Saturated Fatty Acids Undergo Intracellular Crystallization and Activate the NLRP3 Inflammasome in Macrophages


**Objective**—Inflammation provoked by the imbalance of fatty acid composition, such as excess saturated fatty acids (SFAs), is implicated in the development of metabolic diseases. Recent investigations suggest the possible role of the NLRP3 (nucleotide-binding oligomerization domain, leucine-rich repeat and pyrin domain containing 3) inflammasome, which regulates IL-1β (interleukin 1β) release and leads to inflammation, in this process. Therefore, we investigated the underlying mechanism by which SFAs trigger NLRP3 inflammasome activation.

**Approach and Results**—The treatment with SFAs, such as palmitic acid and stearic acid, promoted IL-1β release in murine primary macrophages while treatment with oleic acid inhibited SFA-induced IL-1β release in a dose-dependent manner. Analyses using polarized light microscopy revealed that intracellular crystallization was provoked in SFA-treated macrophages. As well as IL-1β release, the intracellular crystallization and lysosomal dysfunction were inhibited in the presence of oleic acid. These results suggest that SFAs activate NLRP3 inflammasome through intracellular crystallization. Indeed, SFA-derived crystals activated NLRP3 inflammasome and subsequent IL-1β release via lysosomal dysfunction. Excess SFAs also induced crystallization and IL-1β release in vivo. Furthermore, SFA-derived crystals provoked acute inflammation, which was impaired in IL-1β-deficient mice.

**Conclusions**—These findings demonstrate that excess SFAs cause intracellular crystallization and subsequent lysosomal dysfunction, leading to the activation of the NLRP3 inflammasome, and provide novel insights into the pathogenesis of metabolic diseases.

**Visual Overview**—An online visual overview is available for this article. (Arterioscler Thromb Vasc Biol. 2018;38:744-756. DOI: 10.1161/ATVBAHA.117.310581.)

Key Words: crystallization ◼ cytokines ◼ fatty acids ◼ inflammasome ◼ macrophages
Fatty Acid Crystals and NLRP3 Inflammasome

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Nonstandard Abbreviations and Acronyms

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<tr>
<th>Abbreviation</th>
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<tr>
<td>ASC</td>
<td>apoptosis-associated speck-like protein containing a caspase recruitment domain</td>
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<tr>
<td>Ch-C</td>
<td>cholesterol crystal</td>
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<tr>
<td>EP</td>
<td>ethyl palmitate</td>
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<tr>
<td>FFA</td>
<td>free fatty acid</td>
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<tr>
<td>IL</td>
<td>interleukin</td>
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<td>NF-κB</td>
<td>nuclear factor-κB</td>
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<td>NLRP3</td>
<td>nucleotide-binding oligomerization domain, leucine-rich repeat and pyrin domain containing 3</td>
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<tr>
<td>OA</td>
<td>oleic acid</td>
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<td>OA-OD</td>
<td>OA-derived oil droplet</td>
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<td>PA</td>
<td>palmitic acid</td>
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<td>ROS</td>
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<td>SA</td>
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<td>Scd1</td>
<td>stearoyl-CoA desaturase-1</td>
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<td>SFA</td>
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<td>TLR</td>
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<td>TNF-α</td>
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<td>USFA</td>
<td>unsaturated fatty acid</td>
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Inflammasome activation. However, the precise mechanism by which SFAs activate the NLRP3 inflammasome is involved in the PA-mediated NLRP3 inflammasome. These findings describe the novel mechanism by which SFAs activate the NLRP3 inflammasome. Additionally, we suggest new potential molecular targets for the treatment of metabolic diseases.

