Inhibition of Long-Chain Acyl Coenzyme A Synthetases During Fatty Acid Loading Induces Lipotoxicity in Macrophages

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Objectives—Obesity is often associated with hypertriglyceridemia and elevated free fatty acids (FFAs), which are independent risk factors for cardiovascular disease and diabetes. Although impairment of cholesterol homeostasis is known to induce toxicity in macrophages, the consequence of altered fatty acid homeostasis is not clear.

Methods and Results—Long-chain acyl CoA synthetases (ACSLs) play a critical role in fatty acid homeostasis by channeling fatty acids to diverse metabolic pools. We treated mouse peritoneal macrophages (MPMs) with VLDL or FFAs in the presence of triacsin C, an inhibitor of the 3 ACSL isoforms present in macrophages. Treatment of macrophages with VLDL and triacsin C resulted in reduced TG accumulation but increased intracellular FFA levels, which induced lipotoxicity characterized by apoptosis. Treatment of MPMs with the saturated fatty acid stearic acid in the presence of triacsin C increased intracellular stearic acid and induced apoptosis. Stromal vascular cells collected from high-fat diet–fed mice displayed foam cell morphology and exhibited increased mRNA levels of macrophage markers and ACSL1. Importantly, all of these changes were associated with increased FFA level in AT.

Conclusions—Inhibition of ACSLs during fatty acid loading results in apoptosis via accumulation of FFAs. Our data have implications in understanding the consequences of dysregulated fatty acid metabolism in macrophages. (Arterioscler Thromb Vasc Biol. 2009;29:1937-1943.)

Key Words: VLDL foam cells free fatty acids triacsin C long chain acyl Co synthetases stearic acid apoptosis

Obesity and the associated metabolic dysregulations such as dyslipidemia and elevated plasma free fatty acids (FFAs) contribute to increased incidence of cardiovascular disease and type 2 diabetes.1,2 Macrophages are cells of the innate immune system, traditionally thought to participate predominantly in immune disorders. However, in the past 2 decades, a role for macrophages in lipid homeostasis and in metabolic diseases has been established. It is well known that free cholesterol induces an inflammatory response and apoptosis in macrophages, and that apoptotic macrophages contribute to atherosclerotic lesion formation3; however, the consequences of FFA accumulation in macrophages are not clear.

Long-chain acyl CoA synthetases (ACSLs) play a crucial role in regulating fatty acid metabolism by converting FFAs into fatty acyl CoA derivatives via a process called fatty acid activation. This modification is required for any FFA to undergo further metabolism. Activated fatty acids can enter several metabolic pathways such as β-oxidation, desaturation, or esterification into triglycerides, phospholipids, or cholesterol esters. Because of the critical role of ACSLs in activating fatty acids, and in partitioning them to diverse metabolic pools, we hypothesized that inhibition of ACSLs would impair fatty acid homeostasis in macrophages.

Five different isoforms of ACSL—1, 3, 4, 5, and 6—have been identified in humans and rodents.4 Mouse peritoneal macrophages (MPMs) predominantly express ACSL1, although ACSL 3 and 4 are also expressed to some extent.5 Triacsin C has been shown to inhibit ACSL 1, 3, and 4 but does not inhibit ACSL 5 or 6.6,8 Triacsin C is a potent inhibitor of ACSLS, and the inhibitory potential of triacsin C varies among the different ACSL isoforms. Triacsin C has been shown to inhibit ACSL 1, 3, and 4 but does not inhibit ACSL 5 or 6.6,8 Thus, triacsin C can inhibit all of the isoforms of ACSL present in macrophages.

Taking advantage of this inhibitor, we demonstrate that blocking the activity of ACSLs during fatty acid loading leads to induction of apoptosis which is attributable, at least in part, to accumulation of intracellular FFAs. We also show that stromalvascular cells (SVCs) derived from obese adipose tissue (AT) display foam cell morphology and exhibit increased mRNA levels of macrophage markers and ACSL1. All of these changes were associated with increased local FFA levels in AT. These findings highlight the importance of ACSLs in regulating fatty acid homeostasis in macrophages and have implications for potential mechanisms by which AT macrophages respond to increased fatty acid flux in obese AT.
Methods

