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

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Objective—Saturated fatty acids, such as palmitic and stearic acid, cause detrimental effects in endothelial cells and have been suggested to contribute to macrophage accumulation in adipose tissue and the vascular wall, in states of obesity and insulin resistance. Long-chain fatty acids are believed to require conversion into acyl-CoA derivatives to exert most of their detrimental effects, a reaction catalyzed by acyl-CoA synthetases (ACSLs). The objective of this study was to investigate the role of ACSL1, an ACSL isoform previously shown to mediate inflammatory effects in myeloid cells, in regulating endothelial cell responses to a saturated fatty acid-rich environment in vitro and in vivo.

Methods and Results—Saturated fatty acids caused increased inflammatory activation, endoplasmic reticulum stress, and apoptosis in mouse microvascular endothelial cells. Forced ACSL1 overexpression exacerbated the effects of saturated fatty acids on apoptosis and endoplasmic reticulum stress. However, endothelial ACSL1 deficiency did not protect against the effects of saturated fatty acids in vitro, nor did it protect insulin-resistant mice fed a saturated fatty acid-rich diet from macrophage adipose tissue accumulation or increased aortic adhesion molecule expression.

Conclusion—Endothelial ACSL1 is not required for inflammatory and apoptotic effects of a saturated fatty acid-rich environment. (Arterioscler Thromb Vasc Biol. 2013;33:232-240.)

Key Words: acyl-CoA synthetase ■ apoptosis ■ endothelium ■ inflammation

The endothelium protects the organism from a number of diseases, including vascular disease and insulin resistance. However, diabetes mellitus and cardiovascular risk factors cause changes in the endothelium, leading to endothelial dysfunction. Elevated levels of fatty acids are generally believed to result in endothelial dysfunction. Palmitic acid (16:0) and stearic acid (18:0) are 2 common saturated fatty acids that cause endothelial dysfunction in vitro, including proinflammatory signaling and apoptosis. Increased activation of the ceramide pathway and toll-like receptor 4 (TLR4) have been implicated in the pro-inflammatory changes elicited by palmitate. However, the mechanism by which TLR4 is modulated by saturated fatty acids remains controversial. Saturated fatty acids also induce apoptosis by a pathway that appears to require endoplasmic reticulum (ER) membrane saturation and subsequent ER stress, as well as activation of nuclear factor-xB signaling. ER stress induces apoptosis through activation of the protein kinase c-Jun N-terminal kinase (JNK) in many cells. These mechanisms require conversion of the free saturated fatty acids to their acyl-CoA derivatives.

Long-chain acyl-CoA synthetase (ACSL) isoforms ligate long-chain fatty acids to the CoA moiety. Conversion of free fatty acids into their acyl-CoA derivatives is required for channeling of fatty acids into cellular lipid pools and β-oxidation, and most of the biological actions of fatty acids in cells. ACSL1, ACSL3, ACSL4, and ACSL5 are expressed in endothelial cells (ECs). Specific functions of these ACSL isoforms in ECs are unknown.

Forced overexpression of ACSL1 in the heart causes cardiac lipotoxicity and increased apoptosis, whereas ACSL1 deficiency in myeloid cells protects against the inflammatory effects of diabetes mellitus in monocytes and macrophages, as well as diabetes-accelerated atherosclerosis. We therefore hypothesized that ACSL1-dependent acyl-CoA synthesis mediates the detrimental effects of saturated fatty acids in ECs in vitro and in vivo.

Our findings show that forced overexpression of ACSL1 in ECs indeed exacerbates saturated fatty acid–induced apoptosis.
and ER stress. However, ACSL1-deficiency does not protect ECs against the proinflammatory and proapoptotic effects of saturated fatty acids in vitro, nor does it protect against increased adipose tissue macrophage accumulation or aortic expression of vascular cell adhesion molecule 1 (Vcam1) in a saturated fat-fed mouse model of insulin resistance. These findings suggest that when overexpressed, ACSL1 is sufficient to exacerbate the effects of saturated fatty acids on ER stress and apoptosis, but that normally, ACSL1 does not contribute in major ways to these effects of saturated fatty acids, or to inflammatory changes associated with high-fat diet feeding.