Materials and Methods

Reagents

The FA-free BSA (A7030), PA (P5585), SA (S4751), linoleic acid (L1376), linolenic acid (L2376), lipopolysaccharide (L4391), and cytochalasin D (C8273) were all from Sigma (St Louis, MO). The OA (153–01241), lecinthin (124–05031), and CA-074 Me (334–43231) were from Wako Chemicals (Osaka, Japan). Orlistat (sc-203108) was from Santa Cruz Biotechnology (Dallas, TX). Triasin C (BML-E1218-0100) was from Enzo Life Sciences (Farmingdale, NY). Tripalmitin (G3096), triolein (G0089), ethyl palmitate (EP; P0003), and ethyl oleate (O0143) were from Tokyo Chemical Industry (Tokyo, Japan). For preparation of the FA-containing medium, 200 mmol/L stock solution of PA or SA in ethanol was heated at 60°C and added to 0.5% BSA/RPMI (Roswell Park Memorial Institute) 1640 medium. For preparation of the 8 mmol/L stock solution, OA was dissolved in 10% BSA/RPMI1640 medium. To dissolve the FFAs, the media were sonicated for 1 hour and shaken overnight at 37°C. Then, the media were filtered with a 0.20-μm filter. For preparation of FA-derived crystals, 50 mmol/L stock solution of FFAs in ethanol was evaporated using a centrifugal evaporator (Tomy Seiko, Tokyo, Japan) at room temperature. Hydrated cholesterol was crystallized from the mixture of cholesterol (10 mg/mL) and distilled water (10:1). The deposited crystals were suspended by sonication in PBS for 2 minutes. For preparation of EP and ethyl oleate–containing emulsion, each lipid was emulsified by sonication in PBS for 0.1% BSA/RPMI1640 medium. To dissolve the FFAs, the media were sonicated for 1 hour and shaken overnight at 37°C. Then, the media were filtered with a 0.20-μm filter. For preparation of FA-derived crystals, 50 mmol/L stock solution of FFAs in ethanol was evaporated using a centrifugal evaporator (Tomy Seiko, Tokyo, Japan) at room temperature. Hydrated cholesterol was crystallized from the mixture of cholesterol (10 mg/mL) and distilled water (10:1). The deposited crystals were suspended by sonication in PBS for 2 minutes. For preparation of EP and ethyl oleate–containing emulsion, each lipid was emulsified by sonication in PBS for 2 minutes. For preparation of EP and ethyl oleate–containing emulsion, each lipid was emulsified by sonication in PBS for 2 minutes. For preparation of EP and ethyl oleate–containing emulsion, each lipid was emulsified by sonication in PBS for 2 minutes. For preparation of EP and ethyl oleate–containing emulsion, each lipid was emulsified by sonication in PBS for 2 minutes. For preparation of EP and ethyl oleate–containing emulsion, each lipid was emulsified by sonication in PBS for 2 minutes. For preparation of EP and ethyl oleate–containing emulsion, each lipid was emulsified by sonication in PBS for 2 minutes. For preparation of EP and ethyl oleate–containing emulsion, each lipid was emulsified by sonication in PBS for 2 minutes. For preparation of EP and ethyl oleate–containing emulsion, each lipid was emulsified by sonication in PBS for 2 minutes.

Animals

The C57BL/6J mice were purchased from SLC, Inc (Shizuoka, Japan). The NLRP3−/− and IL-1β−/− mice were provided by Dr Visha M. Dixit and Dr Yoichiro Iwakura, respectively. Elov16-deficient (Elov16−/−) mice were generated as previously described. Only male mice were used to exclude influences by the female hormonal cycle. For isolation of thyroglobulin-elicted macrophages, mice were injected intraperitoneally with 1 mL of 4% Brewer thyroglobulin paste (211716; Becton Dickinson, Franklin Lakes, NJ), and peritoneal cells were collected 4 days after injection. Cells were plated on 24-well plates in 10% FCS/RPMI 1640 medium. After 2 hours, the nonadherent cells were washed out, and the adherent cells were used as peritoneal macrophages. In lipid emulsion infusion experiment, 200 μL of EP or ethyl oleate emulsion was injected intravenously every 2 hours. In peritonitis model, crystals were resuspended in PBS (1 mg/mL) and injected intraperitoneally (2 mg/mouse). All animal experiments were approved by the Use and Care of Experimental Animals Committee of the Jichi Medical University Guide for Laboratory Animals and were performed in accordance with the Jichi Medical University guidelines.

