Abstract Chronic inflammation contributes to cardiovascular disease. Increased levels of the inflammatory cytokine, TNF-α, are often present in conditions associated with cardiovascular disease risk, and TNF-α induces a number of pro-atherogenic effects in macrovascular endothelial cells, including expression of adhesion molecules and chemokines, and lipidprotein uptake and transcytosis to the subendothelial tissue. However, little is known about the roles of acyl-CoA synthetases (ACSLs), enzymes that esterify free fatty acids into their acyl-CoA derivatives, or about the effects of TNF-α on ACSLs in endothelial cells. Therefore, we investigated the effects of TNF-α on ACSLs and downstream lipids in cultured human coro- nary artery endothelial cells and human umbilical vein endothelial cells. We demonstrated that TNF-α induces ACSL1, ACSL3, and ACSL5, but not ACSL4, in both cell types. TNF-α also increased oleyl-CoA levels, consistent with the increased ACSL3 expression. RNA-seq-sequencing demonstrated that knockdown of ACSL3 had no marked effects on the TNF-α transcriptome. Instead, ACSL3 was required for TNF-α-induced lipid droplet formation in cells exposed to oleic acid. These results demonstrate that increased acyl-CoA synthesis as a result of ACSL3 induction is part of the TNF-α response in human macrovascular endothelial cells. ---Jung, H. S., M. Shimizu-Albergine, X. Shen, F. Kramer, D. Shao, A. Vivekanandan-Giri, S. Pennathur, R. Tian, J. E. Kanter, and K. E. Bornfeldt. TNF-α induces acyl-CoA synthetase 3 to promote lipid droplet formation in human endothelial cells. J. Lipid Res. 2020. 61: 33-44.

Supplementary key words cytokines • fatty acid/metabolism • fatty acid/oxidation

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Abbreviations: ACSL, acyl-CoA synthetase; ADRP, adipose differen- tiation-related protein; CS, citrate synthase; Ct, cycle threshold; FAO, fatty acid oxidation; FCCP, carbonyl cyanide-4-(trifluoromethoxy)-phenylhydrazone; FDR, false discovery rate; LD, lipid droplet; OCR, oxygen consumption rate; PDI, protein disulfide isomerase; PGE2, prostaglan- din E2; PGL3, prostacyclin; RNA-seq, RNA-sequencing.

The data discussed in this publication have been deposited in NCBI’s Gene Expression Omnibus (Bornfeldt, 2019) and are accessible through GEO Series accession number GSE134489 (http://www.ncbi.nlm.nih. gov/geo/query/acc.cgi?acc=GSE134489).

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The online version of this article (available at http://www.jlr.org) contains a supplement.
Free fatty acids of 12–20 carbons (long-chain fatty acids) are readily taken up by cells and then rapidly converted into their acyl-CoA derivatives by a group of five long-chain acyl-CoA synthetases (ACSLs) (ACSL1, ACSL3, ACSL4, ACSL5, and ACSL6) (9) as well as by other enzymes with ACSL activity. Any given cell type typically expresses several of the ACSL isoforms (10). The acyl-CoA derivatives undergo compartmentalized processes, and different ACSL isoforms have been suggested to channel acyl-CoAs to different fates in the cell, such as neutral lipid storage and fatty acid β oxidation (11). Endothelial cells express ACSL1, ACSL3, ACSL4, and ACSL5 (12), but the regulation and function of ACSL isoforms in this cell type are largely unknown.

Because ACSL1 is induced by TNF-α and a number of other inflammatory mediators in macrophages (13–15), we set out to investigate regulation of ACSLs in response to TNF-α in human macrovascular endothelial cells. We show that ACSL3 induction is part of the TNF-α response in both HUVECs and human coronary artery endothelial cells (HCAECs), and that ACSL3 induction is required for TNF-α-induced formation of LDs.