Fatty Acid Treatment
We previously reported that fatty acids at 90 μmol/L concentration induce a proinflammatory response or apoptosis in endothelial cells. Therefore, in most of the experiments, MPMs were treated with individual FFAs at 90 μmol/L concentration or an equimolar mixture of the long-chain fatty acids palmitic acid, stearic acid, oleic acid, and linoleic acid at a total final concentration of 90 μmol/L. The fatty acids were first dissolved in ethanol and then added to DMEM with 5% FBS and MPMs were treated with fatty acids for 24 hours in the presence or absence of triacsin C (5 μmol/L). This resulted in a fatty acid to albumin ratio of 3:1, which is within a physiological range. This method of fatty acid treatment was used for most of the experiments unless otherwise indicated. In separate experiments, MPMs were also treated with FFAs complexed to fatty acid free BSA using serum-free DMEM as described earlier. Briefly, fatty acids were first dissolved in ethanol and pre-equilibrated with BSA at 37°C for 1.5 hour at a molar ratio of 5:1 (fatty acid:albumin). Fatty acid-albumin complex solution was freshly prepared before each experiment. Other methods are described in detail in the supplemental data (available online at http://atvb.ahajournals.org).

Results

Inhibition of ACSLs During VLDL Loading Reduces Triglyceride Accumulation and Increases Intracellular FFA Concentrations in MPMs
MPMs were incubated with VLDL in the presence or absence of triacsin C, and lipid accumulation was analyzed by Oil Red O staining. As we previously reported, incubation of MPMs with VLDL by itself resulted in a dramatic increase in neutral lipid accumulation. However, staining was greatly reduced in cells treated with both VLDL and triacsin C (5 μmol/L), suggesting that inhibition of ACSL activity nearly abolished the accumulation of neutral lipids (Figure 1A). Analysis of the lipid profiles in these cells by gas chromatography (GC) revealed that treatment with VLDL plus triacsin C reduced triglyceride (TG) accumulation by 73% (P<0.01) compared to treatment with VLDL alone. In contrast, intracellular FFAs were increased 2-fold (P<0.001) compared to the VLDL-treated group (Figure 1B). The levels of cholesterol esters, unesterified cholesterol, and phospholipids were not altered by treatment with VLDL and triacsin C.

Inhibition of ACSLs During VLDL Loading Induces Apoptosis in MPMs
Treatment of MPMs with VLDL in the presence of 2.5 and 5 μmol/L triacsin C for 6 hours increased the cleavage of the apoptotic marker procaspase 3 (35 kDa), to its 17-kDa active form in a dose-dependent manner (Figure 2A). Along with the activation of caspase 3, cleavage of Poly (ADP-ribose) polymerase (PARP, 116 kDa), another apoptotic marker, to its 89-kDa fragment was observed in MPMs treated with VLDL in the presence of triacsin C. Apoptotic death was also confirmed by immunohistochemical staining of cleaved caspase 3 in MPMs treated with VLDL in the presence of triacsin C (Figure 2B). Quantification of cleaved caspase 3–stained cells revealed that 8.4±0.7% of cells were apoptotic in the VLDL plus triacsin C–treated cells compared to less than 2% of cells in the control groups (Figure 2C, P<0.001).
To determine whether accumulation of intracellular FFAs, as seen in stearic acid plus triacsin C–treated cells, also induces apoptosis in MPMs, MPMs were pretreated for 30 minutes with 2.5 μmol/L or 5 μmol/L triacsin C followed by cotreatment with 100 μg/mL of VLDL for an additional 6 hours. A, Western blot analysis was performed for markers of apoptosis. Representative samples from 2 experiments performed in triplicate wells are shown. B, Immunohistochemical analysis for cleaved caspase 3. Arrows indicate cells positive for cleaved caspase 3. C, Cleaved caspase 3–positive cells were counted in 3 fields from 3 different wells for each treatment group. Data are expressed as percentage of total cells. VL indicates VLDL; TC, triacsin C.

Similar to stearic acid treatment using DMEM with 5% FBS, stearic acid complexed to BSA and treated using serum-free DMEM also significantly increased both the total intracellular FFA fraction as well as the specific stearic acid accumulation (Figure 2D and 2E, P<0.001 versus control). Interestingly, oleic acid accumulation was significantly increased not only in oleic acid plus triacsin C–treated cells but also in stearic acid plus triacsin C–treated cells (Figure 2F, P<0.05). Taken together, stearic acid plus triacsin C led to a significant increase in total intracellular FFA content and specific stearic acid accumulation regardless of the treatment method.

