Loss of Stearoyl-CoA Desaturase-1 Attenuates Adipocyte Inflammation
Effects of Adipocyte-Derived Oleate

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Background and Purpose—Adipose inflammation is crucial to the pathogenesis of metabolic disorders. This study aimed at identify the effects of stearoyl-CoA desaturase-1 (SCD1) on the inflammatory response of a paracrine network involving adipocytes, macrophages, and endothelial cells.

Methods and Results—Loss of SCD1 in both genetic (Agouti) and diet-induced obesity (high-fat diet) mouse models prevented inflammation in white adipose tissue and improved its basal insulin signaling. In SCD1-deficient mice, white adipose tissue exhibited lower inflammation, with a reduced response to lipopolysaccharide in isolated adipocytes, but not in peritoneal macrophages. Mimicking the in vivo paracrine regulation of white adipose tissue inflammation, SCD1-deficient adipocyte-conditioned medium attenuated the induction of tumor necrosis factor (TNF) α/interleukin 1β gene expression in RAW264.7 macrophages and reduced the adhesion response in endothelial cells. We further demonstrated that the adipocyte-derived oleate (18:1n9), but not palmitoleate (16:1n7), mediated the inflammation in macrophages and adhesion responses in endothelial cells.

Conclusions—Loss of SCD1 attenuates adipocyte inflammation and its paracrine regulation of inflammation in macrophages and endothelial cells. The reduced oleate level is linked to the inflammation-modulating effects of SCD1 deficiency. (Arterioscler Thromb Vasc Biol. 2010;30:31-38.)

Key Words: SCD1 ▪ adipocyte ▪ macrophage ▪ endothelial cells ▪ inflammation ▪ oleate ▪ palmitoleate

Chronic inflammation plays a causative role in the emergence of various metabolic disorders, including type 2 diabetes mellitus, insulin resistance, and atherosclerosis.1 This inflammatory condition is provoked by diverse factors, including reactive oxygen species, endoplasmic reticulum stress, hypoxia, lipotoxicity, and protein kinase C isoforms.2–4 More important, lipids have been implicated in the coordinate regulation of metabolism and inflammatory and immune responses.5 The modulation of inflammation by lipids has been further demonstrated by a recent study that showed that fatty acids are ligands for Toll-like receptor 4 (TLR4) in macrophages.6

In the initiation of chronic inflammation, white adipose tissue (WAT) plays a central role, although other tissues, such as liver, might also be involved.7 The cross talk among adipocytes, macrophages, and endothelial cells in WAT orchestrates the inflammatory response in this tissue. The subsequent increased production of proinflammatory cytokines and chemokines, such as tumor necrosis factor (TNF) α, interleukin (IL) 6, plasminogen activator inhibitor (PAI)-1, and monocyte chemoattractant protein (MCP) 1 in WAT, leads to insulin resistance and increases the risk of cardiovascular disease associated with obesity.8,9 Therefore, the prevention of WAT inflammation is potentially beneficial for controlling chronic inflammation.

Stearoyl-CoA desaturase (SCD) catalyzes the rate-limiting step in the conversion of saturated to monounsaturated fatty acids (MUFAs) (mainly oleate [18:1n9] and palmitoleate [16:1n7]). This enzyme plays a central role in lipogenesis in rodents and humans.10 Several SCD gene isoforms (SCD1–4) have been identified in the mouse, and two SCD isoforms (SCD1 and SCD5) that are highly homologous to the mouse SCDs are well characterized in humans. Despite the high abundance of oleate (18:1n9) as a major MUFA from diet, the expression of SCD is highly regulated by developmental, dietary, hormonal, and environmental factors. Because of the involvement of MUFAs in the regulation of diverse processes, including signal transduction, cell differentiation, and neuronal development,11–13 SCD is regarded as an important enzyme in the

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regulation of normal and pathophysiological processes. Altered SCD activity has been implicated in a variety of morbidities, such as obesity, diabetes, atherosclerosis, cancer, and immune disorders.13

Although SCD1 has been well established as a key regulator of metabolism, recent studies14,15 have also reported its influence on inflammatory processes. However, the function of SCD1 expression in regulating adipocyte and WAT inflammation is poorly understood. In the current study, we demonstrate that SCD1 deficiency prevents WAT inflammation and improves insulin signaling under the challenge of obesity. For the first time to our knowledge, we demonstrate the effects of SCD1 deficiency on adipocyte inflammation, and reveal unique functions of oleate as a lipid inflammation mediator in linking the network of adipocytes, macrophages, and endothelial cells.

Methods

Animals and Diets
All mice used in the study were C57BL6/J males. The homozygous SCD1-deficient mice were generated, genotyped, and maintained as described.16 The breeding of mice was in accordance with the protocols approved by the animal care research committee of the University of Wisconsin, Madison. A standard Purina formula 5008 chow diet was used as regular food. Male chow-fed mice, aged 12 to 15 weeks, were used for primary cell preparation. For studies in genetically obese mice, the SCD1 deficiency was introduced into Agouti mice by crossing Agouti:SCD1-deficient or Agouti:SCD1-nondeficient with SCD1-deficient mice and generating Agouti:SCD1-deficient mice. Wild-type (WT) control mice, Agouti mice, and Agouti:SCD1-deficient mice were fed ad libitum with chow diet until the age of 24 weeks. In diet-induced obesity, 8-week-old WT and SCD1-deficient mice were fed a high-fat diet (HFD) (Research Diets, RD12492) until the age of 24 weeks.

Isolation of Primary Adipocytes, Stromal Vascular Cells, and Resident Peritoneal Macrophages
Primary adipocytes, WAT stromal vascular cells (SVCs), and peritoneal macrophages were isolated as described,17 with minor modifications. Age-matched WT and SCD1-deficient mice were used in the isolation. The WAT used in all studies was from an epididymal fat pad.

Statistical Analysis
Values shown in the study were expressed as mean±SEM. Statistical analysis with three or more groups was done using one-way ANOVA analysis of variance with a Bonferroni Post test, and the difference between the two groups was tested by two-tail, unpaired, Student t test; in both cases, significance was considered as P<0.05.