MATERIALS AND METHODS

Cells

HCAECs were purchased (LifeLine Cell Technology, Frederick, MD) from two different donors: a 28-year-old man and a 40-year-old woman (lot number 05079 and 05154, respectively). The cells were grown in VascuLife® VEGF endothelial medium (LL-0003; VascuLife Cell Technology) and used at passages 5–9. For experiments, the medium was replaced with medium without FGF, IGF, and VEGF 4 h before stimulation with human recombinant TNF-α (20 ng/ml; PeproTech Inc., Rocky Hill, NJ). HUVECs were isolated from fresh human umbilical cords by collagenase digestion, as described by Surapistchat et al. (16). At least two cords were used for each preparation, and HUVECs from >10 donors were used for the experiments of this study. Briefly, the umbilical vein was perfused with collagenase (Worthington Biochemical Corp., Lakewood, NJ) and incubated at 37°C for 25 min. Digested HUVECs were flushed out, collected, and resuspended in growth medium (RPMI 1640, 20% FBS, 5% heparin, and 1% bovine pituitary extract (Sigma), as a source of endothelial cell growth factor) and plated in 2% gelatin-coated (gelatin type A from porcine skin; Sigma) culture flasks. HUVECs were used at passages 1–6. For experiments, cells were stimulated with human TNF-α (20 ng/ml) in the presence of RPMI 1640 (11.1 mmol/l glucose), 5% FBS, 0.1% fatty acid-free BSA, 50 μmol/l L-carnitine, heparin, and endothelial cell growth factor. In some experiments, the cells were treated with the p38 MAPK inhibitor, SB202190 (10 μM; Santa Cruz Biotechnology, Dallas, TX), the JNK inhibitor, SP600125 (10 μM; Santa Cruz Biotechnology), the IKK/NFκB inhibitor, Bay11-7082 (2 μM; Santa Cruz Biotechnology), or the PI3K inhibitor, LY294002 (10 μM; Cell Signaling Technology, Danvers, MA) in the presence or absence of 20 ng/ml TNF-α for 18 h. The inhibitors were dissolved in DMSO, and DMSO vehicle was used in control cells. Expression of the endothelial cell marker von Willebrand factor was confirmed in both HUVECs and HCAECs. Isolation of cells from humans was approved by the Institutional Review Board of the University of Washington.

Phoenix amphototropic cells (Orbigen, San Diego, CA) used for generation of retrovirus were maintained in DMEM, 25 mmol/l glucose, 10% FBS, nonessential amino acids, 100 Units/ml penicillin, and 100 μg/ml streptomycin. After transfection, 1 μg/ml puromycin was added in order to select for cells expressing retroviral vectors.

Creation of retroviral vectors for ACSL1, ACSL3, and ACSL5 overexpression

The cDNA clones for human ACSL1 variant 2 (NM_001995.2), human ACSL3 variant 1 (NM_004457.3), and human ACSL5 variant 1 (NM_016234) were obtained in pCMV expression vectors (OriGene, Rockville, MD). ACSL1, ACSL3, and ACSL5 cDNAs were cloned into the retroviral pBabe-RES-PURO (pBabe) vector (generously provided by Dr. Gary Nolan), as previously described (17). All vectors were sequenced to verify correct directionality by using an ABI 3730XL high-throughput capillary DNA analyzer. Phoenix amphotropic cells were transfected with empty vector (pBabe), pBabe-ACSL1, pBabe-ACSL3, or pBabe-ACSL5 vectors, as previously described (17). The ACSL3 virus was handled according to BSL2+ regulations.

Overexpression of ACSL1, ACSL3, and ACSL5

HUVECs were grown on 100 mm tissue culture dishes. Upon reaching 60–70% confluence, cells were incubated with retroviral supernatants for 18–24 h at 37°C. The cells were then treated with 2 μg/ml puromycin for 48–48 h for positive selection. The puromycin-resistant cell cultures were allowed to grow to full confluence and used for experiments.

Analysis of long-chain acyl-CoA levels and ACSL activity

Long chain fatty acyl-CoAs were quantified by LC-ESI-MS/MS, as previously described (13, 17). ACSL activity was determined as previously described (13, 17), using [9,10(α)-3H]C16:0 (GE Healthcare, Piscataway, NJ) as a substrate. The results were corrected for blanks (samples without cell lysates added and samples analyzed in the absence of CoA or ATP) and for protein content. All reactions were confirmed to occur within the linear range.

Knockdown of ACSL3 by siRNA

HCAEC cultures (80–90% confluence) were trypanosized and resuspended (5 × 10^3 cells/μl) in Amaxa HCAEC Nucleofector solution (Lonza, Cologne, Germany) containing 300 nM siRNA. The cells were applied to Amaxa Nucleofector I for electroporation using program S405, and then plated. Cells were harvested 72 h after electroporation. Two siRNAs for ACSL3 (s4997 and s4998; Silencer Select®) were prespecified and validated (Ambion, Grand Island, NY). After testing, s4997 was selected for further studies due to higher efficiency. Negative siRNA control #2 (Ambion) was used as a control.