**Inhibition of ACSLs During Saturated but not Unsaturated Fatty Acid Loading Induces Apoptosis in MPMs**

To determine whether accumulation of intracellular FFAs, as seen in stearic acid plus triacsin C–treated cells, also induces lipotoxicity, we analyzed markers of apoptosis. In addition to stearic acid, MPMs were treated with other free fatty acids such as oleic acid (18:1) or linoleic acid (18:2) individually, or with a mixture of fatty acids in the presence of triacsin C for 24 hours (all at 90 μmol/L). Cell viability, as determined by MTT assay, was dramatically reduced in cells treated with stearic acid plus triacsin C (P<0.001) but was not reduced by treatment with any other fatty acid tested (Figure 4A). In line with the MTT assay, a profound increase in lactate dehydrogenase (LDH) release, a marker of cell death, was exhibited by cells treated with stearic acid and triacsin C for 24 hours (P<0.001). A mild increase in LDH release was also seen in cells treated with triacsin C alone as compared to untreated control cells. In addition, MPMs treated with a mixture of fatty acids in the presence of triacsin C showed a small but significant (P<0.05) decrease in LDH activity compared to controls (Figure 4B).

Cells treated with stearic acid and triacsin C demonstrated cleavage of caspase 3 and PARP; however, neither individual unsaturated fatty acids nor mixtures of fatty acids induced apoptosis in the presence of triacsin C (Figure 4C). Quantification of cleaved caspase 3 stained cells revealed that 20.0±1.9% of cells exhibited features of apoptosis on treatment with both stearic acid and triacsin C (P<0.001, Figure 4D).

We have also determined the dose-dependent effect of stearic acid in inducing apoptosis in the presence of triacsin C and noted that stearic acid induced apoptosis at a concentration ranging from 30 to 90 μmol/L in the presence of triacsin C (supplemental Figure II).

**High-Fat Diet Feeding Leads to Increased FFAs and Macrophage Accumulation in AT**

Because ACSLs play an important role in regulating fatty acid homeostasis in macrophages, we next attempted to determine the physiological significance of these enzymes on macrophage fatty acid homeostasis. It is well established that AT in obesity releases excessive FFAs and that macrophages accumulate in greater numbers in obese AT. We fed wild-type C57BL/6 mice with a low-fat (LF) or a high-fat (HF) diet for 16 weeks and analyzed the perigonadal AT. As expected, the morphology of AT derived from HF diet–fed mice analyzed by TBO staining suggests the presence of more macrophages compared to LF diet–fed mice (Figure 5A and 5B). Further analysis of AT sections for F4/80, CD68, and Mac-1 demonstrated that AT derived from HF diet–fed mice exhibited features of foam cell morphology after staining with Oil Red O (Figure 6A). In addition, there was an increase in the mRNA levels of the macrophage markers F4/80, CD68, and Mac-1. More importantly, the SVCs derived from the AT of HF-fed mice exhibited foam cell–like morphology associated with increased ACSL1.

Interestingly, SVCs from HF diet–fed mice exhibited features of foam cell morphology after staining with Oil Red O (Figure 6A). In addition, there was an increase in the mRNA levels of the macrophage markers F4/80, CD68, and Mac-1. More importantly, the SVCs derived from the AT of HF-fed mice exhibited foam cell–like morphology associated with increased ACSL1.

**SVCs From HF Diet–Fed Mice Exhibit Foam Cell–Like Morphology Associated With Increased ACSL1**

Interestingly, SVCs from HF diet–fed mice exhibited features of foam cell morphology after staining with Oil Red O (Figure 6A). In addition, there was an increase in the mRNA levels of the macrophage markers F4/80, CD68, and Mac-1. More importantly, the SVCs derived from the AT of HF-fed mice exhibited foam cell–like morphology associated with increased ACSL1.
diet–fed mice displayed a significant increase in the expression of ACSL1 and a trend toward an increase in ACSL 3 and 4 (Figure 6B). Thus, the macrophage enriched SVCs collected from HF diet–fed mice exhibited signs of lipid deposition, which was also associated with an increased expression of ACSL1.

Discussion

We and others have previously reported that macrophages efficiently take up FFAs derived from lipoprotein lipase–mediated hydrolysis of TG-rich VLDL, which, in turn, are esterified and stored as intracellular TGs.13,19–22 Because conversion of fatty acids into fatty acyl CoAs is required for their esterification into TGs, we attempted to determine the impact of inhibiting fatty acid activation on fatty acid homeostasis in macrophages. In the current study, fatty acid loading was achieved using both TG-rich VLDL and albumin-bound FFAs, and fatty acid activation was inhibited by triacsin C, which inhibits all 3 ACSL isoforms expressed in macrophages. We demonstrate that VLDL treatment in the presence of triacsin C leads to accumulation of intracellular FFAs and induction of apoptosis. Furthermore, we show that not only VLDL but also the FFA, stearic acid, in the presence of triacsin C, leads to accumulation of intracellular FFAs and induction of apoptosis. In addition, we demonstrate that SVCs derived from obese AT exhibit foam cell morphology, and show increased mRNA expression of ACSL1, and these changes occur concurrently with increased local FFA levels in AT. Our current data suggest that ACSLs play a critical role in regulating fatty acid homeostasis in macrophages and have implications in understanding the mechanisms by which AT macrophages respond to elevated local FFA levels.