For additional methods and details, please refer to http://atvb.ahajournals.org for supplemental materials.

Results

Loss of SCD1 Prevents WAT Inflammation in Obesity Mouse Models
WAT is a critical site in the initiation of chronic inflammation in obesity.18 We first examined the effects of SCD1 deficiency on the overall inflammatory status of WAT in both genetic Agouti mutation induced (Agouti) and HFD-induced obese mouse models. The activation of nuclear factor κB (NF-κB), a master proinflammation transcriptional factor, has been linked to adipose tissue inflammation in obesity.7 In the Agouti and HFD-fed mice, SCD1 deficiency substantially reduced WAT inflammation, which was shown by a decrease in the DNA binding activity of NF-κB p65/50, the transcriptional active dimer complex of NF-κB (Figure 1A). The expression of MCP-1, TNF-α, PAI-1, and vascular cell adhesion molecule (VCAM)-1, as well as CC-chemokine receptor 2 and colony-stimulating factor 1 receptor, was also reduced (Figure 1B and supplemental Figure 1). In addition, cell death in WAT, which is closely correlated with WAT inflammation, was also prevented in SCD1-deficient mice, as shown by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining of WAT sections (supplemental Figure 1). These results further indicate that loss of SCD1 suppresses obesity-associated WAT inflammation.

Because inflammation has been linked to insulin resistance,7 we next analyzed basal insulin signaling in WAT from SCD1-deficient mice. The decreased expression of insulin signaling components, such as insulin receptor β
subunit, has been observed in obesity-related chronic insulin resistance. In SCD1-deficient mice, the decreased protein level of insulin receptor subunit in WAT from Agouti and HFD-fed WT mice was improved (Figure 1C). Consistently, the levels of serine 473 phosphorylation (pSer473) of Akt were increased (Figure 1C). Furthermore, the decreased mRNA levels of insulin receptor and insulin receptor substrate (IRS)-1 in WAT from obese mice were also prevented with loss of SCD1 in these mice (supplemental Figure 1). These results suggest that, consistent with the prevention of WAT inflammation, SCD1 deficiency improves basal WAT insulin signaling under the challenges of obesity.

Loss of SCD1 Reduces Inflammation in WAT and Primary Adipocytes, But Not in Peritoneal Macrophages

To test the role of SCD1 in WAT inflammation, we next analyzed the inflammatory response in WAT from WT and SCD1-deficient mice, which were fed a standard laboratory chow diet. The DNA binding activity of NF-κB p65/50 was reduced in WAT from SCD1-deficient mice (Figure 1C). Consistently, the levels of serine 473 phosphorylation (pSer473) of Akt were increased (Figure 1C). Furthermore, the decreased mRNA levels of insulin receptor and insulin receptor substrate (IRS)-1 in WAT from obese mice were also prevented with loss of SCD1 in these mice (supplemental Figure 1). These results suggest that, consistent with the prevention of WAT inflammation, SCD1 deficiency improves basal WAT insulin signaling under the challenges of obesity.

Figure 2. Loss of stearoyl-CoA desaturase-1 (SCD1) reduces inflammation in white adipose tissue (WAT) and adipocytes. A, Nuclear factor (NF) κB DNA binding activity in white adipose tissue (WAT). B, Baseline and lipopolysaccharide (LPS; 10 ng/mL)-induced inflammation in adipocytes. C, Messenger RNA (mRNA) level of Toll-like receptor (TLR) 4 and TLR2 in adipocytes. D, Protein levels of TLR4 in WAT. *Wild type (WT) vs SCD1 deficient and #, LPS vs untreated (UT).

In isolated peritoneal macrophages, the baseline and LPS-stimulated expression of proinflammatory genes (MCP-1, TNF-α, cyclooxygenase (COX)-2, and IL-6) was not significantly different between WT and SCD1-deficient mice (supplemental Figure 2). A similar phenomenon was observed on treatment with lipoteichoic acid, a TLR2 ligand. In addition, no genotypic difference was detected in the expression of TLR4 and TLR2, two key receptors mediating inflammation. These data indicate that the modulation of inflammation by SCD1 deficiency is cell-type selective for adipocytes that express high levels of SCD1.

Furthermore, SCD1-deficient adipocytes displayed significantly lower TLR4, but not TLR2, gene expression (Figure 2C). In parallel, the TLR4 protein level was also lower in WAT from SCD1-deficient mice (Figure 2D). This selective downregulation of TLR4 expression in SCD1-deficient adipocytes may be one of the causative factors in attenuating the inflammatory responses.

SCD1-Deficient Adipocyte-Conditioned Medium Induces Lower Inflammation in RAW264.7 Macrophages

WAT inflammation is attributable to the interaction between adipocytes and resident macrophages in a paracrine manner. We collected the WT and SCD1-deficient adipocyte-conditioned media (CM) that contain adipocyte-derived soluble factors, and tested their effects on inflammation. The induction of proinflammatory genes TNF-α and IL-1β was significantly lower in RAW264.7 macro-
phages treated with SCD1-deficient CM, compared with the treatment with WT CM (Figure 3A). As the two adipocyte CMs were further diluted by 2- and 4-fold with basal media, the difference in the induction of TNF-α and IL-1β genes was reduced, and comparable induction was observed at 4-fold dilution (Figure 3A). These data indicate that adipocyte-derived soluble factors are the likely mediators of inflammation in macrophages, and that the levels of these factors are lower in SCD1-deficient CM.

SVCs from WAT contain multiple cell types, including tissue macrophages, endothelial cells, preadipocytes, and others. With SCD1 deficiency, these cells, which are subject to paracrine regulation by adipocytes in WAT in vivo, showed reduced expression of proinflammatory genes IL-6 and MCP-1 under both basal and LPS-stimulated conditions (Figure 3B).