RNA sequencing

HCAEC RNA was isolated by using NucleoSpin® RNA plus kit (Macherey-Nagel, Bethlehem, PA), according to the manufacturer’s protocol. The RNA samples went through quality control, including RNA quantification using the Quanti-IT RNA assay (Invitrogen) and RNA integrity analysis using a fragment analyzer (Advanced Analytical). For library production, total RNA was normalized to 10 ng/μl in a total volume of 50 μl on a Perkin Elmer Janus Workstation (Janus II; Perkin Elmer). Poly-A selection and cDNA synthesis were performed using the TrueSeq Stranded mRNA kit as outlined by the manufacturer (illumina; RS-122-2103). All steps were automated on the Perkin Elmer Sciclone NGSx Workstation to reduce batch to batch variability and to increase sample throughput. Final RNASeq libraries were quantified using the Quanti-it dsDNA High Sensitivity assay, and library insert size distribution was confirmed using a fragment analyzer.
(Advanced Analytical; DNF474). Samples in which adaptor dimers constituted more than 4% of the electropherogram area were failed prior to sequencing. Technical controls (K562, AM7832; Thermo Fisher Scientific) were compared with expected results. Successful libraries were normalized to 10 nM for submission to sequencing. Barcoded libraries were pooled using liquid handling robotics prior to clustering (Illumina cBot) and loading. Massively parallel sequencing-by-synthesis with fluorescently labeled reversibly terminating nucleotides was carried out on the HiSeq sequencer at the Northwest Genomics Center at the University of Washington. The processing pipeline consisted of the following elements: 1) base calls generated in real-time on a HiSeq4000 instrument (RTA 2.7.6); 2) demultiplexed unaligned BAM files produced by Picard ExtractIlluminaBarcodes and IlluminaBasecallsToSam were converted to FASTQ format using SamTools bam2fq (v1.4); 3) sequence read and base quality were confirmed using the FASTX-toolkit (v0.10.13); and 4) sequences were aligned to GRCh38 with reference transcriptome GENCODE release 26 (GRCh38.p10) using STAR (v2.5.3a). Transcript-level expression quantification was generated with RNA-SeQC (v1.1.9) and RSEM (v1.3.0). Reads per kilo base per million mapped reads (RPKM), fragments per kilo base per million mapped reads (FPKM), and transcripts per kilobase million (TPM) for transcript isoforms were quantitated on the merged lane-level data with RSEM (v1.3.0). Gene-level RPKMs were quantitated with RNA-SeQC (v1.1.9) on a standard collapsed reference annotation. Key expression quality control metrics were reviewed for outliers. Marginal outliers in quality were identified using the interquartile ranges (~1.5 x interquartile range) and flagged for further review. PCA plots of meta data were generated using PCA analysis in Python for detection of confounding effects. Differential expression from RNA-sequencing (RNA-seq) experiments was determined for FPKM, normalizing for gene length and sequencing depth. Data are reported using cutoffs of 2 for the log2 of the ratio between experimental and control samples. Statistical testing was done in GraphPad Prism using a 5% false discovery rate (FDR). Pathway enrichment analysis was performed using DB String, version 11.0 (18). The RNA-seq data have been deposited in NCBi’s Gene Expression Omnibus and are accessible through GEO Series accession number GSE134489.

Real-time PCR
RNA isolation and the real-time PCR protocol were performed as described (19). Briefly, RNA was isolated by using NucleoSpin® RNA plus kits according to the manufacturer’s protocol. Real-time PCR was performed by using the SYBR Green 1 detection method (Thermo Fisher Scientific). Cycle threshold (Ct) values were normalized to RN18S, and the results were presented as fold over control. Primers were designed to detect each ACSL isform (17) and inflammatory mediators (20). Primer sequences are available upon request.

Determination of ACSL mRNA copy number was performed as follows. In order to generate standard curves for ACSL1, ACSL3, ACSL5, and ACSL6, cDNA from HCAECs and HUVECs (ACSL1, ACSL3, ACSL4, and ACSL5) and HEK293 cells (ACSL6) were used for reactions with Phusion polymerase (Thermo Fisher Scientific) and the primer sets ACSL1-forward (F), AACAAGCG-GAAGCCCAAGGC; ACSL1-reverse (R), TCGGTGAGTGACCATTTCC- GCCT (102 bp amipicon); ACSL4-F, GCCCGTAACTGTGCTGCTGCTG; ACSL4-R, TCCGCCGTGGAATGTTTGT (78 bp amipicon); ACSL5-F, CTCCGGTTTTGACACTGCT (84 bp amipicon); ACSL6-F, TTAGAAGGCCTGGAAAAGA; ACSL6-R, AGAATACCCACCCACGAC- GCTTC (205 bp amipicon). The reactions were run at 98°C for 3 min, and then 35 cycles of 98°C for 10 s, 64°C for 20 s, 72°C for 7 s, and 72°C for 4 min. After the PCR products were verified on a gel as a single product, they were purified by a QIAquick PCR purification kit (Qiagen) and the concentration of each product was measured. The gene copy numbers of each ACSL were calculated by the size of product and the concentration of solution.

A pool of the PCR products containing five ACSLs together [each 1 x 10^11 gene copies per microliter (GC/µl)] was made and further diluted to 1 x 10^6 to 10 GC/µl gene copies per microliter (seven increments) for standard curves. To assess gene copy numbers of ACSLs in HCAECs and HUVECs, pools of RNA (0.1 µg) collected from two to four different donors for each cell type were used for a reverse transcriptase reaction. The cDNAs of each cell type and PCR product pool (dilution 1 x 10^6 to 10 GC/µl) were simultaneously run for PCR with each ACSL primer set and SybrGreen reagent. Standard curves were plotted as gene copy numbers to Ct values. The gene copy numbers in cell samples were determined by obtained Ct values on a standard curve of each ACSL.