Materials and Methods

Generation of Conditional ACSL1 Endothelial and Hematopoietic-Deficient Mice

Acsl1fl/fl mice were generated, as described previously, and back-crossed 10 generations into the C57BL/6 background. These mice were then crossed with Tie2-Cre mice (The Jackson Laboratory, Bar Harbor, ME) on a C57BL/6 background to obtain mice with endothelial and hematopoietic ACSL1 deficiency. Female breeders were Cre-negative to avoid germ-line transmission. The resulting Acsl1fl/fl Tie2-Cre+ mice were termed ACSL1 endothelial and hematopoietic-deficient mice (ACSL1E/H–/– mice). Littermate controls carried the Cre transgene, but were wild-type (WT) for Acsl1 (Acsl1fl/fl Tie2-Cre–). In addition, Acsl1fl/fl ‘Tie2-Cre’ control mice were used for some experiments.

Insulin-Resistant Obese Mouse Model

Male ACSL1E/H–/– mice and WT littermate controls (10–12 weeks of age) were fed chow diet or a diabetogenic diet with added cholesterol (DDC) rich in saturated fatty acids for 20 weeks, as previously described. Glucose tolerance tests were performed at week 18, as previously described.

Isolation and Culture of Endothelial Cells

Mouse microvascular endothelial cells (MMECs) were isolated by a fluorescence-activated cell-sorting method from lungs or hearts, or by the magnetic bead separation method between passage 3 and 5. Most of the experiments were performed on heart MMECs isolated by the magnetic bead separation method between passage 3 and 6. Bovine aortic endothelial cells (BAECs) were purchased from Cambrex Bioscience (Walkersville, MD).

Additional methods are described in the online-only Data Supplement.

Results

Palmitate and Stearate Promote Inflammatory Changes and Apoptosis in Mouse Microvascular Endothelial Cells

MMECs exposed to palmitate (16:0) or stearate (18:0) bound to BSA at indicated molar ratios for 24 hours exhibited dose-dependent increases in secretion of the chemokines CCL2 (chemokine [C-C motif] ligand 2) and CXCL1 (chemokine [C-X-C motif] ligand 1), as well as increased shedding of soluble vascular cell adhesion molecule 1 (sVCAM-1) and soluble intracellular adhesion molecule 1 (sICAM-1), as shown in Figure 1A through 1D. Significant effects of 16:0 were observed at 1:2 and 1:3 BSA:fatty acid molar ratios, corresponding to 156 μmol/L and 234 μmol/L at 16:0, respectively, whereas 18:0 was more potent and induced significant changes at 1:0.5 to 1:1 BSA:fatty acid molar ratios, corresponding to 39 μmol/L and 78 μmol/L, respectively. These concentrations of 18:0 are in the physiological range for human plasma.

Stearate and palmitate also induced increased caspase 3 activity in these MMECs (Figure 1E) and apoptosis (Figure 1F). Again, stearate was more potent than palmitate (Figure 1F). Thus, saturated fatty acids, and particularly stearate, induce an inflammatory response and apoptosis in MMECs, consistent with studies on ECs from other vascular beds and species.

Forced Overexpression of ACSL1 Results in Increased Palmitoyl-CoA and Stearoyl-CoA levels, and Exacerbated Apoptosis, ER Stress, and JNK Activation in Palmitate-Stimulated Bovine Aortic Endothelial Cells