SCD1-Deficient Adipocyte-Conditioned Media Reduces the Adhesion Response of Endothelial Cells

The paracrine regulation of WAT inflammation involving adipocytes also influences adhesion pathways in endothelial cells, which recruit circulating monocytes into WAT.21 We next investigated the effects of SCD1-deficient CM on adhesion of mouse monocytes to mouse aortic endothelial cells. Compared with the endothelial cells incubated with WT CM, SCD1-deficient CM significantly decreased the adhesion of monocytes to endothelial cells, as assessed by the expression of leukocyte-specific gene CD45 after the adhesion assay (Figure 4A). The mRNA levels of adhesion molecules inter-cellular adhesion molecule (ICAM)-1 and P-selectin were also consistently significantly lower in endothelial cells treated with SCD1-deficient CM, as shown in Figure 6C. Furthermore, the SVCs from the WAT of SCD1-deficient mice displayed a lower ratio of CD68 (macrophage marker gene) expression to adipocyte number and reduced expression of Mac1 (another macrophage marker gene) (Figure 4B and supplemental Figure 7), suggesting less abundance of macrophages in the WAT.

SCD1-Deficient Mice Are Resistant to Obesity-Associated Macrophage Infiltration Into the WAT

Given the reduced adhesion response in endothelial cells on treatment with SCD1-deficient CM, we further tested the effects of SCD1 deficiency on macrophage infiltration into WAT in obesity. SCD1-deficient mice, under two different obesity challenges (the genetic model Agouti22 and the HFD model), exhibited significantly decreased macrophage abundance, as shown by Emr1 (F4/80) staining in WAT sections (Figure 4C) and lower expression of Emr1 (F4/80) and CD68 (supplemental Figure 1). In addition, analysis of the cell morphology in WAT from SCD1-deficient mice also re-
revealed diminished multinucleated cell structures, which are typical of the infiltrated macrophages in the WAT of obese mice (supplemental Figure 1).

Loss of SCD1 Leads to Reduced Content of Unsaturated Fatty Acids in Adipocytes and the Adipocyte Conditioned Media

Next, we set out to identify the adipocyte-derived inflammatory mediators in the CM that were affected by loss of SCD1. Adiponectin and leptin are two adipocyte-specific adipokines that are known to exert endocrine and paracrine regulation on inflammation.9 The levels of adiponectin in CMs were not significantly different between WT and SCD1-deficient adipocytes (supplemental Figure 6). Levels of leptin in WT CM tended to be slightly higher, but were not statistically different from that in SCD1-deficiency.

Free fatty acids (FFAs), which are released by adipocytes, have been well established as active regulators for inflammation.6,20,23 SCD1 is a key lipogenic enzyme responsible for the de novo synthesis of MUFAs, mainly oleate (18:1n9) and palmitoleate (16:1n7) in triglyceride (TG) fraction in primary adipocytes. Contents of oleate and palmitoleate from TG fraction in white adipose tissue (WAT) from obese mice, *, # P<0.05, wild type (WT) vs SCD1-deficiency.

Figure 5. Stearyl-CoA desaturase 1 (SCD1) deficiency reduces levels of unsaturated fatty acids. A, Free fatty acid profile in adipocyte-conditioned medium (CM). B, Contents of oleate (18:1n9) and palmitoleate (16:1n7) in triglyceride (TG) fraction in primary adipocytes. C, Contents of oleate and palmitoleate from TG fraction in white adipose tissue (WAT) from obese mice. *, # P<0.05, wild type (WT) vs SCD1-deficiency.

Indeed, compared with WT CM, SCD1-deficient CM exhibited significantly lower levels of palmitoleic (16:1n7) and oleic (18:1n9) acids, but comparable levels of the corresponding saturated FFAs, palmitic (16:0) and stearic (18:0) acids, respectively (Figure 5A). These FFAs were mainly derived from adipocytes; their levels in plain 10% Fetal Bovine Serum (FBS)/DMEM media were substantially lower (supplemental Figure 7). Consistently, the contents of oleate (18:1n9) and palmitoleate (16:1n7) were also significantly reduced in SCD1-deficient adipocytes (Figure 5B). Furthermore, WAT from Agouti and HFD-fed mice with SCD1 deficiency exhibited decreased oleate (18:1n9) and palmitoleate (16:1n7) levels (Figure 5C). The level of linoleic acid (18:2n6) was also reduced in the CM of SCD1-deficient adipocytes (Figure 4A), but the content of this FA in WAT was comparable in WT and SCD1-deficient mice (data not shown). In parallel with the reduced concentrations of FFAs in adipocyte CM, WAT and adipocytes from SCD1-deficient mice exhibited decreased levels of lipoprotein lipase, the enzyme responsible for fatty acid uptake in various tissues, including adipose tissue (supplemental Figure 7).

Oleate (18:1n9), But Not Palmitoleate (16:1n7), Contributes to the Induction of TNF-α in RAW264.7 Macrophages Treated With Adipocyte CM

Herein, we asked whether the decreased levels of unsaturated FFAs in SCD1-deficient adipocyte CM were linked to the reduced inflammation in macrophages. Treatment of RAW264.7 macrophages with increasing doses (50 and 200 μmol/L) of oleate (18:1n9) induced significantly
higher expression of TNF-α, whereas the same doses of palmitoleic (16:1n7) or linoleic (18:2n6) acid were ineffective (Figure 6A). Given this selective effect of oleate (18:1n9), we subsequently supplemented SCD1-deficient CM with 50 μmol/L of oleate, and found that it significantly enhanced the expression of TNF-α in RAW macrophages to a level comparable to the treatment with WT CM (Figure 6B). The observed reduction in LPL activity in SCD1-deficient CM did not alter the induction of TNF-α in RAW cells (supplemental Figure 7). These data indicate a unique role of oleate in modulating macrophage inflammation, which is not shared by palmitoleate, another enzymatic product of SCD1.