Immunoblot
Total cell lysates (10–20 µg) were loaded onto SDS-PAGE gels, separated, and transferred onto nitrocellulose membranes. Detection was accomplished by using a rabbit polyclonal ACSL3 antibody (1.0 µg/ml; Thermo Fisher Scientific; PA5-42883) and a mouse monoclonal β-actin antibody (1:100,000 dilution; Santa Cruz Biotechnology; sc-47778).

Analysis of TNF-α-mediated signal transduction
Activation of different signaling pathways was evaluated using PathScan inflammation multi-target sandwich ELISA kits (Cell Signaling Technology). Briefly, cells were treated with 20 ng/ml TNF-α for 0 or 20 min before they were harvested in cell lysis buffer containing 1 µmol/l PMSF, according to the manufacturer’s instructions. Protein (75–300 µg) was loaded onto plates to detect changes in phospho-NF-κB p65 (Ser-536), phospho-SAPK/JNK (Thr-183/Tyr-185), and phospho-p38 MAPK (Thr-180/Tyr-182). All results were normalized to protein content.

Immunocytochemistry and confocal microscopy
After transfecting HCAECs with control siRNA or siACSL3, the cells were cultured in 8-well chamber slides (BioCoat poly-lysine culture slide, CB364588; Corning) for 2–3 days. The cells were then treated with oleic acid at the indicated concentrations (pre-bound to BSA at a ratio of 1:4 BSA:oleic acid for 1 h at 37°C) for 3 or 18 h. Human TNF-α (20 ng/ml) was added to cells with or without indicated concentrations of oleic acid-BSA complexes for 18 h. At the end of the treatments, cells were fixed with formalin for 10 min at room temperature. The fixed cells were blocked in PBS containing 3% normal goat serum, 100 mg/ml BSA, and 100 µg/ml saponin for 1 h, incubated with primary antibodies in BSA/PBS overnight at room temperature, washed four times, incubated with fluorescent labeled secondary antibodies (anti-mouse Alexa 546 or anti-rabbit Alexa 488) in BSA/PBS for 1 h, and washed four times. The primary antibodies used were a mouse anti-ACSL3 antibody (H00002181-B01P; Abnova) at a 1:100 dilution, a rabbit anti-adipose differentiation-related protein (ADRP)/perilipin2 antibody (152941-AP; Proteintech) at a 1:300 dilution; a rabbit anti-protein disulfide isomerase (PDI) antibody (11245-1- AP; Proteintech) at a 1:200 dilution, and a rabbit anti-citrate synthase (CS) antibody (16131-1-AP; Proteintech) at a 1:200 dilution. Lipid droplets were visualized by BODIPY 493/503 (D932; Invitrogen). BODIPY was added together with the secondary antibody solution at a final concentration of 10 µg/ml. After the final
washing, cells were mounted with Prolong Diamond antifade mountant with DAPI (P36971; Invitrogen). Stained cells were imaged on a Nikon A1R confocal microscope with a ×60 water objective.

LD measurements

Confocal images of BODIPY staining were converted to black and white 8-bits. To determine the edge of an individual cell, the output threshold setting of an image was lowered in order to show features of a whole cell, and then the cell was outlined (supplemental Fig. S1). Pixel numbers of the BODIPY-unstained cellular area were measured by ImageJ. The black and white image was then transposed and pixel numbers of the BODIPY-stained area were measured. The percentage of cellular BODIPY-positive area was calculated as: BODIPY-positive pixels/(BODIPY-positive pixels + BODIPY-negative pixels). Thirty-five to 50 cells were analyzed for each experimental condition.

Determination of CCL2 and prostaglandin release

HCAEC conditioned media were collected after stimulation with TNF-α. CCL2 levels were measured using a human CCL2 ELISA kit (eBioscience, San Diego, CA). Release of prostaglandin E2 (PGE2) and prostacyclin (PGI2) were measured with ELISA kits for PGE2 and 6-keto prostaglandin F1α, respectively (Cayman Chemical Co., Ann Arbor, MI). All results were normalized to cellular protein contents.

Mitochondrial respiration and endogenous fatty acid oxidation measurements

HCAECs were transfected with siRNA, incubated for 72 h, and then transferred to XF 24-well cell-culture microplates (Agilent Technologies, Santa Clara, CA) at 7.5 × 10^4 cells/well. After overnight incubation, the growth medium was replaced with assay buffer (Seahorse XF base medium; Agilent Technologies; 103335-100) containing 2 mM pyruvate, pH 7.4), and the cells were incubated in the absence of CO2 for 1 h. Then oxygen consumption rate (OCR) of HCAECs was calculated as: BODIPY-positive pixels/(BODIPY-positive pixels + BODIPY-negative pixels). Thirty-five to 50 cells were analyzed for each experimental condition.