The proinflammatory and proapoptotic effects of saturated fatty acids are likely to be mediated by the acyl-CoA derivatives of these fatty acids, as discussed above. To investigate the effect of ACSL1 on saturated fatty acid-induced changes in ECs, we next stably overexpressed ACSL1 by using a retroviral vector in BAECs. BAECs were used for these experiments because of their greater proliferative capacity compared with MMECs. Both 16:0 and 18:0 increased chemokine (Ccl2) expression and cell death in these cells, like in MMECs, whereas oleate (18:1) prevented these effects (Figure IA and IB in the online-only Data Supplement). The proinflammatory and proapoptotic effects of 16:0 were also prevented by triacsin C, an acyl-CoA synthetase inhibitor that inhibits most ACSL isoforms, consistent with previous studies (Figure IC–IE in the online-only Data Supplement). BAECs infected with the ACSL1 retroviral vector exhibited clearly detectable myelocytomatosis oncogene-tagged ACSL1 protein compared with the empty vector (pBM)-infected controls (Figure 2A). Consistent with these observations, cell extracts from BAECs overexpressing ACSL1 exhibited a significant increase in palmitoyl-CoA synthesis (Figure 2B). Whereas there were no significant differences in acyl-CoA levels between ACSL1-overexpressing cells and controls under basal low-serum conditions, both palmitoyl-CoA and stearoyl-CoA levels increased in palmitate-stimulated cells overexpressing ACSL1 (Figure 2C). Forced ACSL1 overexpression exacerbated palmitate-induced BAEC cell death and caspase 3 activity (Figure 2D and 2E). Saturated fatty acids can promote JNK activation, thereby inducing apoptosis and proinflammatory changes in different cell types. To investigate whether ACSL1 overexpression exacerbates the effects of 16:0 on JNK activation, BAECs were stimulated with 16:0 for 4 hours. Cells stimulated with 16:0 demonstrated an increased JNK phosphorylation, and ACSL1 overexpression further increased 16:0-induced JNK phosphorylation (Figure 2F and 2G).

ER stress is often linked to JNK activation. Therefore, Hspa5 (Grp78) mRNA, an ER stress marker, was used to evaluate ER stress in control and ACSL1-overexpressing BAECs. Consistent with the JNK activation, Hspa5 mRNA levels were increased by palmitate-stimulation, and overexpression of ACSL1 resulted in a further elevation of Hspa5 mRNA levels (Figure 2H). Increased ER stress has been shown to be associated with an increased presence of saturated fatty acids in the membrane, and consistently, palmitate-stimulated BAECs
exhibited a marked increase in membrane palmitate levels, which was exacerbated by ACSL1 overexpression (Figure 2I). Thus, ACSL1 overexpression results in membrane fatty acid saturation after palmitate-stimulation, and accompanied ER stress, JNK activation, and increased apoptosis.

To investigate whether the increased JNK activation contributes to apoptosis in palmitate-stimulated BAECs, we used the cell-permeable JNK Inhibitor I (EMD Millipore). As shown in Figure 2I, the JNK inhibitor partially, but significantly, protected palmitate-stimulated BAECs as well as ACSL1-overexpressing BAECs from apoptosis, as measured by reduced DNA fragmentation (TUNEL). Similar results were obtained using an unrelated JNK inhibitor (SP600125, Biomol; data not shown), suggesting that activation of the JNK pathway is, in part, responsible for the increased apoptosis in palmitate-stimulated BAECs and BAECs overexpressing ACSL1.

Finally, overexpression of ACSL1 did not significantly alter oxygen consumption, suggesting that the metabolic state of the ECs was not markedly different depending on ACSL1 levels (Figure 2K).

Endothelial ACSL1-Deficiency Does not Provide Protection From Saturated Fatty Acid-Induced Proapoptotic or Proinflammatory Changes

To investigate whether intrinsic ACSL1 promotes proapoptotic and proinflammatory changes in saturated fatty acid-exposed ECs, we next generated mice with endothelium and hematopoietic-targeted ACSL1 deficiency, Acsl1<sup>lox/lox</sup> Tie2-Cre<sup>Tg</sup> (ACSL1<sup>E/H--/-</sup>) mice and control littermate WT controls (Acsl1<sup>lox/lox</sup> Tie2-Cre<sup>Tg</sup>/+). As shown in Figure 3A, the ECs from ACSL1<sup>E/H--/-</sup> mice demonstrated selective loss of ACSL1 (Figure 3B), ACSL1 protein was also reduced in MMECs from ACSL1<sup>E/H--/-</sup> mice (Figure 3C).

