Supplement Material

Supplemental Materials and Methods

Materials: Triacsin C was purchased from Biomol (Plymouth Meeting, PA). An apoptosis sampler kit (Cell Signaling, Danvers, MA) was used to detect caspase 3 and Poly (ADP-ribose) polymerase (PARP). Antibody to β-actin was purchased from Cell Signaling (Danvers, MA). Fatty acids were obtained from Nu-chek Prep (Elysian, MN).

Macrophage collection: Mouse peritoneal macrophages (MPMs) were collected from female C57BL/6 mice 3 days after peritoneal injection of 3% thioglycollate medium. Cells were washed and plated using Dulbecco's Modified Eagle's Medium (DMEM) containing 10% FBS. After incubation at 37°C for 4 h, non-adherent cells were removed and adherent cells were incubated for an additional 48 h at 37°C before treatment.

VLDL treatment: Plasma VLDL (d<1.019 g/ml) for these studies was isolated from fasted human donors by ultracentrifugation as described previously.[1] After centrifugation, the lipoprotein fraction was filtered and endotoxin content in the lipoprotein preparations was tested using the LAL assay (Cambrex, East Rutherford, NJ). All samples were found to contain <10 pg endotoxin /mg protein. Protein concentration was determined using a modified Lowry assay, and MPMs were treated with 100 μg/ml VLDL for 6 h as we reported previously. [2]

Oil Red O Staining: MPMs were plated in 8-well chamber slides and pretreated with triacsin C (5 μM) for 30 min followed by VLDL treatment for 6 h. The cells were washed and fixed with 4% paraformaldehyde, followed by staining with Oil Red O for 4 h. The cells were counterstained with Hematoxylin and Eosin.

Gas chromatography: MPMs were plated at 3 x 10^6 cells per well of 6-well plates and treated with either VLDL or FFAs in the presence or absence of triacsin C. After 24 h of exposure, the medium was removed and macrophage monolayers were washed once with 0.1 % BSA in PBS followed by 2 more washes with warm PBS. The cells were harvested in PBS, and the lipid profile was analyzed by gas chromatography (GC) as described.[2]

Immunoblotting: MPMs were plated at 1.5 x 10^6 cells per well of 12-well plates and treated with either VLDL or FFAs in the presence or absence of triacsin C. Cells were collected in lysis buffer containing 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.1% Nonidet P-40, 2.5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, and 0.5 mM PMSF. A modified Lowry protein estimation was performed, and 10 µg of protein was electrophoresed through 4–12% SDS gels (Invitrogen), transferred to nitrocellulose membranes, and immunoblotted for proteins with appropriate antibodies.

Cell viability: Cell viability was determined using the MTT cell proliferation assay kit (Cayman Chemical, Ann Arbor, MI). MPMs were seeded in 48-well dishes at 3x10^5 cells per well and treated with different fatty acids in the presence or absence of triacsin C. After 24 h, the experimental media was replaced with fresh DMEM containing 5% FBS. MTT reagent was added and MPMs were incubated for an additional 4 h and the assay was performed per the manufacturer's instructions. Lactate dehydrogenase (LDH) assay was performed using LDH cytotoxicity assay kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's instructions. Briefly, MPMs were plated and treated as described for the MTT assay. At the end of 24 h, the plate was centrifuged at 1200

rpm for 5 minutes and 100 µl of media supernatant was transferred to a 96-well plate and 100 µl of reaction mixture was added. The absorbance was read at 490 nm.

Immunohistochemistry: MPMs, plated in 4-well chamber slides and treated with stearic acid in the presence or absence of triacsin C, were fixed in 10% buffered neutral formalin for 1 min. Immunohistochemical staining for cleaved caspase 3 was carried out using 1:600 dilution of anti-cleaved caspase 3 antibody (Promega, Madison, WI).

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR): Total RNA was isolated from the SVCs using TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RNA was reverse-transcribed to cDNA using the iQ cDNA synthesis kit. From this cDNA, real-time PCR analysis was carried out for different genes using the iQ5 multicolor real-time PCR detection system (BioRad, Hercules, CA). Primer-probe sets (Assays-on-Demand) were purchased from Applied Biosystems (Foster City, CA).