Statistical analysis

Statistical analyses for all studies were performed using GraphPad Prism 7 software (La Jolla, CA). Unpaired two-tailed Student’s t test was used to compare two conditions, while multiple groups were compared by ANOVA with Tukey’s post hoc test or the Holm-Sidak multiple comparison method. The Wilcoxon matched-pairs signed rank test was used for FAO data when the same samples were compared under different conditions. The RNA-seq data were analyzed using a 5% FDR. Discoveries were determined using the two-stage linear step-up procedure of Benjamini, Krieger, and Yekutieli (GraphPad Prism 7 software). Each row was analyzed individually, without assuming a consistent SD. Error bars indicate SEM. *P < 0.05; **P < 0.01; ***P < 0.001.

RESULTS

TNF-α stimulation increases ACSL3 mRNA levels and acyl-CoA levels consistent with increased ACSL3 activity in HUVECs

Because endothelial cells from different vascular beds show differences in ACSL expression patterns (12), we first analyzed expression of ACSL isoforms in HUVECs by real-time PCR. We initially used HUVECs because large quantities of these cells are readily available. These measurements showed that ACSL1, ACSL3, ACSL4, and ACSL5 are expressed in HUVECs. Furthermore, TNF-α increased gene expression of ACSL1, ACSL3, and ACSL5 after an 18 h stimulation, but had no effect on ACSL4 (Fig. 1A). Consistent with an increased activity of ACSLs in TNF-α-stimulated HUVECs, acyl-CoA levels were increased following TNF-α stimulation, with the most marked effect on oleoyl-CoA (18:1-CoA) levels (Fig. 1B). TNF-α also increased ACSL activity in HUVEC lysates from 1,800 ± 58 acyl-CoA pmol/min/µg protein to 2,300 ± 58 acyl-CoA pmol/min/µg protein (mean ± SEM; n = 3; P = 0.004; two-tailed unpaired t test).

Next, we overexpressed ACSL1, ACSL5, and ACSL3 in HUVECs using a retroviral approach to assess differential effects on acyl-CoA levels. Overexpression of ACSL3 resulted in markedly increased ACSL3 mRNA levels as compared with the empty pBMB vector control, without affecting levels of ACSL1, ACSL4, or ACSL5 mRNA (Fig. 1C). Overexpression of ACSL3 in HUVECs mimicked the effect of TNF-α, with a significant increase in 18:1-CoA levels (Fig. 1D). These results suggest that induction of ACSL3 in HUVECs by TNF-α increases ACSL activity and subsequently oleoyl-CoA levels. We next overexpressed ACSL1 and ACSL5 in separate experiments. ACSL1 overexpression increased ACSL1 mRNA levels by 32 ± 7-fold (mean ± SEM; P = 0.0001; n = 6) and ACSL5 overexpression increased ACSL5 mRNA levels by 317 ± 59-fold (mean ± SEM; P < 0.0001; n = 6) without affecting other ACSLs. Overexpression of ACSL1 showed an increase in 18:1-CoA levels, like ACSL3 overexpression, whereas overexpression of ACSL5 primarily resulted in increased levels of saturated 18:0-CoA (Fig. 1E). Because little is known about the role of ACSL3 in endothelial cells, and because the effects of TNF-α on acyl-CoA profiles mimicked that of ACSL3, we focused our subsequent functional studies on this ACSL isoform.

TNF-α stimulation increases ACSL3 mRNA and ACSL3 protein levels in HCAECs

Further experiments were performed with HCAECs because HCAECs are more relevant to atherosclerosis. We studied HCAECs isolated from two different donors and
observed consistent results. Just like HUVECs, HCAECs expressed ACSL1, ACSL3, ACSL4, and ACSL5 mRNA (Fig. 2A). The relative pattern of expression of ACSLs was similar between the two endothelial cell types, with ACSL4 exhibiting the highest mRNA copy number, followed by ACSL3, and ACSL6 expression being very low or undetectable, consistent with previous studies (12) (supplemental Fig. S2A). Absolute levels of ACSL1, ACSL3, and ACSL4 were higher in HUVECs than in HCAECs. In HCAECs, like in HUVECs, an 18 h stimulation with TNF-α significantly induced mRNA levels of ACSL1, ACSL3, and ACSL5, however, not that of ACSL4 (Fig. 2A). CCL2 expression was measured as a positive control. TNF-α did not markedly alter gene expression of the SLC27A family members, SLC27A1 (FATP1), SLC27A3 (FATP3), or SLC27A4 (FATP4), fatty acid transport proteins that can act together with ACSLs or alone to promote vectorial acylation and acyl-CoA synthesis (21) (supplemental Fig. S2B, C). When tested. The most effective of these ACSL3 siRNAs markedly altered the ACSL3 siRNA (supplemental Fig. S3D).

Suppression of ACSL3 expression in HCAECs has no major effect on the TNF-α transcriptome

We next explored expression of the inflammatory mediators CCL2, ICAM1, and VCAM1 in cells stimulated with TNF-α in the absence or presence of ACSL3 knockdown. Expression of these inflammatory mediators was markedly induced by TNF-α as expected, but was not altered by loss of ACSL3 (supplemental Fig. S4A, B). Accordingly, TNF-α-induced CCL2 secretion from HCAECs was not influenced by ACSL3 expression levels (supplemental Fig. S4C).