As a second approach, ACSL1 expression was knocked down by small interfering RNA (siRNA). Acsl1 siRNA reduced Acsl1 mRNA levels by 80% to 90% in MMECs, under both basal and fatty acid-stimulated conditions (Figure 3D). ACSL1 protein levels were also reduced, whereas no significant compensatory upregulation was found for ACSL3 or ACSL4 protein (Figure 3E). However, fatty acid stimulation, and especially stearate, upregulated Acsl3 and Acsl4 mRNA levels in MMECs (Figure 3F), suggesting that these ACSL isoforms might take on a more important role in saturated fatty acid-stimulated MMECs.

No compensatory upregulation of Acsl3, Acsl4, or Acsl5 was observed in MMECs treated with Acsl1 siRNA, under basal or fatty acid-stimulated conditions (Figure 3F).

We next investigated fatty acid and glucose utilization in MMECs deficient in ACSL1. ACSL1 knockdown did not result in a measurable reduction in palmitate β-oxidation (Figure IIIA in the online-only Data Supplement), no differences in glucose consumption, and a slight increase in lactate production (Figures IIIB and IIIC in the online-only Data Supplement). These experiments also demonstrated that these MMECs are highly glycolytic cells.

Cultured MMECs from WT and ACSL1<sup>E/H--/-</sup> mice responded to saturated fatty acid stimulation with an increased secretion of CCL2 (data not shown), and increased shedding of sVCAM-1 and sICAM-1 (Figure 4A and 4B). ACSL1 deficiency did not protect against these effects, but rather tended to exacerbate the stearate-induced effects. Furthermore, palmitate- and stearate-induced ER stress, as measured by increased Hspa5 (Grp78) and Ddit3 (Chop) mRNA levels and Xhp1 splicing (Figure 4C and 4D and Figure IV in the online-only Data Supplement). ACSL1 deficiency did not protect the cells from the stearate- or palmitate-induced ER stress response, but rather increased levels of the chaperone Hspa5 (Figure 4C). ACSL1 deficiency also did not protect against saturated fatty acid-induced apoptosis, measured as DNA fragmentation (Figure 4E). The lack of effects of ACSL1 deficiency was confirmed in MMECs in which ACSL1 had been downregulated.
by siRNA. Consistent with the results on MMECs from ACSL1E/H–/– mice, Acsl1 siRNA did not result in an inhibition of stearate-induced sVCAM-1 or sICAM-1 shedding, ER stress response genes, or apoptosis, but rather an increase in these responses was observed (Figure 4F–4J). Thus, ACSL1 is not required for saturated fatty acid-induced proinflammatory and proapoptotic effects in cultured MMECs.

Saturated fatty acids have been shown to mediate inflammatory effects through TLR4 in ECs, and we therefore repeated some of these experiments in MMECs isolated from hearts of Tlr4+/+ and Tlr4 –/– mice. In agreement with previous findings, TLR4 deficiency exerted a protective effect on stearate-induced CCL2 secretion, and sVCAM-1 and sICAM-1 shedding (Figure 5A–5C), whereas it did not significantly protect against saturated fatty acid-induced apoptosis (Figure 5D). Thus, in contrast to ACSL1 deficiency, TLR4 deficiency blunts the inflammatory effects of stearate. The effects of palmitate were weaker than those of stearate and were not affected by TLR4 deficiency. It is possible that palmitate mediates its effects, in part, through other mechanisms, such as through activation of TLR2.