**Supplementation of Oleate (18:1n9), But Not Palmitoleate (16:1n7), in SCD1-Deficient Adipocyte CM Enhances the Adhesion Response of Endothelial Cells**

Next, we examined the effects of decreased oleate and palmitoleate levels on the adhesion response of endothelial cells. Endothelial cells incubated with WT CM exhibited significantly higher expression levels of adhesion molecules, such as ICAM-1, P-selectin, and E-selectin, compared with treatment with basal media, whereas SCD1-deficient CM led to significantly lower expression levels of ICAM-1 and P-selectin and a lower tendency of E-selectin in these cells (Figure 6C). Supplementation of oleate, 50 μmol/L, but not of palmitoleate (50 μmol/L), to SCD1-deficient CM significantly enhanced the expression of these adhesion molecules in endothelial cells. A similar pattern was consistently observed on monocyte/endothelial cell adhesion (Figure 6D). In these experiments, the reduced LPL activity in SCD1-deficient CM did not alter the adhesion responses in endothelial cells (data not shown). These data indicate that differential regulation on endothelial cell adhesion response exists between oleate and palmitoleate.

To further test the in vivo effects of SCD1 deficiency on endothelial inflammation, we analyzed the expression of adhesion molecules in WAT-derived SVC, which contains multiple cell types (including endothelial cells). The expression of ICAM-1, VCAM-1, and P-selectin was significantly lower in SVCs from the WAT of SCD1-deficient mice (supplemental Figure 8). Endothelial inflammation and dysfunction are closely associated with insulin resistance. SVCs with SCD1 deficiency exhibited significantly lower expression of endothelial dysfunction markers, Nox4 (NADPH oxidase 4), and NOS3 (nitric oxide synthase), and a lower tendency of endothelin 1 expression (supplemental Figure 8). These data suggest that endothelial cells in the WAT from SCD-deficient mice may have reduced inflammation with enhanced insulin sensitivity.

**Discussion**

Ever since the first finding that TNF-α is overproduced by adipocytes in obesity, WAT has been regarded as a critical site for promoting chronic and systemic inflammation and thereby contributing to the development of atherosclerosis. The present investigation has demonstrated that the inflammation in WAT can be attenuated by SCD1 deficiency under the challenge of obesity. Using isolated primary adipocytes, we showed a reduced response to inflammation with SCD1 deficiency directly in the adipocytes and a decreased paracrine regulation on macrophages and endothelial cells. We further showed that the decreased production of oleate (18:1n9), but not of palmitoleate (16:1n7), by SCD1-deficient adipocytes contributed to these effects. Palmitoleate has been recently demonstrated to be an adipocyte-derived lipokine that improves muscle insulin sensitivity and suppresses hepatosteatosis. However, the effects of oleate were not measured in the study. Given that both oleate and palmitoleate are the enzymatic products of SCD1, the current study using SCD1-deficient mice affords a unique model to demonstrate the functional difference of these two MUFAs in regulating inflammation.

It is becoming increasingly clear that in WAT inflammation, adipocytes interact with macrophages and endothelial cells in a paracrine manner. An array of protein factors, which include cytokines (TNF-α and IL-6), chemokines (MCP-1), adipokines (adiponectin and leptin), and others, are released by WAT and are involved in regulating inflammation in obesity. However, it was reported that the adipocyte-secreted TNF-α and IL-6 were unlikely to mediate the proinflammatory effects and, therefore, other unknown adipocyte-derived soluble factors may exert these effects. Under our experimental settings, we did not detect significant difference in the levels of adiponectin and leptin in WT and SCD1-deficient CMs. However, because these experiments were done in an in vitro context and unable to replicate the hormonal or other physiological regulations in vivo, we cannot rule out the possibilities that the above protein factors released by adipocytes may contribute to the in vivo regulation of inflammation.

In addition to secreting adipokines and cytokines, adipocytes also actively release FFAs through lipolysis. Recent studies have demonstrated the potent regulation by FFAs on the immune response of macrophages through members of TLR family. We were, therefore, prompted to explore the function of this class of soluble mediators in regulating the inflammation in an SCD1-deficient system. Although FFAs, most notably saturated FAs, such as palmitate (16:0), are important adipocyte-derived mediators in promoting macrophage inflammation, the precise contribution of MUFAs, mainly oleate (18:1n9) and palmitoleate (16:1n7), to the cross talk among adipocytes, macrophages, and endothelial cells has remained largely understudied. Given that SCD1 is highly expressed in adipocytes and has direct impact on the FA profile, we hypothesized that the alteration in the profile of secreted FAs from adipocytes lacking SCD1 might contribute to the reduced paracrine inflammatory regulation on the network of adipocytes, macrophages, and endothelial cells.

Our results demonstrated that oleate, but not palmitoleate, promotes macrophage inflammation and endothelial inflammation, which is not shared by palmitoleate, another enzymatic product of SCD1.
lial adhesion response in a paracrine fashion. SCD1-deficient adipocytes released similar levels of saturated FFAs as WT adipocytes. However, the levels of unsaturated FFAs, including palmitoleic (16:1n7), oleic (18:1n9), and linoleic (18:2n6) acids, were detected to be lower. In this regard, the SCD1-deficient CM model provides us with a unique opportunity to selectively examine the roles of these unsaturated FFAs in regulating inflammation independent of the effects from saturated FFAs. Oleic, but not palmitoleic or linoleic, acid contributes to the inflammation in both RAW macrophages and endothelial cells with the doses and time frame of our treatments. In another study, oleic was reported to consistently and significantly promote inflammation in RAW264.7 macrophages. Furthermore, of particular interest is the fact that both oleate and palmitoleate are enzymatic products of SCD1, and the only chemical structural difference is the two additional carbons present in the fatty acyl chain of the former. Performing an examination of how this subtle structural difference in the FAs leads to differential outcomes of cell signaling may yield important findings.

In parallel with the reduced paracrine inflammation by SCD1-deficient adipocytes, these adipocytes exhibit a reduced inflammatory response to LPS. We showed that the gene expression level of TLR4 was lower in SCD1-deficient than WT adipocytes. The role of TLR4 in mediating FFA-induced inflammation has been well established. In adipocytes, another recent study further demonstrated that FAs enhance the expression of TLR4 and induce inflammation through the TLR4/NF-κB cascade. In addition to the reduced TLR level, we further observed a substantially lower level of lipids (triglycerides) in SCD1-deficient adipocytes than WT adipocytes (supplemental Figure 3) and decreased NF-κB DNA binding activity in WAT. Taken together, these data suggest that reduced lipid levels in SCD1-deficient adipocytes might lead to reduced expression of TLR4, and subsequently to a decreased TLR4/NF-κB pathway, resulting in lower inflammation. In line with this reduced TLR4/NF-κB signaling, the basal and LPS-stimulated expression of proinflammatory factors (MCP-1 and IL-6) is lower in SCD1-deficient adipocytes than in WT adipocytes.