Mice and diet: Two month old male C57BL/6 mice were fed a low fat (LF, 10% fat) or high fat (HF, 60% fat) diet for 16 wk (Research Diets, New Jersey). At the end of the feeding period, the mice were sacrificed and perigonadal AT samples were collected for the study.

AT histology: AT collected in 10% formalin was embedded in paraffin and 7 µm sections were collected. To determine the morphology of the AT, the sections were stained with toluidinie blue O (TBO). Immunohistochemistry was performed to detect AT macrophages using anti rat F4/80 antibody (ABCAM, Cambridge, MA). Stromal vascular cells (SVCs) were collected from the AT[3] and stained with Oil Red O.[2]

Statistical analysis: Results are presented as the mean ± SEM. Data were analyzed with Prism Graphpad using ANOVA followed by Tukey's *post-hoc* test to compare the mean responses among different treatment groups unless otherwise indicated. A statistical probability of $P<0.05$ was considered significant.

References

1. Farkas MH, Swift LL, Hasty AH, Linton MF, Fazio S. The recycling of apolipoprotein E in primary cultures of mouse hepatocytes. Evidence for a physiologic connection to high density lipoprotein metabolism. *J Biol Chem.* 2003;278:9412-9417.
2. Saraswathi V, Hasty AH. The role of lipolysis in mediating the proinflammatory effects of very low density lipoproteins in mouse peritoneal macrophages. *J Lipid Res.* 2006;47:1406-1415.
3. Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW, Jr. Obesity is associated with macrophage accumulation in adipose tissue. *J Clin Invest.* 2003;112:1796-1808.

Supplemental Figure Legends

Figure I. MPMs treated with stearic acid for 6 h accumulates intracellular stearic acid both in the presence or absence of triacsin C

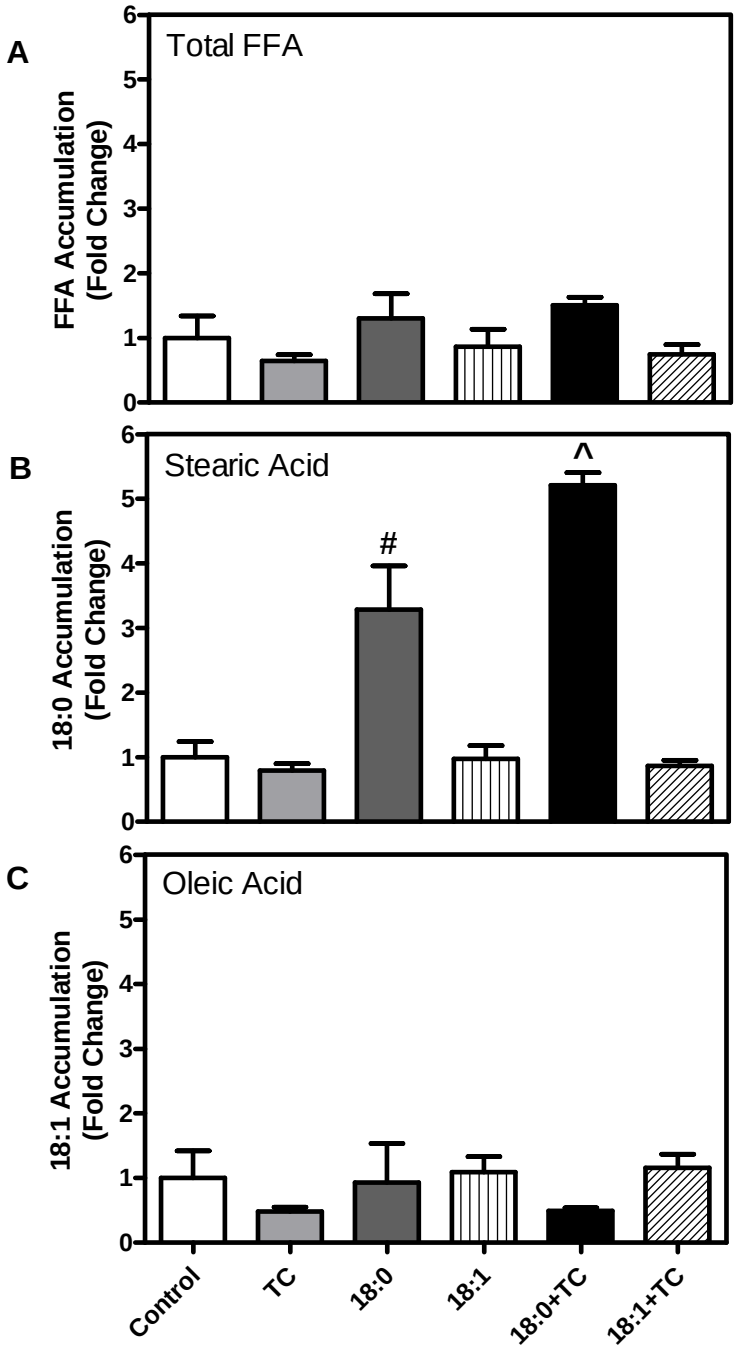
MPMs were pretreated for 30 min with 5 μM triacsin C followed by co-treatment for 6 h with fatty acids complexed to albumin in serum free DMEM. (A) FFAs, (B) stearic acid, and (C) oleic acid. The stearic and oleic acid levels are expressed as the fold change of the mass amount present in total FFA fraction as compared to control cells. Data are presented as mean ± SEM of 3-6 individual samples.