Figure 2. Acyl-CoA synthetase (ACSL) 1 overexpression exacerbates effects of palmitate in bovine aortic endothelial cells (BAECs). BAECs were transduced with the empty pBM vector, a vector encoding ACSL1-mycelocytomatosis oncogene (myc), or no vector (control). A, Cell lysates (45 μg/lane) were separated on SDS-PAGE gels. Blots were probed with anti–myc and anti–β-actin antibodies. B, ACSL activity was analyzed in cell lysates as the rate of conversion of [3H]-16:0 to [3H]-palmitoyl-CoA. C, BAECs were treated with or without 156 μmol/L 16:0 bound to BSA at a 1:2 BSA: fatty acid ratio for 24 hours. Acyl-CoA species were quantified by liquid chromatography-electrospray ionization tandem mass spectrometry. D, BAECs were treated with 16:0 for 2 days, and cell death was determined as the percentage of floating propidium iodide-positive cells. E, Caspase 3 activity was measured after 16:0 treatment for 24 hours. F and G, Cells infected with pBM or ACSL1 were stimulated with 16:0 for 4 hours. Total cell lysates (40 μg/lane) were separated on 12% SDS-PAGE gels. Blots were probed with antiphospho-JNK and anti-JNK antibodies. H, Hspa5 mRNA was measured by real-time PCR after 24-hour stimulation. I, Membrane fatty acid composition of BAECs was analyzed by gas chromatography-mass spectrometry after 24-hour stimulation with 16:0 and expressed as percentage of total fatty acids analyzed. J, Apoptosis was detected using the HT TiterTACS assay in BAECs incubated in the presence or absence of a cell-permeable JNK inhibitor I (JNK I; 10 μmol/L). K, Oxygen consumption was measured in real-time. Data represents the steady state values of kinetic data, after normalizing to the value obtained at 5 mmol/L glucose without palmitate (the oxygen consumption rate at 5 mmol/L glucose was 1.26±0.35 mmol/min per 10⁵ cells for pBM-transduced BAECs, and 0.69±0.28 mmol/min per 10⁵ cells for BAECs overexpressing ACSL1), n=3 to 6; mean±SEM; white bars indicate cells infected with the empty pBM vector; and gray bars, ACSL1-overexpressing cells.
parameters. Plasma levels of sICAM-1 and sVCAM-1 were not elevated by the DDC, and were not affected by ACSL1 deficiency (Figure 6E and 6F), but aortic levels of Vcam1 mRNA were significantly elevated by DDC-feeding (Figure 6G). Endothelial ACSL1 deficiency did not protect the aorta from DDC-induced Vcam1 induction (Figure 6G).

Macrophages are known to accumulate in adipose tissue in mice fed high-fat diets, including the diet used in the present study.20,21 This macrophage accumulation requires monocytes to bind to and traverse microvessels. We therefore next investigated the effect of DDC and endothelial ACSL1 deficiency in epididymal fat from chow-fed and DDC-fed mice. ACSL1E/H–/– mice exhibited no significant differences in the number or morphology of microvessels in epididymal fat (Figure 6H). As expected, DDC-fed mice had significantly more macrophages in epididymal fat (measured as increased levels of the myeloid cell/macrophage markers Emr1 and Cd11b) and increased levels of Ccl2 mRNA (Figure 6I–6K), as compared with chow-fed mice. Endothelial ACSL1 deficiency had no effect on these parameters (Figure 6I–6K).

Also, there were no differences in ER stress markers between the 4 groups of mice (data not shown).

To evaluate whether the mice had been exposed to elevated saturated fatty acid ratios, fatty acid composition of the chow diet and DDC as well as plasma fatty acid composition in the 4 groups of mice were analyzed by gas chromatography-mass spectrometry. The DDC had higher relative levels of 16:1, 16:0, 18:1, and 18:0, and lower relative levels of 18:2 as compared with the chow diet, and the ratio of saturated fatty acids/unsaturated fatty acids was significantly higher (Figure 6L). Plasma levels of fatty acids in the 4 groups of mice generally reflected the fatty acid composition of the diet. Thus, mice fed DDC exhibited lower relative levels of 18:2 and higher relative levels of 18:1 and 18:0, as compared with chow-fed mice (Figure 6M). There were no differences in 16:0 plasma levels between the different groups. Endothelial ACSL1 deficiency did not alter plasma fatty acid composition (Figure 6M). Finally, cultured MMECs were exposed to fatty acid ratios mimicking those found in plasma of chow-fed and DDC-fed mice. The fatty acids were bound to BSA at a 1:2 BSA:fatty acid ratio. As shown at Figure 6N, sVCAM-1 shedding was the same in MMECs exposed to fatty acid ratios mimicking plasma fatty acid ratios in chow-fed mice and DDC-fed mice. Similarly, CCL2 secretion, sICAM-1 shedding, and apoptosis were the same in both groups (data not shown).