Despite the observed reduced inflammation in WAT and adipocytes of SCD1-deficient mice in our study, a recent study using an SCD1-deficient and low density lipoprotein receptor (LDLR)-deficient mouse model showed that SCD1 deficiency resulted in increased atherosclerosis. The mechanism was attributed to the skin inflammation associated with global SCD1 deficiency. Although increased levels of circulating inflammatory factors were detected in the SCD1-deficient and LDLR-deficient mice in that study, the levels of MCP-1 that are highly produced by WAT were actually lower in these mice. Thus, this result is consistent with the decreased inflammation of WAT and adipocytes observed in our study in SCD1-deficient mice. Interestingly, there is another study suggesting that SCD1 knock-down by targeted antisense oligos resulted in reduced atherosclerosis in a mouse model of chronic intermittent hypoxia. The apparent discrepancy suggests that there are likely other functions of SCD1 in regulating atherosclerosis independent of skin inflammation. In this regard, the pronounced skin inflammation might override the other beneficial effects of SCD1 deficiency on atherosclerosis, including the reduced WAT inflammation observed in our study. Therefore, better models of SCD1 deficiency (eg, tissue-specific deletion of SCD1) might provide more insights on the exact role of SCD1 in atherosclerosis.

Although adipocytes are sensitive to SCD1 deficiency in inflammation, the inflammatory responses of peritoneal macrophages, one of the major immune cells, are not altered by SCD1 deficiency in our study, which is consistent with the observation made in the SCD1-deficient and LDLR-deficient mouse model. Because we detected decreased macrophage infiltration in WAT from SCD1-deficient mice challenged by obesity, adipocytes may likely be the primary cell type regulating WAT inflammation in SCD1-deficient mice. In contrast, a recent study showed increased inflammation response in peritoneal macrophages after treating mice with SCD1-targeted antisense oligos, which may contribute to the increased atherosclerosis in those mice. The discrepancy may be partially the result of different specificities in deleting SCD1 expression (genetic knockout vs antisense oligos knock down), or the varying genetic backgrounds of mouse models used in these studies.

In obesity, increased lipolysis in WAT results in elevated plasma circulating levels of FFAs that cause inflammation and insulin resistance. Given that the content of oleate was reduced in WAT of obese mouse models with SCD1 deficiency, it is tempting to speculate that this would contribute to the prevention of WAT inflammation in these mice. However, because SCD1 deficiency protects mice from HFD-induced obesity or Agouti-induced obesity, our current study was not intended to address the precise contributions of oleate to inflammation independent of the obesity-preventing effects.

In summary, this study is the first demonstration on the molecular mechanism of SCD1 deficiency in modulating the levels of adipocyte-derived FAs on inflammation. An understanding of the differential functions of different types of MUFAs (oleate and palmitoleate) on the inflammatory cross talk among adipocytes, macrophages, and endothelial cells will provide more comprehensive understanding of the relationship between obesity, lipid metabolism, and atherosclerosis.

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Disclosures
None.

References


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In the article, “Loss of Stearoyl-CoA Desaturase-1 Attenuates Adipocyte Inflammation: Effects of Adipocyte-Derived Oleate” by Liu et al, which appeared in the January 2010 issue of the journal (Arterioscler Thromb Vasc Biol. 2010;30:31–38; DOI: 10.1161/ATVBAHA.109.195636), the publisher omitted an important correction from the final published version. On page 33, 2nd column, 1st full paragraph, line 10, the expansion for CPT should have appeared as “carnitine palmitoyl transferase (CPT) 1.”

The online version has been corrected.

The publisher sincerely regrets the error.

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Supplemental Materials

Loss of Stearoyl-CoA desaturase-1 attenuates adipocyte inflammation: effects of adipocyte-derived oleate

Methods:

Animals and diets:
Mice were weaned 2 weeks after born and fed *ad libitum* with food and water, they were housed and bred in a pathogen-free barrier facility of the Department of Biochemistry of the University of Wisconsin-Madison. The facility operated at room temperature with a 12h light/12h dark cycle. The high-fat diet (Research Diets, RD12492) contains 200g Casein, 3g L-Cystine, 125g Maltodextrin 10, 68.8g Sucrose, 50g Cellulose, 25g Soybean Oil, 245g Lard (contains 44% MUFAs), 10g Mineral Mix S10026, 13g DiCalcium Phosphate, 5.5g Calcium Carbonate, 16.5g Potassium Citrate (1 H2O), 10g Vitamin Mix V10001, 2g Choline Bitartrate and 0.05g FD&C Blue Dye#1 out of total 773.85g.

