrity, before measuring the plate in a FusionTM Series Universal Microplate Analyser (Packard Bioscience) with excitation and detection wavelengths of 485 and 535 nm, respectively.

Monocyte chemotaxis assay

The chemotactic activity of the conditioned media from 3T3-L1 adipocytes was studied in a 96-well microchamber (ChemoTx, Neuro Probe) as described previously (7). To collect the conditioned media from 3T3-L1 adipocytes, cells were grown in 5 or 25 mM glucose with or without 250 μM FFAs for 7 days, washed extensively with PBS, and incubated with serum free media for 24 h. To confirm that exogenous FFAs were not carried over into the conditioned medium, FFA levels were measured in the conditioned media by a NEFA kit (Wako) and shown to be undetectable. THP-1 monocyte cells were fluorescently labeled with calcein-AM. Conditioned media from adipocytes or control PBS containing 0.1% endotoxin-free BSA, were placed in the lower wells of a chemotactic chamber (Neuro Probe). THP-1 cells (5 x 10^6/ml) were loaded in the upper wells. The lower and upper wells were separated by nitrocellulose filters with an 8 μm pore size (Neuro Probe). The chamber was incubated for 90 min at 37°C in humidified air with 5% CO_2 and then placed in a multi-well fluorescent plate reader in a FusionTM Series Universal Microplate Analyser (Packard Bioscience) with excitation and detection wavelengths of 485 and 535 nm, respectively. Total migration was defined as the sum of random migration and directed migration, i.e. chemokinesis and chemotaxis, respectively. Chemokinesis was determined after adding the same concentration of chemoattractant into the chamber wells on both sides of the filter. Chemotaxis was determined by subtracting the chemokinesis value from the total migration. The results

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were expressed as a chemotaxis index, which is a ratio of cell migration in response to chemoattractants versus medium control. All assays were performed in triplicate.

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