These studies suggest that endothelial ACSL1 deficiency does not protect mice from a diet rich in saturated fatty acids on adipose tissue macrophage accumulation or aortic VCAM-1 expression, consistent with the in vitro studies.

Figure 3. Acyl-CoA synthetase (ACSL) 1 deficiency in mouse microvascular endothelial cells (MMECs) induced by Tie2-Cre or small interfering RNA (siRNA). A, PCR amplification of Acsl1 LoxP sites and Tie2-Cre recombinase. B, Levels of Acs1 mRNA in heart MMECs and liver from ACSL1E/H–/– mice and wild-type (WT) littermates were measured by real-time qPCR. C, ACSL1 protein levels in lung MMECs from ACSL1E/H–/– mice and WT littermates. ACSL1 runs above a prominent nonspecific band in MMECs. D, Acs1 mRNA levels in heart MMECs grown in 10% serum. Quantification of ACSL1 and ACSL3 protein levels (n=3) is shown below the representative blots. E, Acsl3, Acsl4, and Acs1 mRNA levels in the samples analyzed in D. n=3 to 5; mean±SEM; white bars indicate WT MMECs (B) or MMECs expressing control siRNA (D–F); and gray bars, MMECs expressing Acs1 siRNA (B) or MMECs expressing Acs1 siRNA (D–F).
Discussion

We demonstrate that, whereas forced overexpression of ACSL1 in ECs results in exacerbation of apoptosis, ER stress, and JNK activation induced by saturated fatty acids, ACSL1 deficiency does not protect ECs in vitro or in vivo from the detrimental effects of a saturated fatty acid-rich environment. Thus, overexpression of ACSL1 is likely to cause effects that are not normally conferred by ACSL1, perhaps because...

Figure 4. Acyl-CoA synthetase (ACSL) 1-deficient mouse microvascular endothelial cells (MMECs) are not protected from the proinflammatory or proapoptotic effects of saturated fatty acids. Soluble vascular cell adhesion molecule 1 (sVCAM-1) and soluble intracellular adhesion molecule 1 (sICAM-1) shedding was measured by ELISA after a 24-hour stimulation with 16:0 or 18:0 at a 1:2 BSA:fatty acid molar ratio in MMECs from ACSL1<sup>E/H–/–</sup> and wild-type (WT) mice (A and B) or heart MMECs treated with Acsl1 small interfering RNA (siRNA) or control siRNA (F and G). The ER stress markers Hspa5 and Ddit3 were measured by real-time PCR in MMECs from ACSL1<sup>E/H–/–</sup> and WT mice (C and D) or MMECs treated with Acsl1 siRNA or control siRNA (H and I). Apoptosis was detected using the HT TiterTACS assay in MMECs from ACSL1<sup>E/H–/–</sup> and WT mice (E) or MMECs treated with Acsl1 siRNA or control siRNA (J). The effects of fatty acids were lower (F–J) in electroporated cells, and the palmitate effect was not significant and therefore not shown. n=3 to 5; mean±SEM; white bars indicate WT MMECs (A–E) or MMECs expressing control siRNA (F–J); and gray bars, MMECs from ACSL1<sup>E/H–/–</sup> mice (A–E) or MMECs expressing Acsl1 siRNA (F–J).

Figure 5. Toll-like receptor-4 (TLR4) deficiency exerts protective effects in stearate-stimulated mouse microvascular endothelial cells (MMECs). A–C, CCL2 (chemokine [C-C motif] ligand 2) secretion, soluble vascular cell adhesion molecule 1 (sVCAM-1) shedding and soluble intracellular adhesion molecule 1 (siCAM-1) shedding were measured by ELISA in conditioned media from Tlr4<sup>+/+</sup> or Tlr4<sup>–/–</sup> heart MMECs (passage 4) incubated in the presence or absence of 16:0 or 18:0 bound to BSA at a 1:3 molar ratio, or in the presence of BSA alone for 24 hours (Basal). D, Apoptosis was detected using the HT TiterTACS assay. n=3 to 6; mean±SEM; white bars indicate Tlr4<sup>+/+</sup> MMECs; and black bars, Tlr4<sup>–/–</sup> MMECs.
downstream enzymes that further process the generated palmitoyl-CoA and stearoyl-CoA are not able to efficiently deal with the increased acyl-CoA levels. In addition, overexpression of ACSL1 is likely to result in increased uptake and trapping of saturated fatty acids through vectorial acylation, especially under in vitro conditions in which only saturated fatty acids are provided. Consistent with these findings, overexpression of ACSL1 in the heart causes increased apoptosis and cardiomyopathy,17 and this can be prevented by also overexpressing diacylglycerol acyltransferase 1, which promotes downstream triacylglycerol formation.32 Palmitate has previously been shown to cause ER stress and apoptosis by increasing ER membrane saturation.12 Our finding that ACSL1 overexpression exacerbates palmitate-induced apoptosis, ER stress, and membrane palmitate accumulation suggests that ACSL1 has the ability to enhance this pathway in ECs.