To get an unbiased view of how TNF-α alters the transcriptome in HCAECs and whether the effects are dependent on ACSL3, we performed RNA-seq experiments. We used cutoffs of 2 and −2 for the log2 of the ratio between experimental and control samples and a 5% FDR to discover genes significantly regulated by TNF-α (Fig. 3A). We excluded antisense RNAs, noncoding RNAs, and pseudogenes from subsequent analysis. The RNA-seq experiment revealed 93 genes altered by TNF-α using the above criteria; most genes were upregulated, but 19 genes were
downregulated (Fig. 3B). The upregulated genes included cytokines and chemokines (IL7, LTB, CCL2, CXCL8, and TNFSF8). Accordingly, the transcriptome showed a significant enrichment for genes in the KEGG pathway (Hsa04060) cytokine-cytokine receptor interaction (FDR 0.04). Changes in levels of several of these transcripts were confirmed by real-time PCR in both donors (Fig. 3C).

ACSL3 was also upregulated by TNF-α in the RNA-seq data set, although the increase did not reach statistical significance after multiple comparison correction. Fewer genes were altered by ACSL3 knockdown (52 genes met the criteria above) and only nine of these genes were part of the TNF-α transcriptome (Fig. 3B). Furthermore, none of the validated TNF-α targets were significantly affected by ACSL3 knockdown (Fig. 3C). Together, these results suggest that TNF-α-induced upregulation of ACSL3 does not serve to significantly alter the TNF-α transcriptome.

TNF-α induces oleic acid LD formation in HCAECs through ACSL3

Distinct subcellular localization and substrate preference of ACSL isoforms have been reported, suggesting that ACSL isoforms are regulating acyl-CoA compartmentalization in cells in order to drive an interaction of fatty acid substrates with specific metabolic pathways. Proteomics of isolated LDs in different cell types revealed that, among the ACSL isoforms, ACSL3 is associated with LDs (24–26). Furthermore, high resolution time-lapse video of COS-1 cells revealed that expressed ACSL3 localized to the ER.
ACSL3 mediates lipid droplet formation in human endothelium

Fig. 3. Suppression of ACSL3 expression in HCAECs has no marked effects on the TNF-α transcriptome. A: RNA sequencing was performed in HCAECs from donor 1 stimulated with and without TNF-α (20 ng/ml) for 18 h in the presence of control siRNA or siACSL3. A heat map showing genes altered by TNF-α. Data are reported using cutoffs of 2 and −2 for the log2 of the ratio between experimental and control samples and a 5% FDR. Results are presented as means of triplicate samples. #ACSL3 is shown for validation, but did not meet the significance criteria when corrected for multiple comparisons. B: Venn diagram showing transcripts regulated by TNF-α and ACSL3 knockdown. C: Validation of altered levels of LTB, CXCL8, and ITGB5 mRNA in donor 1 and donor 2 by real-time PCR. Representative experiments from each donor among two to three independent experiments are shown. The results are expressed as mean ± SEM. One-way ANOVA followed by Tukey’s multiple comparison tests was applied (C). *P < 0.05; **P < 0.01; ***P < 0.001 as indicated.
and LDs depending on the supply of fatty acids, and that a tagged ACSL3 was recruited to assemble pre-LDs on the ER as well as for nucleation and maturation of LDs (27). The enzyme has also been shown to be essential for LD formation in COS cells (27, 28). The role of ACSL3 in LD formation and maturation in endothelial cells was unknown.

In order to observe cellular localization of endogenous ACSL3 in HCAECs, the cells were treated with oleic acid (18:1) for 3 h and immunostained with an ACSL3 antibody. ACSL3 immunostaining showed both ring-like and small dot-like structures in the cytoplasm, likely consisting of early LDs and pre-LDs (emerging LDs from the ER), respectively, as well as ER-like structures (Fig. 4A–C). The ACSL3 immunostaining was completely abolished in cells treated with ACSL3 siRNA (Fig. 4D), confirming antibody specificity and siRNA specificity. Cells were next costained with organelle markers of LDs, ER, and mitochondria (Fig. 4A). Distinct roundish structures positive for ACSL3 were costained with the ER marker, PDI, suggesting that a part of LDs, likely pre-LDs, contain both ACSL3 and the ER marker protein (Fig. 4B). Conversely, ACSL3 did not colocalize with CS, a mitochondrial marker (Fig. 4C). These findings demonstrate that ACSL3 is localized to LDs in human endothelial cells, consistent with previous reports (27).