Cell Culture and FFA Treatment

Murine peritoneal macrophages were cultured in RPMI 1640 containing 10% FCS, 1% antibiotics/antimycotics, and 1% GLUTAMAX (35050–061; Thermo Fisher Scientific, Waltham, MA). Murine J774 macrophage cells were maintained with 10% FCS/DMEM. For low-dose (100 ng/mL) lipopolysaccharide priming, cells were starved in 0.1% BSA/RPMI 1640 for 16 hours.

Lentiviral Vector

The polymerase chain reaction–generated cDNAs encoding human full-length IL-1β and mouse Scd1 (stearoyl-CoA desaturase-1) were subcloned into the CS-CA-MCS plasmid (kindly provided by Dr H
Miyoshi, RIKEN BioResource Center, Ibaraki, Japan). HEK293T cells were transfected with CS-CA-MCS, pLP1, pLP2, and pSVSVG using PEI MAX (Polyethyleneimine Max; 24765-2; Polysciences, Warrington, PA). Culture media containing the lentiviral vectors were collected 3 days after transfection. The collected media were filtered with a 0.45-µm filter and ultracentrifuged at 21,000 rpm for 2 hours using a Type 45 Ti rotor (Beckman Coulter, Brea, CA). The pellets were then suspended again in 5% FCS/PBS. The lentiviral titer was measured using a Lentivirus qPCR Titer kit (number LV900; Applied Biological Materials, Richmond, British Columbia, Canada). To transduce the lentiviral vectors, J774 cells were incubated with 8 µg/mL of polybrene.

IL-1β Secretion Assays
The levels of IL-1β and IL-1α were measured by ELISA using a commercial kit (mouse IL-1β: DY401, human IL-1β: DY201, and mouse IL-1α: DY400; R&D Systems, Minneapolis, MN).

Measurement of Caspase-1 Activity
Caspase-1 activity was analyzed using the carbboxylfluorescein FAM-FLICA Caspase-1 Assay kit (98; Immunochemistry Technologies, Bloomington, MN) according to the manufacturer’s instructions. Nuclei were costained with Hoechst33342. The fluorescence was assessed by confocal laser scanning microscopy (FV-10i; Olympus, Tokyo, Japan).

Oil Red O Staining
FFA-treated cells were washed with PBS and then fixed with 10% neutral buffered formalin for 10 minutes at room temperature. After washing with PBS, cells were equilibrated in 60% 2-propanol for 1 minute and then stained with 60% oil red O solution.

Western Blot Analysis
Western blot analysis was performed as described previously.20 Samples were separated by SDS–PAGE and transferred to polyvinylidene fluoride membranes. After blocking with Tris-buffered saline containing 2% casein, the membranes were incubated with the primary antibodies against β-actin (clone AC-15; Sigma), IL-1β (clone 2805; R&D Systems), and NLRP3 (clone Cryo-2; Enzo Life Sciences).

Reverse Transcription and Real-Time Polymerase Chain Reaction
Total RNA was prepared using ISOGEN (311–02501; Nippon Gene, Co, Tokyo, Japan) according to the manufacturer’s instructions. Total RNA was reverse transcribed using a Super Script VILO cDNA Synthesis kit (11754250; Thermo Fisher Scientific). Real-time polymerase chain reactions were performed using SYBR Premix Ex Taq II (RR820A; Takara Bio, Siga, Japan). The primers used in the assay were as follows: Actb forward: CACAGCTTTCTTGCAAGCTTCT, Actb reverse: AAGCAGGGATCATGCTCAT, Scd1 forward: AGATCTCCAGTTCCTACAACAC, Scd1 reverse: CCTTTATGGAGAGGGATGTCT, Lysg forward: GACCTCC TGCAAACACACTACC, Lysg reverse: ACACGATTACAGTGA TCTCAGT, Cxcr2 forward: CTTGTTGAGAACACTGCGGAT TAAG, Cxcr2 reverse: GGTTCTCTGAGTGGCATGGGAC. The obtained data by flow cytometry were analyzed using FlowJo software version 10 (Tree Star, Ashland, OR).