ACSL1 normally expressed in ECs is likely to act in different subcellular compartments or on different fatty acids, as compared with overexpressed ACSL1 because endothelial ACSL1 deficiency did not protect against the effects of obesity and insulin resistance on adipose tissue macrophage accumulation or aortic vascular cell adhesion molecule 1 (VCAM-1) expression. Male wild-type (WT) and ACSL1E/H–/– mice were fed regular chow or diabetogenic diet with added cholesterol (DDC) for 20 weeks. A, Body weights at the end of the study. B, Fasting (5 hours) blood glucose levels at week 18. C, Glucose tolerance tests were performed at week 18 by injecting 1.5 g/kg dextrose into 5-hour fasted mice, and blood glucose was measured at indicated times. D, Plasma cholesterol levels at the end of the study. E and F, Plasma soluble intracellular adhesion molecule 1 (sICAM-1) and soluble vascular cell adhesion molecule 1 (sVCAM-1) levels were measured by real-time PCR. H. Epidymal fat pads were isolated and sectioned. An anti-von Willebrand factor antibody was used to detect endothelial cells (ECs) in microvessels. Scale bar, 20 μm. I–K, The macrophage markers Emr1 (F4/80) and Cd11b, and Ccl2 mRNA were measured in epididymal adipose tissue by real-time PCR. L, Fatty acid composition of the Chow diet and DDC was analyzed by gas chromatography-mass spectrometry (GC-MS) and was expressed as saturated/unsaturated fatty acid ratio. M, Fatty acid composition of plasma samples was measured by GC-MS. N, sVCAM-1 shedding was measured by ELISA from mouse microvascular endothelial cells (MMECs) incubated in the presence of fatty acid ratios reflecting plasma fatty acids in chow-fed and DDC-fed mice. n=5 to 8 per group; means±SEM; white bars indicate WT mice; and gray bars, ACSL1E/H–/– mice (A–G, I–K, M). In L and N, white bars indicate chow diet (L) or a fatty acid ratio mimicking that of chow diet (N) whereas gray bars indicate DDC (L) or a fatty acid ratio mimicking that of DDC (N).
ECs, consistent with results from the present study, which demonstrated that triacsin C prevents the proinflammatory and proapoptotic effects of palmitate in BAECs. ACSL1 acts on a broad range of fatty acids, but is not specific to saturated fatty acids. For example, in hepatocytes, oleoyl-CoA levels are most reduced by ACSL1 deficiency,17 whereas in thioglycollate-elicited macrophages, arachidonoyl-CoA levels are preferentially reduced by ACSL1 deficiency.18 As ECs do not express significant levels of ACSL6, it is possible that the effects of palmitate and stearate are mediated by ACSL4, ACSL3, and ACSL5 in ECs. Consistently, our results suggest an increase in ACSL3 and ACSL4 expression in fatty acid-stimulated ECs. However, part of the effect of saturated fatty acids on release of inflammatory markers and apoptosis might be mediated by the free fatty acids, for example, by altering membrane biophysical properties. The tendency of ACSL1 deficiency to exacerbate the effects of stearate supports this interpretation. Contrary to ACSL1 deficiency, TLR4 deficiency conferred protective effects against the inflammatory effects of stearate, consistent with published studies. Thus, ACSL1 deficiency and TLR4 deficiency exert the opposite effects in stearate-stimulated ECs.