Because TNF-α treatment increased ACSL3 expression in HCAECs, we next evaluated the effect of TNF-α on LD accumulation. HCAECs were treated with oleic acid (100, 200, or 400 μM for 18 h) in order to promote LD formation in the presence or absence of TNF-α. The LDs were stained with BODIPY 493/503, which probes neutral lipids, and the BODIPY fluorescence images were quantified as described in supplemental Fig. S1. The BODIPY-stained area per cell was increased by oleic acid in a dose-dependent manner. The addition of TNF-α significantly augmented LD accumulation for all concentrations of 18:1 (Fig. 4E–G).

We next investigated whether the effect of TNF-α on increased LD formation was due to upregulation of ACSL3 expression in HCAECs. Cells were treated with oleic acid with or without TNF-α for 18 h following transfection with either ACSL3 siRNA or control siRNA. First, we observed that ACSL3 immunoreactivity was more intense and clearly surrounding LDs in TNF-α-stimulated cells, as compared with the cells incubated without TNF-α (Fig. 5A). The TNF-α treatment significantly increased BODIPY-stained area per cell in control siRNA-transfected cells; however, the effects of TNF-α was markedly diminished in the cells treated with ACSL3 siRNA (Fig. 5B, C). These results indicate that upregulation of ACSL3 is necessary for increased LD formation in response to TNF-α.

The effects of TNF-α and ACSL3 on LD formation were most likely due to increased oleoyl-CoA synthesis and resulting increases in triacylglycerol levels, rather than due to transcriptional regulation, because the RNA-seq revealed no overall significant differences in genes involved in LD regulation (GO:0005811), PPAR signaling pathway (GO:0035357), or PPAR binding (GO:0042975) (supplemental Fig. S5).

Because of the possibility that LDs could alter TNF-α transcriptional events, we next measured the TNF-α target protein expression in the presence or absence of TNF-α. Because TNF-α treatment increased ACSL3 expression in HCAECs, we next evaluated the effect of TNF-α on LD accumulation. HCAECs were incubated with oleic acid (400 μM) for 3 h and fixed. The cells were stained with an anti-ACSL3 antibody and either an ADRP antibody (a LD marker) (A), a PDI antibody (an ER marker) (B), or a CS antibody (a mitochondrial marker) (C). D: To induce ACSL3 knockdown, the cells were transfected with ACSL3 siRNA (siACSL3), cultured for 3 days, and stained with anti-ACSL3 antibody. E, F: Representative BODIPY staining is shown in cells treated with 400 μM oleic acid alone (E) and oleic acid plus TNF-α (F). G: HCAECs were treated with or without TNF-α (20 ng/ml) in the presence of oleic acid (100, 200, or 400 μM) for 18 h and fixed. The percent of BODIPY-positive area/cell was measured as described in the Materials and Methods. Statistical significance was determined using two-way ANOVA followed by Holm-Sidak multiple comparison tests. Each row was analyzed individually, without assuming a consistent SD (n = 28–47 cells/experiment). The results are expressed as mean ± SEM. The experiment was repeated twice. ***P < 0.001 as indicated. Scale bar, 10 μm.
genes identified by RNA-seq in HCAECs incubated in the presence of oleate to induce LD formation. LD loading did not significantly alter the ability of TNF-α to induce ACSL3, CCL2, or LTB in cells treated with control siRNA or siACSL3 (supplemental Fig. S6A–C).

**ACSL3 mediates endogenous FAO in HCAECs**

Another role of ACSL3 could include providing cellular energy by generating acyl-CoAs for β-oxidation. To address this possibility, OCR was measured as an index of mitochondrial respiration using a Seahorse analyzer. HCAECs were transfected with ACSL3 siRNA or control siRNA, and then after 3 days were used for the measurement of OCRs in the presence or absence of etomoxir, an inhibitor of carnitine palmitoyltransferase I, in order to access FAO of endogenous fatty acids (Fig. 6A). FAO basal respiration and FAO ATP production were significantly reduced by ACSL3 knockdown, as compared with controls, while FAO maximal respiration was not significantly altered by ACSL3 knockdown (Fig. 6B). There were no significant differences in the ability of etomoxir to suppress OCR in control siRNA-treated cells and siACSL3-treated cells. Basal respiration was suppressed by etomoxir by 31.9 ± 3.5% (mean ± SEM; n = 6) in control siRNA-treated cells and by 28.2 ± 3.9% in siACSL3-treated cells and by 34.5 ± 4.0% in siACSL3-treated cells (P = 0.59); and maximal respiration was suppressed by etomoxir by 34.6 ± 6.1% in control siRNA-treated cells and by 38.0 ± 6.8% in siACSL3-treated cells (P = 0.94). Furthermore, TNF-α did not significantly alter FAO rates (supplemental Fig. S7A). Thus, ACSL3 contributes to endogenous basal FAO and ATP production in endothelial cells, while TNF-α has no significant effect on FAO.