Polarization Microscopy
For analysis of cultured cells, FFA-treated cells were washed with PBS and then fixed with 10% neutral buffered formalin for 10 minutes at room temperature. For analysis of crystallization in tissues, the fresh frozen sections were prepared and stained with hematoxylin after formalin fixation. After washing with PBS, the slides were enclosed in 50% glycerol-PBS and observed with polarization microscopy (BX51; Olympus). For the evaluation of intracellular crystallization, the cells in 3 nonoverlapping areas of each group were analyzed.

X-Ray Analysis for Crystallization
PA-treated J774-hIL1B cells were mounted on a cryoloop (PW-101-004; Protein Wave) and cooled in liquid nitrogen for cryogenic x-ray crystallography at 100 K. Diffraction images were collected using synchrotron x-ray beam at the beamline 17A in Photon Factory (Tsukuba, Japan). The detector used was a Pilatus 6 mol/L (Dectris). The x-ray wavelength, the camera distance, the oscillation angle, and the exposure time were 0.98 Å, 300 mm, 2.5°, and 600 seconds, respectively.

Immunohistochemistry
The fresh frozen sections of the spleen were fixed with 10% neutral buffered formalin for 5 minutes and permeabilized by 0.1% Triton X100 for 10 minutes. After blocking with normal goat serum, slides were incubated with anti-Gr-1 (granulocyte-differentiation antigen-1) antibody (14–5931; eBioscience, San Diego, CA). This was followed by incubation with Histofine Simple Stain Rat MAX PO (414311; Nichirei Corporation, Japan). The immune complexes were detected using DAB Peroxidase (HRP) Substrate Kit (with Nickel), 3,3’-diaminobenzidine (SK-4100; Vector Laboratories). The sections were then counterstained with hematoxylin. No signals were detected when an irrelevant IgG (I-4000; Vector Laboratories) was used instead of the primary antibody as a negative control.

Flow Cytometry
Cells from peritoneal lavage were analyzed using flow cytometry. The cells were labeled with the following antibodies: fluorescein isothiocyanate–conjugated anti-CD45R (14–5931; eBioscience), phycoerythrin-conjugated anti-Ly6G (561104; BD Biosciences), and allopnycacin-conjugated anti-CD45 (17–0451; eBioscience). The cells were examined by flow cytometry (FACS Verse) and analyzed using FlowJo software version 10.

Statistical Analysis
Data are expressed as mean±SE. Mann–Whitney U test was used to compare the 2 groups. Differences between multiple group means were determined by 1-way ANOVA combined with the Tukey–Kramer test or Kruskal–Wallis test. The selected statistical methods were determined by 1-way ANOVA combined with the Tukey–Kramer test or Kruskal–Wallis test. All analyses were performed using the Prism (GraphPad Software, La Jolla, CA). A P value of <0.05 was considered statistically significant.

Results
SFAs Induce NLRP3 Inflammasome Activation in Macrophages
To investigate the effect of FFAs on NLRP3 inflammasome activation, we primed murine peritoneal macrophages with lipopolysaccharide (100 ng/mL) and then treated them with albu- min-conjugated PA or SA. Treatment with PA or SA (20–200 µmol/L) induced IL-1β release in a dose-dependent manner,