Isolation of primary adipocytes, SVC and peritoneal macrophages:
Primary adipocytes, WAT stromal vascular cells (SVC) were isolated as described\(^1\) with modifications. Age-matched wild type (Wt) and SCD1-/- mice were sacrificed by isofluorine overdose, and epididymal WAT was collected immediately followed by mincing into pieces of about 1mm in diameter in the adipocyte isolation buffer containing 119mmol/L NaCl, 4.7mmol/L KCl, 2.6mmol/L CaCl2, 1.2mmol/L KH2PO4, 1.2mmol/L MgSO4, 323mM HEPES (pH7.4), 20mg/ml BSA, 2mmol/L glucose and 1mg/ml collagenase. The minced WAT tissue was then incubated at 37°C with agitation for 25 min. The digested tissue was filtered through a sterile cell strainer (BD Falcon) with
100µm pore size. The cell suspension was seated for 2 min, allowing the adipocytes to float to the top. The lower phase was then carefully collected as SVC, and the floating cells were collected as adipocytes. The adipocytes were further washed 4 times with the adipocyte isolation buffer without collagenase, and the buffer after washing was collected and combined with SVC. The number of adipocytes isolated from WAT of Wt and SCD1-/- mice were not significantly different as counted by a hemocytometer (Supplemental Figure 3B). The cell viability was also comparable as assessed by trypan blue exclusion (data not shown). The adipocytes were then resuspended in 10% FBS/DMEM (Invitrogen). For collection of adipocyte conditioned media, the Wt and SCD1-/- adipocytes were cultured in a cell culture incubator with 10% CO₂ at 37 °C for 18 hours before being collected. For testing the inflammatory response in adipocytes, Wt and SCD1-/- adipocytes were first cultured with 10% CO₂ at 37 °C for 2 hours, and subsequently treated with 10ng/ml of lipopolysaccharide (LPS) (Sigma). The SVC was centrifuged at 1500xg for 3 min and resuspended in 10% FBS/DMEM medium, and cultured in the same incubator for 2 hours, then the un-attached cells and cell debris were removed by replacing with fresh 10% FBS/DMEM medium, 10ng/ml of LPS was added at this point. Adipocytes and SVCs isolated from 5-6 mice per group (Wt or SCD1-/- mice) were treated with LPS or un-treated. Treatments were stopped 8 hours later by adding Tri Reagent to isolate RNA from the adipocytes and SVC. Resident peritoneal macrophages were isolated from age-matched Wt and SCD1-/- mice by lavage of peritoneal cavity as described². Briefly, the peritoneal cavity was flushed with cold sterile PBS 3 times, and the fluid was collected. After centrifugation, cells were resuspended in 10% FBS/DMEM (Invitrogen), counted using a hemocytometer, and
equal numbers of Wt and SCD1-/ macrophages were cultured at 10% CO₂, 37 °C for 2 hours, the un-attached cells were then washed off with medium. The peritoneal macrophages were treated with 10ng/ml LPS, or 1µg/ml lipoteichoic acid (LTA) (Sigma) for 8 hrs before RNA harvest.

**Gel shift assay:**

The DNA binding activity of NF-κB was assayed according to the protocol from Promega Corp. Briefly, the oligo with NF-κB consensus binding element (Promega) was end-labeled by T4 polynucleotide kinase (Promega) using [γP³²]-ATP (BioRad). 30 µg of total tissue extract isolated from white adipose tissue (WAT) using 1X passive lysis buffer (Promega) was mixed with radio-labeled oligo for binding. Unlabeled cold probe was used to compete with the radio-labeled probe to show binding specificity. Then the reaction mixture was loaded to 5% polyacrylamide gel under non-denaturing condition and separated by electrophoresis at 4°C. The gel was then dried and exposed to X-ray film (Kodak) to visualize the binding of NF-κB onto the radio-labeled probe. The binding specificity was shown by blockade of binding with excessive competitive cold probe, and the position of NF-κB p65/50 complex was confirmed by supper shift assay using anti-p65 antibody from Santa Cruz Inc.

**RAW264.7 macrophages culture and treatment with adipocyte-conditioned medium:**

RAW264.7 macrophage cell line was cultured with 10%FBS/DMEM (Invitrogen). Cells were plated into 24-well tissue culture plate with a density of 0.25 x 10⁶ cells/well one day before treatments. Adipocyte-conditioned media were collected by individually
culturing Wt or SCD1-/- primary adipocytes isolated from each of the 6 mice per genotype for 18 hours. The RAW264.7 cells were then treated with either the Wt adipocyte-conditioned or SCD1-/- adipocyte-conditioned media for 12 hours before harvest of RNA by Tri-reagent. In treatments using exogenous fatty acids, fatty acids (oleic, linoleic sodium salts, Sigma-Aldrich) were dissolved in 150mmol/L NaCl at 60°C and conjugated with 12% cold fatty-acid-free BSA (Roche) by gentle shaking to make a stock concentration of 10mmol/L. Palmitoleate (Nu-Chek) was mixed with ethanol and NaOH, after ethanol was evaporated under N2 gas, 150mmol/L NaCl was added and heated at 60°C for 3-5 minutes until dissolved, then conjugated with 12% cold fatty-acid-free BSA by gentle shaking to make a stock concentration of 10mmol/L. RAW264.7 macrophages were treated with these fatty acids for 12 hours before RNA harvest. 4 replicated wells were used for each treatment.

Monocytes/Endothelial cells adhesion assay:

Mouse aortic endothelial cells were generously provided by Dr. Robert Auerbach from the University of Wisconsin-Madison. Endothelial cells were cultured using Clonetics EGM bullet kit containing the recommended supplemental components (Lonza group, Switzerland). The cells were cultured in an incubator with 37°C and 5% CO2. Mouse monocytes were isolated freshly from wild-type C57/BL6/J mice using Percoll (Sigma-Aldrich) density centrifugation following a protocol from Sigma. Excess Percoll was removed by sequential washing with PBS, and the number of monocytes was counted using a hemocytometer. The monocytes/endothelial adhesion assay was performed as previously described with minor modifications. Wt or SCD1-/- adipocyte conditioned
media were collected from 6 individual mice per group. Confluent endothelial cells cultured in 48-well plate were treated with either Wt or SCD1-/- adipocyte-conditioned media, in the absence or presence of supplemented oleic (18:1n9), palmitoleic (16:1n7) or linoleic (18:2n6), for 12 hours. The endothelial cells were then washed with DMEM, and incubated for 2 hours with the freshly isolated mouse monocytes (1 x 10^6/well) resuspended in DMEM. After the incubation, cells were washed four times with PBS to remove non-adherent monocytes. The monocytes adhesion to endothelial cells was then quantified by real-time PCR analysis of the mRNA level of leukocyte gene CD45 that is expressed by monocytes but not endothelial cells.

**Fatty acid profile analysis in adipocyte-conditioned media, adipocytes and adipose tissue:**

Different lipid fractions (FFA in adipocyte-conditioned media or TG in adipocytes and adipose tissue) were separated by thin layer chromatography (TLC). The fatty acid profile of certain lipid fraction was analyzed using gas chromatography as described^4^.