**Suppression of ACSL3 expression in HCAECs modestly enhances TNF-α-induced prostaglandin release from HCAECs**

Finally, because enzymes involved in prostanoid production are present on LDs (29), and in some cells increased LDs provide the means for increasing prostanoid production, we evaluated the role of ACSL3 in PGE2 and PGI2 secretion in HCAECs. Our results revealed that ACSL3 deficiency caused a small but significant increase in PGE2 secretion in response to TNF-α in both donors (supplemental Fig. S7B). In the case of PGI2 secretion, the effect of ACSL3 knockdown was less prominent and was not consistent between the two donors (supplemental Fig. S7C). The effect of ACSL3 on prostanoid secretion did not appear to be due to changes in expression of genes involved in prostanoid synthesis [COX1 (PTGS1), COX2 (PTGS2), PGI2 synthase (PTGIS), or PGE synthase (PTGES)], at least not at the 18 h time-point. TNF-α induced COX2 mRNA levels and suppressed PGI2 synthase levels (supplemental Fig. S7D), consistent with published results (30, 31). Our results indicate that a third function of ACSL3 in human endothelial cells is to dampen TNF-α-induced PGE2 secretion, likely without altering transcriptional events.

**DISCUSSION**

Our results demonstrate that TNF-α promotes accumulation of LDs derived from oleate and that this process is
mediated by induction of ACSL3 in human macrovascular endothelial cells. Previous studies have shown that TNF-α promotes lipoprotein uptake in endothelial cells through upregulation of the LDL receptor and caveolins (5), and that TNF-α supports lipid bodies containing cholesterol in a human dermal microvascular endothelial cell line (32). Furthermore, preventing endothelial cell LDL transcytosis was recently shown to be associated with attenuated atherosclerosis (33).

Kuo, Lee, and Sessa (7) first defined the functions of LDs derived from fatty acids in endothelial cells and suggested that abnormal LDL metabolism may contribute to vascular disease (7). They demonstrated that mouse aortic endothelial cells exposed to increased fatty acids in vivo through olive oil gavage exhibit a transient LD appearance and disappearance (7). They further showed that oleic acid-mediated LD formation in endothelial cells serves a protective function because safe storage of fatty acids in LD triacylglycerol avoids accumulation of detrimental levels of free saturated fatty acids, which induce ER stress in these cells (7). In addition, they described that LDs are used as an energy source in endothelial cells (7). In addition, they described that LDs are used as a source of fatty acids for LD formation in endothelial cells, and because cAMP promotes lipolysis of these cells (43), suppression of PGE2 and PGI2 by TNF-α might serve as additional mechanisms to preserve LDL formation from fatty acids in ER microdomains in several cell types (26–28, 35–38). In HCAECs, ACSL3 immunostaining localized with some of the LDs, but not all. Previous studies have suggested the presence of two LD subpopulations; one relatively small that stays constant in size and a larger population that grows by lipid loading. The larger population contained enzymes for each step of triacylglycerol synthesis (39). In unstimulated cells, ACSL3 localized with small LDs, whereas in TNF-α stimulated cells, its localization was clearly observed surrounding large LDs. ACSL3 therefore appears to be involved in both steps of LD biogenesis, providing acyl-CoAs for triacylglycerol assembly.

In addition to LD accumulation, ACSL3 was shown to promote endogenous FAO. Induction of ACSL3 could serve to increase energy production in activated endothelial cells, or possibly to provide a source of carbon needed for de novo nucleotide synthesis through FAO (40), although TNF-α had no significant effect on endogenous FAO. The effect of ACSL3 knockdown on endogenous FAO is unlikely to result from indirect effects due to reduced LD formation because our FAO experiments were performed in the absence of exogenous fatty acids, under conditions at which LDs are very small or undetectable.

The third function of ACSL3 revealed by our studies is a modest suppression of TNF-α-mediated secretion of PGE2. Interestingly, previous studies have shown that LDs are sites of PGE2 synthesis (29). However, this mechanism does not appear to explain the effect of ACSL3 on PGE2 secretion because ACSL3 both promoted LD formation and suppressed PGE2 secretion. Instead, it is likely that ACSL3 suppresses prostanoid production by acylating free arachidonic acid, the precursor of eicosanoids. ACSL4, which has a high preference for arachidonic acid as a substrate, suppresses PGE2 release from human arterial smooth muscle cells (17). ACSL3 can use arachidonic acid as a substrate in some tissues, even though its selectivity for arachidonic acid is lower than that of ACSL4 (41, 42). Because PGE2 and PGI2 enhance receptor-mediated increases in cAMP in endothelial cells, and because cAMP promotes lipolysis of LDs in these cells (43), suppression of PGE2 and PGI2 by ACSL3 might serve as an additional mechanism to preserve LDs. It is therefore possible that TNF-α-induced ACSL3 serves both to increase LD formation and expansion and to prevent their lipolysis.

Why would formation and maintenance of fatty acid-derived LDs be important in macrovascular endothelial cells? Lipolysis products from lipoprotein particles might be a source of fatty acids for LD formation in endothelial cells...
Acyl-CoAs were analyzed through the Molecular Phenotyping Core, Michigan Nutrition and Obesity Center (P30DK089503).

REFERENCES