Assessment of Lysosomal Dysfunction
Primary macrophages were labeled with Alexa 488–conjugated dextran (D22910, molecular weight=10000; Thermo Scientific) at 50 µg/mL for 2 hours and then treated with FFAs. The J774 expressing human IL1B gene (J774-hIL1B) cells were stained with AcidFluor ORANGE for 2 hours (GC301; Goryo Kayaku, Sapporo, Japan) to assess lysosome integrity. The fluorescence was analyzed by confocal laser scanning microscopy and flow cytometry (FACSVerse; BD Biosciences).
indicating the inflammasome activation in response to SFAs (Figure IA and IB in the online-only Data Supplement). Because USFAs have been shown to protect from SFA-induced lipotoxicity, we next examined the effect of OA on SFA-induced inflammasome activation and IL-1β release. The OA treatment significantly inhibited PA- or SA-induced IL-1β release in a dose-dependent manner (20–200 µmol/L; Figure 1A and 1B). Similarly, the other USFAs, such as linoleic acid (18:2; n–6) and linolenic acid (18:3; n–3) inhibited PA-induced IL-1β release (Figure IC and ID in the online-only Data Supplement). The inhibitory effects of OA on the SFA-induced inflammasome activation were further confirmed by the detection of the

Figure 1. Saturated fatty acids induce inflammasome activation in macrophages. A through D, Primary peritoneal macrophages isolated from wild-type (WT) mice were rested or primed with lipopolysaccharide (LPS) for 16 h and then treated with palmitic acid (PA), stearic acid (SA), and oleic acid (OA) for 24 h. A and B, The levels of IL-1β (interleukin-1β) in the supernatants were assessed (n=3). C, Caspase-1 activity was visualized by FLICA assay. D, Quantitative analysis of relative fluorescence units (RFU) was performed (n=25–40). E through H, J774-hIL1B cells were treated with PA, SA, and OA for 24 h. E and F, The levels of IL-1β in the supernatants were assessed (n=3). G, Oil red O staining performed. H, Intracellular triglyceride (TG) levels were quantified (n=3). Data are expressed as mean±SEM. *P<0.05, **P<0.01, ***P<0.005 as determined by 1-way ANOVA with Tukey–Kramer test or Kruskal–Wallis test. Data are representative of 2 independent experiments.
fluorescent cell-permeable probe that specifically binds to activated caspase-1 (FLICA assay; Figure 1C and 1D). The SFA-induced inflammasome activation is NLRP3 dependent because PA-induced IL-1β release is blunted in NLRP3-deficient (NLRP3<sup>−/−</sup>) macrophages (Figure IE in the online-only Data Supplement). However, OA had no effect on ATP and nigericin-induced IL-1β release (Figure IF in the online-only Data Supplement). Because SFAs are delivered as triglyceride in lipoprotein, we examined whether triglyceride-rich lipid emulsion induces IL-1β release. Triglyceride emulsion made of tripalmitin promoted IL-1β release, which was inhibited by a lipase inhibitor orlistat (Figure IG and IH in the online-only Data Supplement), indicating that its effect is dependent on FFA.

The SFAs reportedly stimulate a TLR2/TLR4 signaling and subsequently induce Il1β expression at transcriptional levels in macrophages. Actually, we confirmed that PA treatment tended to increase mRNA expression of Il1β and other inflammatory cytokines (Figure IIA through IIC in the online-only Data Supplement). Because IL-1β release is regulated by transcriptional Il1β induction and inflammasome activation to process pro-IL-1β into its mature form, we established murine J774 macrophages that stably expressing human IL1β gene (J774-hIL1B) using a lentiviral transduction. The J774-hIL1B cells allowed us to assess whether SFAs directly activated the inflammasome by measuring human IL-1β release. When stimulated with the well-known NLRP3 inflammasome activator, nanosilica, in the presence of long chain acyl-CoA synthase, on SFA-induced IL-1β release, which was dose dependently inhibited by OA (Figure 1E and 1F). Oil red O staining was performed to assess the accumulation of lipids in J774-hIL1B cells. Less lipid droplets were visualized in PA-treated cells than in OA-treated cells (Figure 1G).

