SUPPLEMENTAL MATERIAL

Endothelial Acyl-CoA Synthetase 1 is not Required for Inflammatory and Apoptotic Effects of a Saturated Fatty Acid-Rich Environment

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Supplemental Methods

General methods
Real-time PCR, reverse transcriptase PCR, Western blot analysis, and ELISAs were performed as described previously.\textsuperscript{1,2} CCL2 ELISAs were obtained from eBioscience (San Diego, CA), and sICAM-1, sVCAM-1 and CXCL1 ELISAs from R&D Systems Inc. (Minneapolis, MN). The following ACSL antibodies were used for Western blot analysis: anti-ACSL1 (Cell Signaling, #4047, 1:1,000 dilution), anti-ACSL3 (Aviva, ARP46453_P050, 1:250 dilution), anti-ACSL4 (Aviva, ARP49775_P050 at a 1:1000 dilution), and anti-ACSL5 (Novus, NB100-2918, 1:1,000 dilution). Anti-phospho-SAPK/JNK (Thr183/Tyr185), anti-SAPK/JNK, and anti-myc antibodies were obtained from Cell Signaling (Beverly, MA). Primer sequences for real-time PCR and reverse transcriptase PCR (for Xbp1) are listed in Table S1. Epididymal blood vessels were detected by using the endothelium marker von Willebrand factor (vWF) for immunohistochemistry. In short, paraffin-embedded sections were incubated with an anti-vWF antibody (DAKO cat#A0082) at a 1:1000 dilution following proteinase K antigen retrieval (20 µg/mL in 10 mmol/L Tris, 1 mmol/L EDTA, pH 8.0). Rabbit IgG (Invitrogen) was used as a negative control, and resulted in no staining. Oxygen consumption by WT and ACSL1\textsuperscript{E/H-} cells in the presence of 5 mmol/L glucose with and without 156 µmol/L palmitate, was measured as described previoulsy.\textsuperscript{3}

Endothelial culture and fatty acid stimulation
The fluorescence-activated cell sorting method of MMEC isolation was based on the method described by Ieronimakis et al.,\textsuperscript{4} but modified slightly. In short, lungs or hearts were digested, and the CD45-negative CD31-positive population was used as ECs. The CD45^-CD31^ cell
population was expanded in culture and used for experiments. MMECs were isolated from WT Cre<sup>+</sup> control littermate mice and mice with endothelial and hematopoietic ACSL1-deficiency. MMECs from lung and heart showed similar results. Mouse heart and lung endothelial cells isolated by FACS as CD31<sup>+</sup> CD45<sup>−</sup> cells were cultured in high glucose Dulbecco’s Modified Eagle’s Medium (DMEM), 10% fetal bovine serum (FBS), penicillin (60 units/mL) and streptomycin (0.5 µg/mL), non-essential amino acids (Sigma), 100 mg/mL endothelial cell growth supplement (ECGS, Biomedical Technologies, Inc., Stoughton, MA, #BT-203-50MG) and 10 ng/mL recombinant vascular endothelial growth factor (VEGF; R&D Systems).

Mouse heart endothelial cells isolated by positive magnetic bead selection using an ICAM2 antibody<sup>5-6</sup> (eBioscience #13-1021-84) were cultured as described above, except that normal glucose DMEM and 10% heat-inactivated horse serum (Sigma) was used instead of FBS. BAECs were cultured in normal glucose DMEM with 10% fetal bovine serum (FBS) and nonessential amino acids in the presence of penicillin (60 units/mL) and streptomycin (0.5 µg/mL). For experiments, mouse endothelial cells were starved in the presence of 1% serum without ECGS and VEGF for 4 h prior to experiments, and experiments were performed in the same medium. BAECs were starved in the presence of 0.5% serum.