For FFA profile analysis in adipocyte-conditioned media and the content of oleate (18:1n9) and palmitoleate (16:1n7) in TG fraction of primary adipocytes, adipocytes isolated from 6 mice per genotype were used. For analysis of the content of oleate (18:1n9) and palmitoleate (16:1n7) in TG fraction of WAT from Agouti and HFD-fed mice, 3 to 4 mice per genotype were studied.

**Real-time PCR:**
The level of gene expression was tested by real-time PCR using A&B Applied Biosystems 7500 Fast Real-time PCR System and Power SYBR green master mix (Applied Biosystems). To perform real-time PCR, RNAs were isolated from snap-frozen tissue or cultured cells using Tri-Reagent (Molecular Research Center, Inc), followed by DNase I (Ambion) digestion to remove residual genomic DNA. cDNA was then synthesized using SuperScript III First Strand Synthesis System (Invitrogen). To normalize the variation in cDNA quantities, 36B4 gene expression was used as an internal control. Primer sequences are available upon request. The mRNA levels in WAT under obese states were expressed using chow-fed Wt mice as baseline. In the analysis of TNFα induction in RAW macrophages and the induction of adhesion molecules in endothelial cells, 6 individual adipocyte CMs from Wt or SCD1/-/- mice were used for each treatment.

**Histological analysis:**

The epididymal adipose tissue was collected from mice and fixed immediately in 10% buffered formalin. The tissues were then paraffin-embedded and sliced into sections. The WAT sections were stained by hematoxylin and eosin to visualize the morphology of cells present in adipose tissue. The immunohistological staining was performed as described with minor modifications. Briefly, the tissue sections were de-paraffined and rehydrated. After being blocked by 5% goat serum, the sections were incubated with anti-Emr1 (F4/80) antibody (1:100) (Serotec Inc) at 4°C overnight. Then the sections were washed and stained with FITC-conjugated secondary antibody (1:200) (Serotec Inc) for 30 min at room temperature, followed by washing. Finally, the sections were mounted
using ProLong Gold anti-fade mounting solution (Invitrogen) which contains the nucleus staining dye DAPI. The stained adipose sections were examined with fluorescence microscopy using Nikon Eclipse E800 microscope (Nikon) with 200X magnification. The quantity of macrophages was examined by counting Emr1-positive cells from four random areas in a selected section, the average was made to represent macrophage quantity in the WAT from individual mouse. Three sections were taken from each WAT, and 3 to 5 mice were used in each group. Statistical analysis was done by Student’s t-test, \( p<0.05 \) was set to be significant.

**TUNEL assay:**

The TUNEL assay was performed using DeadEnd Colorimetric TUNEL system (Promega). The adipose tissue sections were obtained as described above. The TUNEL staining procedure followed the manual from the kit. The sections were subsequently counter stained by hematoxylin for visualization of normal nucleus. The stained tissue slides were then mounted in Gel Mount Aqueous Mounting medium (Sigma). The slides were examined for TUNEL-positively stained nucleus using Nikon microscope under 40X bright field.

**Western Blotting:**

WAT was collected immediately after mice were sacrificed by isofluorine overdose, and snap frozen in liquid nitrogen. Total proteins were isolated from WAT using 1X passive lysis buffer containing 2mmol/L \( \text{Na}_3\text{VO}_4 \), 2mmol/L PMSF, 2% protease inhibitor cocktail from Sigma (St. Louis, MO). The protein concentration was determined by
Bradford protein assay kit (BioRad), and equal amount of proteins either individual mouse or pooled from 3 mice were used for Western analysis. The proteins with 5x loading buffer were heat-denatured at 100°C for 5 min, then loaded onto SDS-PAGE gel and separated by electrophoresis. Proteins in gel were transferred to a PVDF membrane (Millipore). After blocking, this membrane was incubated with the primary antibody of interest at 4°C overnight. The antibodies used to detect status of phosphorylation or protein expression level was from Santa Cruz Biotech. The target proteins were then detected by horseradish peroxidase-conjugated secondary antibodies, and signals were visualized by adding ECL™ Western Blotting Detection Reagents (Pierce). For analysis of IRβ and Akt Ser473 phosphorylation levels, WAT proteins were pooled from 3 mice per genotype. Data were quantified using ImageQuant and shown at the bottom of each lane.

**Lipoprotein lipase (LPL) activity assay:**

LPL activity in the adipocyte-conditioned was assayed using commercial LPL assay kit (Roar Biomedical, Inc.). A fluorescently labeled LPL substrate was used in the assay, the activity of LPL was measured by the intensity of fluorescence using fluorescent plate reader. The linear range of dose responses for this assay was first established using a purified LPL from *Pseudomonas* (Sigma-Aldrich). The amount of the adipocyte-conditioned medium used for the assay fell into the linear detection range of this kit. The LPL activity in fresh 10%FBS/DMEM medium was used as background activity and subtracted from that of adipocyte-conditioned mediums to show the activity of adipocyte-derived LPL. For experiments using supplementation of LPL, the purified LPL (Sigma-
Aldrich) was added to the SCD1-/- adipocyte-conditioned media by a final concentration of 0.5µg/ml.