In addition, treatment with OA restored the lipid droplet formation in the PA-treated cells. Because SFA-induced lipotoxicity is ameliorated by triglyceride synthesis and accumulation, we assessed the content of triglyceride and found a similar trend in triglyceride content with lipid droplet formation (Figure 1H). These findings suggest that PA and SA induced NLRP3 inflammasome activation and subsequent IL-1β release, which were alleviated by OA in macrophages. In addition, the inhibitory effect of OA was accompanied by triglyceride accumulation.

**Intracellular FFAs Contribute to SFA-Induced Inflammasome Activation**

Because it was recently demonstrated that ceramide, a metabolized product from PA, activates NLRP3 inflammasome, we postulated that lipid metabolites derived from SFAs could mediate SFA-induced inflammasome activation. To test this possibility, we examined the effect of triacsin C, an inhibitor of a long chain acyl-CoA synthase, on SFA-induced IL-1β release. Although triacsin C inhibited OA-induced lipid droplet formation at 5 μmol/L in J774 macrophages, it also suppressed J774-hIL1B cells released human IL-1β (Figure IID and IIE in the online-only Data Supplement). Consistent with the findings of murine primary macrophages, treatment with PA and SA clearly induced human IL-1β release in the J774-hIL1B cells, which was dose dependently inhibited by OA (Figure 1E and 1F). Oil red O staining was performed to assess the accumulation of lipids in J774-hIL1B cells. Less lipid droplets were visualized in PA-treated cells than in OA-treated cells (Figure 1G).

**Figure 2.** Intracellular free fatty acids (FFAs) contribute to saturated fatty acid-induced inflammasome activation. A, J774-hIL1B cells were pretreated with triacsin C (1 and 5 μmol/L) for 30 min and then treated with palmitic acid (PA) and oleic acid (OA) for 24 h. The levels of IL-1β (interleukin-1β) in the supernatants were assessed (n=3). B, Primary macrophages isolated from wild-type (WT) or Elovl6-deficient (Elovl6<sup>−/−</sup>) mice were rested or primed with lipopolysaccharide (LPS) for 16 h and then treated with PA and OA for 24 h. The levels of IL-1β in the supernatants were assessed (n=3). C and D, J774-hIL1B cells were transduced with hKO1 (humanized Kusabira-Orange 1) or stearoyl-CoA desaturase-1 (Scd1) and treated with PA, stearic acid (SA), and OA for 24 h. C, Scd-1 expression was analyzed by real-time polymerase chain reaction. D, The levels of IL-1β in the supernatants were assessed (n=3). E, J774-hIL1B cells were pretreated with triacsin C (1 and 5 μmol/L) for 30 min and then treated with PA and OA for 24 h. Intracellular contents of FFAs were quantified (n=3–4). F, J774-hIL1B cells were treated with PA, SA, and OA for 24 h. Intracellular contents of FFAs were quantified (n=3). Data are expressed as the mean±SEM. *P<0.05, **P<0.01, ***P<0.005 as determined by 1-way ANOVA with Tukey-Kramer test. Data are representative of 2 independent experiments.
lipopolysaccharide-induced Il1b expression (Figure IIIA through IIID in the online-only Data Supplement). Therefore, we used J774-hIL1B cells to determine its direct effects on inflammasome activation. Unexpectedly, triacsin C not only increased IL-1β release, but it also enhanced PA-induced IL-1β release in J774-hIL1B cells (Figure 2A). Furthermore, the IL-1β release in response to triacsin C was inhibited by OA treatment. To further assess whether altered metabolisms of palmitoyl-CoA affect SFA-induced inflammasome activation, we used primary Elovl6-deficient (Elovl6−/−) macrophages and found no difference in IL-1β release between wild-type and Elovl6−/− macrophages (Figure 2B). Furthermore, overexpression of Scd1 failed to rescue PA- or SA-induced IL-1β release in J774-hIL1B cells (Figure 2C and 2D), indicating little contribution of FA metabolites to this process. Therefore, we measured intracellular FFAs in triacsin C–treated cells and found that intracellular levels of FFAs tended to be increased in triacsin C–treated cells (Figure 2E). Moreover, PA treatment significantly increased intracellular FFA levels, which was prevented by OA treatment (Figure 2F). These findings suggest

![Figure 3](image-url)
that intracellular FFAs, rather than the metabolites of FA acyl-CoA, contribute to SFA-induced IL-1β release in macrophages.