Because TG-rich VLDL is an important physiological source of FFAs for macrophages,13,19–22 we first treated macrophages with VLDL in the presence or absence of triacsin C. We found that VLDL treatment in the presence of triacsin C results in redistribution of cellular fatty acids by decreasing TGs and increasing FFAs (Figure 1). Our data also show that partitioning of fatty acids into a “free” pool rather than into the storage form as TGs, leads to lipotoxicity in macrophages (Figure 2). Traditionally, TG accumulation itself has been considered to be responsible for lipotoxicity. However, several reports suggest that TG accumulation may actually protect cells from the toxic effects of lipids by preventing the accumulation of FFAs and their metabolites such as ceramide and diacylglycerol.23,24 This notion is further supported by the observation that even prolonged exposure to high levels of TG-rich VLDL in the absence of triacsin C failed to reduce cell viability in MPMs.25 These data have implications for situations in which macrophage ACSL activity is insufficient for the fatty acid load, especially
when macrophages are exposed to high levels of fatty acids, such as in obesity or hypertriglyceridemia.

Comparison of the FFA accumulation under different conditions (VLDL versus FFA exposure and 6 hours versus 24 hours) reveals important biological information regarding the toxicity of intracellular FFA accumulation. First, 6-hour exposure of MPMs to stearic acid in the presence of triacsin C led to a 5.1-fold increase in stearic acid accumulation in the cells (supplemental Figure I), and no increase in total FFA accumulation, yet did not induce apoptosis (data not shown). Second, treatment of MPMs with VLDL plus triacsin C for 6 hours led to a 5.2-fold increase in total FFAs and extensive apoptosis (Figures 1B and 2). Third, treatment with stearic acid plus triacsin C for 24 hours led to accumulation of both total FFAs (2.3-fold) and stearic acid (4.0-fold) and also resulted in extensive apoptosis (Figures 3A, 3B, 4C, and 4D). In fact, the extent of apoptosis induced by stearic acid plus triacsin C treatment was much higher compared to treatment with VLDL plus triacsin C. One possible reason for this is the difference in exposure time between these 2 treatment conditions. Fourth, treatment of oleic acid plus triacsin C for 24 hours led to a 2-fold increase in oleic acid and minimal increases in total FFA and did not induce apoptosis (Figures 3A, 3C and 4C). Taken together, these data indicate that 3 elements of fatty acid exposure are important for their lipotoxic effects in MPMs: the source of fatty acids, the amount of total FFAs that accumulate, and the length of exposure time to intracellular FFAs.

Because the lipotoxic effect was seen only with stearic acid, it is possible that the apoptotic response we noted with VLDL treatment may also be caused by the stearic acid present in VLDL triglycerides. We previously reported that palmitic acid, stearic acid, oleic acid, and linoleic acid were the predominant fatty acids found in VLDL used for treating macrophages. The amount of stearic acid in the VLDL triglyceride was 15 μmol/L, whereas the minimum concentration of stearic acid we used to induce apoptosis in the current study was 30 μmol/L. However, even with a lower stearic acid content (15 μmol/L) and at an earlier time point (6 hours), VLDL plus triacsin C treatment led to stearic acid accumulation to levels only slightly lower than those in cells treated with stearic acid (90 μmol/L) plus triacsin C treatment for 24 hours (2.24 ± 0.28 versus 3.2 ± 0.47 μg/mg protein, respectively). Thus, stearic acid accumulation accompanied by an increase in total FFA concentration may contribute to the apoptotic response seen in VLDL plus triacsin C–treated macrophages.

![Figure 4](image-url) Differential effects of long-chain fatty acids on cell viability and apoptosis during ACSL inhibition. MPMs were pre-treated for 30 minutes with 5 μmol/L triacsin C followed by cotreatment with 90 μmol/L stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2), or a mixture (22.5 μmol/L each of palmitic acid, stearic acid, oleic acid, and linoleic acid) for an additional 24 hours. Cell viability was determined by (A) MTT assay and (B) LDH assay. Data are expressed as mean ± SEM of 4 to 6 individual samples. C, Western blot analysis was carried out for cleaved caspase-3 and cleaved PARP. Representative bands from 2 experiments performed in triplicate are shown. D, Immunohistochemical analysis for cleaved caspase-3. Cleaved caspase-3-positive cells were counted in 3 fields from 3 different wells for each treatment group. Data are expressed as percentage of total cells. TC indicates triacsin C; FAM, fatty acid mixture. *P < 0.01 vs all. #P < 0.01 vs TC and P < 0.001 vs all others. $P < 0.01 vs 18:0 + TC and P < 0.001 vs all others. $P < 0.05 vs control.