Supplemental Fig.1
Supplemental Figure 1. SCD1 deficiency prevents the obesity-related inflammation, macrophage infiltration and cell death in WAT.  A and B, The elevated expression of proinflammatory genes PAI-1, VCAM-1, Csf-1R and macrophage marker gene CD68 and Emr1 (F4/80) in WAT from Agouti or HFD-fed Wt mice was prevented when SCD1 was deficient in these mice.  C, The decrease in the expression of IRβ and IRS-1 in WAT from the two obese mouse models was improved with SCD1 deficiency.  In A, B and C, gene expressions were quantified by real-time PCR. Results are averages of 4-7 mice per genotype, * p<0.05. D, H&E staining of WAT tissue sections from Agouti, Agouti:SCD1-/- mice, and HFD-fed Wt and SCD1-/- mice. Black arrows represent multi-nucleated cell morphology, typical for infiltrated macrophages in WAT from obese mice.  E, Cell death in WAT detected by TUNEL assay. Black arrows indicate the TUNEL-positive staining of the nucleus from dead cells.
Supplemental Figure 2. The separation efficiency of adipocytes and stromal vascular (SVC) cells from WAT and the inflammation response of peritoneal macrophages to LPS and LTA. A, The separation efficiency of adipocytes and SVC from WAT of Wt and SCD1−/− mice was shown by the expression of leukocyte marker gene CD45 and the adipocyte specific gene leptin. Gene expression was quantified by real-time PCR using
the level from Wt mice as baseline. Results are averages of 3 to 4 mice per genotype. *, p<0.05, adipocytes vs. SVC from Wt mice; #, p<0.05, adipocytes vs. SVC from SCD1/-/ mice. B and C, gene induction of TNFα, Cox-2, MCP-1 and IL-6 after treatment with LPS (10ng/ml) or LTA (1 µg/ml), and the expression level of TLR4 and TLR2 in Wt and SCD1/-/ peritoneal macrophages. Gene expressions were quantified by real-time PCR using the level in Wt macrophages as baseline. Results are averages of 5 mice per genotype.

**Supplemental Figure 3.** The cell morphology, cell number and the triglyceride content of isolated primary adipocytes from Wt and SCD1/-/ mice. A, The microscopic morphology of adipocytes in culture media. B, The number of isolated adipocytes from
Wt and SCD1-/- mice. Adipocytes were counted using a hemocytometer. Results are averages of 9-10 mice per genotype. C, The triglyceride (TG) content of Wt and SCD1-/- adipocytes. TG is the predominant form of lipids in adipocytes (data not shown). The contents of TG in adipocytes were assayed using WAKO TG Assay Kit. Results are averages from 3-4 mice per genotype and expressed as relative to that in Wt adipocytes. *, p<0.05.

Supplemental Fig.4

**Supplemental Figure 4.** Expression of genes in adipocyte metabolic function and mitochondrial metabolism were not significantly altered in the isolated adipocytes from
SCD1-/- mice compared to that in Wt mice. The expression of genes studied were PPARγ and its target gene PEPCK, Glut4, ADRP, aP2; Fatty acid oxidation genes (also target genes of PPARα) L-CPT1 and MCAD; Genes of mitochondrial metabolism, cytochrome oxidase IV (Cyox IV) and cytochrome c, somatic (Cycs). Gene expression was quantified by real-time PCR using 36B4 mRNA level as the internal control, results are averages from 5 individual mice for each genotype.

Supplemental Fig.5

**Supplemental Figure 5.** The gene expression of oxidative stress markers SOD1, SOD2, Gpx-1 and ER stress marker GPR78 in the isolated adipocytes from Wt and SCD1-/- mice. No significant difference was found between Wt and SCD1-/- adipocytes on the expression of SOD1, SOD2 and GRP78. Glutathione peroxidase 1 (Gpx-1) expression appears to be significantly lower in SCD1-/- adipocytes. However, given the unchanged expression of other markers of oxidative stress, ER stress, mitochondrial function, and adipocyte function, decreased Gpx-1 expression alone may not reflect altered oxidative
stress response. Gene expression was quantified by real-time PCR using 36B4 mRNA level as the internal control, results are averages from 5 individual mice for each genotype. *, p<0.05.

Supplemental Fig.6

Supplemental Figure 6. The levels of adiponectin and leptin in Wt and SCD1-/- adipocyte conditioned media. A, The adiponectin levels were analyzed by Western Blotting. Equal volume of Wt or SCD1-/- adipocyte conditioned media was loaded on a 10% SDS-PAGE gel. An anti-adiponectin antibody was used to detect the levels of adiponectin. B, Leptin levels were analyzed using a mouse Leptin ELISA kit from Millipore. Adipocyte CMs from 7 Wt and 6 SCD1-/- mice were analyzed.

Supplemental Fig.7
Supplemental Figure 7. SCD1-/- adipocytes exhibit lower LPL expression and SVC from WAT of SCD1-/- mice has decreased macrophage marker expression. A, The gene expression of LPL is lower in SCD1-/- adipocytes compared to Wt adipocytes. 5 mice per genotype were analyzed, * p<0.05. B, SCD1-/- adipocyte CM has lower LPL activity than that of Wt adipocyte CM, and addition of purified exogenous LPL at 0.5µg/ml final concentration in SCD1-/- adipocyte CM achieves similar LPL activity as that in Wt adipocyte CM. 6 mice per genotype were analyzed, * p<0.05. C, WAT from SCD1-/- mice exhibits lower protein levels of LPL than that from Wt mice. LPL protein levels were analyzed by Western Blotting, GAPDH levels were used as loading control. D, Supplementation of LPL by 0.5µg/ml final concentration in SCD1-/- adipocyte CM does not alter the TNFα induction in RAW macrophages. Gene expression was quantified by
real-time PCR using 36B4 mRNA level as the internal control, results are averages of CMs from 6 individual mice for each genotype. *, p<0.05. E, The basal FFA concentration in basal media (10%FBS/DMEM). The FFA fraction in basal media was separated by thin layer chromatography (TLC) and the profile was quantified by gas chromatography. F, The expression of macrophage marker gene Mac-1 in SVC from WAT of Wt and SCD1-/- mice. 3-4 mice per genotype were analyzed, * p<0.05.

Supplemental Fig. 8

A

ICAM-1

B

VCAM-1

C

P-selectin

D

Nox4 (NADPH oxidase 4)
**Supplemental Figure 8.** Stromal vascular cells (SVC) isolated from WAT of SCD1-/- mice exhibits lower expression of endothelial adhesion molecules and markers for endothelial dysfunction. A, B, C, the expression of adhesion molecules, ICAM-1, VCAM-1 and P-selectin. D, E, F, the expression of markers of endothelial dysfunction, Nox4 (NADPH oxidase), Nos3 (NO synthase 3) and Edn1 (Endothelin 1). The above gene expression in SVC from WAT of Wt and SCD1-/- mice was quantified by real-time PCR using 36B4 mRNA level as internal control, results are averages of 5 Wt mice and 6 SCD1-/- mice, * p<0.05.

**References:**