Sodium salts of fatty acids (Nucheck Prep, Elysian, MN) were dissolved in distilled H<sub>2</sub>O and diluted to a final concentration of 78 µmol/L in sterile DMEM containing 78 µmol/L fatty acid-free BSA (Sigma). This mixture was equilibrated for 1 hr at 37°C, 5% CO<sub>2</sub>, prior to addition to the cells, allowing BSA-fatty acid complexes to form. Fatty acid incubations were carried out with fatty acids pre-bound to low-endotoxin (≤0.1 ng/mg endotoxin) fatty acid-free BSA (product number A8806; Sigma, St. Louis, MO) at BSA:fatty acid molar ratios of 1:0 to 1:4. The concentrations of fatty acid and BSA were based on a physiological ratio between fatty acid and the carrier protein (BSA) and the estimated concentration of albumin present in the extracellular fluid of the intimal portion of the arterial wall.<sup>7</sup> Control cells were incubated with the same concentrations of BSA alone. In some experiments, the ECs were treated with the cell-permeable JNK Inhibitor I, (L)-Form, 10 µmol/L (EMD Millipore) or an unrelated JNK inhibitor (SP600125, 50 µmol/L, Biomol). These two unrelated inhibitors both suppressed palmitate-induced apoptosis, which makes it likely that the effects were mediated by JNK inhibition and not by off-target effects, although off-target effects cannot be completely ruled out. Thapsigargin treatment (Sigma) at 100 nmol/L for 4 h was used as a positive control for Xbp1 splicing. Media and reagents were analyzed by the chromogenic endpoint LAL test (Cambrex Corporation, East Rutherford, NJ) to exclude endotoxin contamination. Endotoxin levels were below 50 pg/mL.
Overexpression of ACSL1 in BAECs
Murine ACSL1 with an N-terminal Myc epitope tag\(^8\) was subcloned into the pBM retroviral vector and used to transfect amphotropic or ecotropic Phoenix cells to produce retrovirus. The infectious medium from the Phoenix cells was collected and added to BAECs. Puromycin was used for selection of transduced cells, as described previously.\(^5\)

Knock-down of ACSL1 in MMECs
MMECs were grown in T75 flasks. Upon reaching 80-90% confluence, cells were transfected with siRNA using an Amaxa Nucleofector kit (Lonza, Cologne, Germany). Briefly, cells were trypsinized and counted. Then, 5x10\(^5\) cells were pelleted and resuspended in Nucleofector solution containing 300 nmol/L siRNA. Cells were applied to Amaxa Nucleofector I and electroporated using program L-29. Cells were then transferred and plated for experiments, and were left resting for 24 h before further treatments. RNA samples were harvested or stimulated with fatty acids 20-24 h after electroporation. The siRNAs were Silencer Select\(^\text{®}\) pre-designed and validated siRNAs (Ambion; Grand Island, NY). Three siRNAs were tested for ACSL1 and the first one was selected for higher efficiency: ACSL1 s65834, s65832 and s65833. Negative siRNA control #2 (Ambion; Grand Island, NY) was used as control for all experiments.

Analysis of ACSL enzymatic activity, long-chain acyl-CoA species, beta-oxidation, glucose consumption and lactate production
ACSL enzymatic activity and long-chain acyl-CoA species were analyzed as described previously.\(^1\) [1-14C]-palmitic acid (1.48-2.22GBq/mmol; Perkin Elmer, Waltham, MA) was used for evaluation of beta-oxidation through measurements of acid-soluble metabolites in MMECs incubated in 10% serum.\(^1\) Glucose consumption and lactate production were measured in parallel experiments in MMECs in 10% serum, as described previously.\(^9\)

Apoptosis assays
Apoptosis/cell death was measured by three different methods. Floating cells were counted as a measure of cell death. ECs were plated in 24-well plates (60,000 cells/well) and treated with fatty acids or BSA alone for 2 or 3 days. Both floating and attached cells were counted by a Coulter Counter. Cell death was calculated as floating cells as percentage of the sum of dead and alive cells. Propidium iodide staining confirmed that the floating cells were dead. A caspase-3 fluorometric assay (R&D Systems) was used to measure caspase-3 activity, and the HT TiterTACS\(^\text{TM}\) assay kit (Trevigen, Inc., Gaithersburg, MD) was used to quantify apoptotic
cells through DNA fragmentation or terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL).

**Statistical analyses**
The results are expressed as mean ± SEM. Statistical analysis was performed using Student’s t-test (for comparison of two groups), one-way ANOVA with Neuman-Keuls Multiple Comparison post hoc test, or two-way ANOVA. Probabilities <0.05 were considered statistically significant and were denoted as *p<0.05, **p<0.01, ***p<0.001.