Rather than mediating effects of saturated fatty acids, ACSL1 appears to have other biological functions in ECs. ACSL1 has a prominent role in mediating β-oxidation in many tissues depending on fatty acids as an energy source, such as heart, liver, and adipose tissue. Conversely, we have recently demonstrated that in macrophages, ACSL1 does not contribute to β-oxidation, but has a prominent role in mediating inflammatory changes in response to diabetes mellitus, most likely by acting on arachidonic acid. ACSL1 also does not appear to contribute to β-oxidation in ECs, at least not under the conditions tested. Overall, these studies support the hypothesis that ACSL isoforms may have distinct biological functions that are context- and cell type-dependent, and perhaps dependent on the availability of different fatty acid substrates under different conditions, as well as the metabolism of the cell. The current work is the first study to investigate the biological role of any of the ACSL isoforms in ECs in vivo. Future studies on endothelial ACSL1 include its potential role in atherosclerosis, vasoreactivity, and blood pressure regulation, as well as evaluation of potential effects of ACSL1 on endothelial nitric oxide synthase activity. The study also raises more complex questions. For example, to what extent do plasma levels of saturated fatty acids contribute to inflammatory reactions in vivo? Ratios of fatty acids mimicking those found in plasma of mice fed the high-fat diet used in the present study did not promote inflammatory changes or apoptosis, as compared with ratios of fatty acids reflecting plasma levels in chow-fed mice. It is possible that the inflammatory effects of diets rich in saturated fatty acids are due to associated systemic factors, such as cholesterol, or to increased tissue levels of saturated fatty acids or acyl-CoAs. In this context, it is interesting to speculate that upregulation of ACSL1 or other ACSL isoforms under inflammatory conditions might lead to increased trapping of fatty acids as acyl-CoAs in tissues, regardless of plasma fatty acid levels.

In conclusion, this study shows that although overexpressed ACSL1 can act to exacerbate detrimental effects of saturated fatty acids in ECs, normally, ACSL1 does not mediate the inflammatory or apoptotic effects of a saturated fatty acid-rich environment.

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**Disclosures**

None.

**References**


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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 previously.\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 \textit{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\textsuperscript{−}CD31\textsuperscript{+} cell
population was expanded in culture and used for experiments. MMECs were isolated from WT Cre\(^{+}\) 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\(^{+}\) CD45\(^{-}\) 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\(^{5-6}\) (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\(_2\)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\(^{\circ}\)C, 5% CO\(_2\), 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.\(^7\) 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 tag⁸ 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.⁸

**Knock-down of ACSL1 in MMECs**

MMECs were grown in T75 flasks. Upon reaching 80-90% confluence, cells were transfected with siRNA using an Amaza Nucleofector kit (Lonza, Cologne, Germany). Briefly, cells were trypsinized and counted. Then, 5x10⁵ cells were pelleted and resuspended in Nucleofector solution containing 300 nmol/L siRNA. Cells were applied to Amaza 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® 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™ 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 Acsl1 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 Acsl1 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
Figure S2

Acsl1 isoforms

Acsl1
Acsl3
Acsl4
Acsl5
Acsl6

0
2
4
6
200
400
600
800

Acsl mRNA (fold over EC)

Heart

MMECs

Whole heart

Acsl mRNA (fold over MMECs)

* MMECs
Whole heart

Acs1
Acs1
Acs1
Acs1

0
2
4
6

Acs1
Acs1
Acs1
Acs1
A. ASM production (siRNA)  B. Glucose cons. (siRNA)  C. Lactate production (siRNA)
Figure S4

Spliced Xbp1

Control siRNA

AcsL1 siRNA

Total Xbp1

Control siRNA

AcsL1 siRNA

Control siRNA

AcsL1 siRNA

Spliced Xbp1

Total Xbp1

Control siRNA

AcsL1 siRNA

Spliced Xbp1

Total Xbp1

Control siRNA

AcsL1 siRNA

+ + - - + + - - + + - - + + + +