^P<0.01 vs 18:0 and P<0.001 vs all others

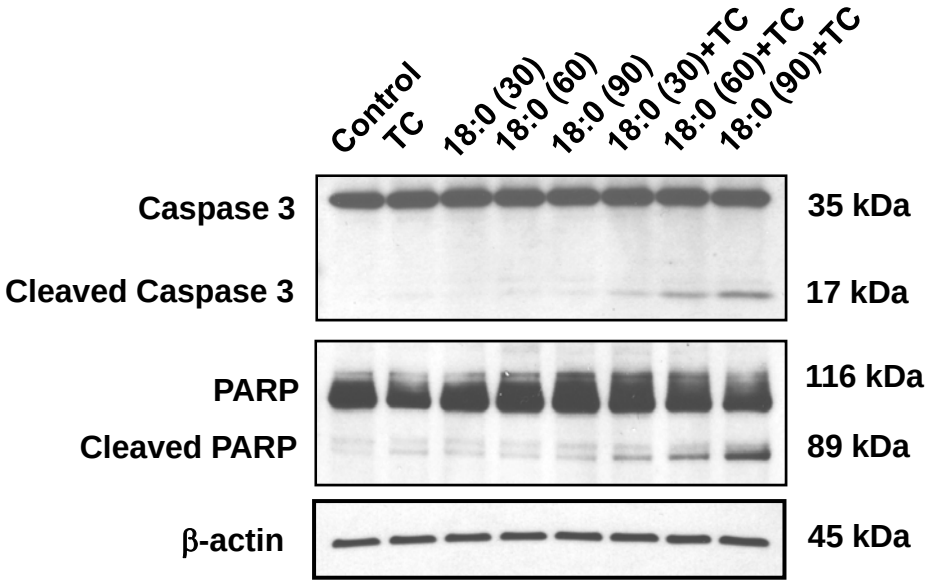
$^{\#}P$<0.01 vs 18:0+TC and P<0.001 vs all others

Figure II. Stearic acid treatment for 24 h in the presence of triacsin C induces apoptosis in a dose-dependent manner

MPMs were pretreated for 30 min with 5 μM triacsin C followed by co-treatment for 24 h with different concentrations (in μM) of stearic acid (18:0) complexed to albumin in serum free DMEM. Cell lysates were prepared and western blot analysis was carried out for cleaved caspase 3 and cleaved PARP as described in the Methods. Representative bands from three experiments performed in duplicate are shown.



Saraswathi, Supplemental Figure 1



Saraswathi, Supplemental Figure II