**Supplemental References**


**Supplemental Figure Legends**

**Figure S1.** Palmitate and stearate exert pro-inflammatory and pro-apoptotic effects in bovine aortic endothelial cells, and the effects are prevented by oleate and an acyl-CoA synthetase inhibitor. **A,** BAECs were treated with 156 µmol/L of each fatty acid for 24 h (fatty acid:BSA ratio 2:1 or 4:1), and Ccl2 mRNA was measured by real time PCR. **B,** BAECs were incubated in the absence or presence of 156 µmol/L of each fatty acid for 2 days (fatty acid:BSA ratio 2:1 or 4:1). Cell death was calculated as % floating cells out of total cells. **C,** BAECs were stimulated with 156 µmol/L 16:0 bound to BSA at a 2:1 molar ratio, with or without triacsin C (Tri-C; 0.3 µmol/L) for 24 h. Ccl2 gene expression was evaluated by real-time PCR. **D,** BAECs were stimulated with 156 µmol/L 16:0 bound to BSA at a 2:1 molar ratio, with or without triacsin C (Tri-C; 0.3 µmol/L) for 24 h. Caspase 3 activity was measured by a caspase-3 fluorometric assay (R&D Systems). **E,** ACSL activity was measured as the rate of conversion of [3H]-16:0 to [3H]-palmitoyl-CoA. N=3-6; mean ± SEM

**Figure S2.** Relative levels of Acsl mRNA in mouse whole heart and heart MMECs. RNA was extracted from whole hearts or isolated mouse heart MMECs, reverse transcribed and subjected to real time PCR. All values were normalized to Rn18s and expressed as fold over MMEC levels for each Acsl isoform using the ΔΔCt method. N=3; *p<0.05; 2-way ANOVA followed by Bonferroni post hoc tests.

**Figure S3.** ACSL1-deficiency does not affect beta-oxidation of palmitate in MMECs. **A,** Beta-oxidation of [1,14C]-palmitate was measured as production of acid-soluble metabolites (ASM) in heart MMECs treated with Acsll siRNA or control siRNA. **B,** Glucose consumption was measured as loss of glucose from the conditioned media during a 24 h incubation in the presence of 10% serum. **C,** Lactate release was measured in the same samples from heart MMECs (passage 4). N=3-5; mean ± SEM; white bars, MMECs expressing control siRNA; gray bars, MMECs expressing Acsll siRNA.
Figure S4. ACSL1-deficient MMECs are not protected from saturated fatty acid-induced Xbp1 splicing. Xbp1 splicing was determined by reverse transcriptase PCR in heart MMECs treated with control siRNA or Acsl1 siRNA, rested for 24 h, and then incubated in the presence or absence of 16:0 or 18:0 bound to BSA at a 1:2 or 1:3 molar ratio, or in the presence of BSA alone for 20 h. Primers specific for spliced Xbp1 and total Xbp1 were used. The primers for total Xbp1 detect both the spliced and unspliced form. Middle panel, the samples were run on the same gel. Lower panel, this experiment included basal and MMECs treated with 18:0 at a 1:3 fatty acid:BSA molar ratio. B; basal BSA alone.
Supplemental Table 1: Primers for real-time PCR and reverse transcriptase PCR

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Figure S1

A. *Ccl2* mRNA

B. Cell death

C. *Ccl2* mRNA

D. Caspase 3 activity

E. ACSL activity

### A. *Ccl2* mRNA

![Bar graph showing Ccl2 mRNA expression](image)

- **Basal 18:1**
- **16:0**
- **16:0 + 18:0**

### B. Cell death

![Bar graph showing cell death](image)

- **Basal 18:1**
- **16:0**
- **16:0 + 18:0**

### C. *Ccl2* mRNA

![Bar graph showing Ccl2 mRNA expression](image)

- **Basal 16:0**
- **Tri-C 16:0**
- **Tri-C 16:0 + Tri-C**

### D. Caspase 3 activity

![Bar graph showing caspase 3 activity](image)

- **Basal 16:0**
- **Tri-C 16:0**
- **16:0 + Tri-C**

### E. ACSL activity

![Bar graph showing ACSL activity](image)

- **Basal**
- **Tri-C**

Note: The graph shows statistical significance levels indicated by asterisks: ***p < 0.001, **p < 0.01, *p < 0.05.
Figure S2

Acsl isoforms: Acsl1, Acsl3, Acsl4, Acsl5, Acsl6

Acsl mRNA (fold over EC)

Heart

MMECs

Whole heart

Acs1 mRNA (fold over MMECs)

*
A. ASM production (siRNA)  B. Glucose cons. (siRNA)  C. Lactate production (siRNA)
Figure S4

Control siRNA

Acs1 siRNA

Spliced Xbp1

Total Xbp1

Control siRNA

Acs1 siRNA

Control siRNA

Acs1 siRNA

Spliced Xbp1

Total Xbp1

Control siRNA

Acs1 siRNA

+ + - - + + - - + +