![Figure 5](image-url) Analysis of macrophage markers and FFA content in AT. A through D, Histological analysis of AT morphology. Wild-type C57BL/6 mice were fed low-fat (A and C) or high-fat (B and D) diet for 16 weeks. Perigonadal AT samples collected at sacrifice were fixed and paraffin embedded. Sections were stained with TBO (A and B), or immunostained with an antibody against F4/80 (C) and D, 20× magnification. E, Concentration of FFAs in AT of low-fat and high-fat diet–fed mice as measured by GC analysis. Student t test was carried out for statistical analysis. Values are mean ± SEM of 6 samples in each group. $P < 0.01.
cells at 6 hours. However, the role of palmitic acid, another saturated fatty acid present in VLDL, cannot be ruled out.

Another interesting observation of our current study is that when MPMs were treated with fatty acids in serum free medium, a significant increase in oleic acid accumulation was noted in stearic acid plus triacsin C treated cells (Figure 3F). The increased oleic acid levels in stearic acid plus triacsin C–treated cells suggests the involvement of stearoyl coA desaturase-1 (SCD-1) in converting stearic acid to oleic acid via a desaturation reaction. Because desaturation reactions also require fatty acid activation, our data indicate that even when ACSLs are inhibited, stearic acid activation occurs to some extent. In fact, fatty acid activation can also be carried out by another family of enzymes, the very long-chain acyl CoA synthetases (ACSVLs), which are not inhibited by triacsin C. These enzymes can activate exogenous fatty acids during their transmembrane transport. On the other hand, in the absence of ACSL activity, the endogenous oleic acid produced intracellularly from stearic acid via SCD-1 activity may not be activated and metabolized further, thus leading to their intracellular accumulation.

It should also be noted that oleic acid accumulated to a lesser degree in oleic acid plus triacsin C–treated cells compared to stearic acid accumulation in stearic acid plus triacsin C–treated cells. As mentioned, even in the absence of ACSL activity, a portion of exogenous oleic acid can still be activated via ACSVLS. It is possible that the activated oleic acid may be preferentially partitioned to \( \beta \)-oxidation as opposed to stearic acid, which appears to go through a desaturation reaction, thus accounting for the difference in the extent of its accumulation. However, we cannot rule out the possibility that the degree of inhibition by triacsin C may not be the same for stearic acid and oleic acid substrates. Further studies are needed to better understand the inhibitory potential of triacsin C on different fatty acids.

To gain insight into the role of ACSLs in regulating fatty acid homeostasis in vivo, we analyzed SVCs collected from lean and obese AT and noted that SVCs from obese AT...
exhibited features of foam cell transformation. As SVCs contain several other cell types, we analyzed them for markers of macrophages and found that all the macrophage markers were significantly higher in SVCs from obese AT compared to lean AT. SVCs from obese AT also exhibited an increased mRNA expression of ACSL1. Overexpression of ACSL1 has been shown to increase TG synthesis in liver. Because the increased ACSL1 expression in SVCs is also associated with increased neutral lipid accumulation and an increase in local FFA levels in AT, our data suggest that AT macrophages respond to increased FFA levels in AT by overexpressing ACSL1, which, in turn, regulates fatty acid homeostasis in AT macrophages.

Taken together, we have demonstrated that inhibition of ACSLs during fatty acid loading leads to intracellular FFA accumulation which results in apoptosis. Our data also demonstrated that increased local FFA levels in obese AT were associated with foam cell transformation of AT macrophages and these changes were accompanied by an increased expression of ACSL1. These findings provide insight into the biological importance of ACSLs in regulating fatty acid homeostasis in macrophages found in sites that are exposed to very high levels of FFAs or TGs, and indicate that perturbations in ACSL activity may lead to lipotoxic effects of FFAs in the context of obesity.

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Disclosures

None.

References

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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. 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.

**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 $3 \times 10^6$ 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 $1.5 \times 10^6$ 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 $3 \times 10^5$ 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 toluidine 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 and stained with Oil Red O.
**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**


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.
Total FFA

Stearic Acid

Oleic Acid