**SFAs Cause Lysosomal Dysfunction in Macrophages**

To investigate the potential mechanism by which SFAs activate the inflammasome in macrophages, we explored whether SFAs could cause lysosomal dysfunction, which has been shown to be a common pathway for NLRP3 inflammasome activation. Pretreatment with CA-074 Me, a specific inhibitor of the lysosomal enzyme cathepsin B, almost completely inhibited PA-induced IL-1β release in lipopolysaccharide-primed primary macrophages (Figure 3A). Meanwhile, the PA-induced IL-1β release was not affected by the actin-disrupting agent cytochalasin D, which blocks macrophage phagocytosis (Figure 3B). Because we and other investigators showed that several particulate matters, including cholesterol crystals, tricalcium phosphates, and nanosilica, are phagocytosed by macrophages to induce NLRP3 inflammasome activation, this result suggests that lysosomal dysfunction is involved in PA-induced inflammasome activation in the absence of phagocytosis. To assess whether PA actually causes lysosomal dysfunction, we labeled the lipopolysaccharide-primed primary macrophages with Alexa488-conjugated dextran, which is incorporated into lysosome and leaked into cytosol when the lysosome is ruptured. The time-lapse imaging revealed that PA induced lysosomal rupture (Figure 3C; Movie I in the online-only Data Supplement), which was inhibited in the presence of OA (Figure IVA in the online-only Data Supplement). To further confirm the lysosomal rupture induced by SFAs, we used AcridiFluor ORANGE, which exhibits fluorescence under acidic conditions. As expected, acidic lysosomes were not detected in the cells treated with bafilomycin A1, an inhibitor of lysosomal acidification. Compared with the control cells, PA treatment significantly increased negatively stained cells indicating lysosomal dysfunction, which was inhibited by OA treatment (Figure 3D through 3F). Recent studies demonstrated that mitochondrial ROS and GPR (G-protein–coupled receptor)-mediated signals are involved in FFA-mediated inflammasome regulation. Although PA induced mitochondrial ROS production, a mitochondrial ROS inhibitor MitoTEMPO failed to inhibit PA-induced IL-1β release (Figure IVB and IVC in the online-only Data Supplement). Furthermore, a GPR120 inhibitor AH-7614 had no effect on IL-1β release (Figure IVD in the online-only Data Supplement). These results suggest that SFAs induce inflammasome activation through lysosomal dysfunction.

**SFAs Cause Intracellular Crystallization in Macrophages**

The involvement of lysosomal dysfunction without phagocytosis in SFA-induced inflammasome activation prompted us to examine whether excess SFAs caused intracellular crystallization in macrophages. Indeed, polarized light microscopy identified crystallization in PA- or SA-treated primary macrophages (Figure 4A through 4D). According to the inhibitory effect of OA on IL-1β release, PA- or SA-induced crystallization was dose dependently decreased by treatment with OA. In addition, linoleic acid and linolenic acid inhibited PA-induced crystallization (Figure VA in the online-only Data Supplement). Crystallization by PA and SA was also detected in J774-hIL1B cells, and it was significantly decreased by treatment with OA (Figure 4E and 4F; Figure VB and VC in the online-only Data Supplement). Interestingly, crystallization was not detected in other types of the cells, such as Hepa1-6 hepatocytes and H9c2 cardiomyocytes (Figure V1A and V1B in the online-only Data Supplement). Because massive influx of lipid into lysosome occurs during the process of phagocytosis, the contribution of lipid droplets was examined. However, BODIPY staining showed no lipid in PA-treated macrophages (Figure V1A and V1B in the online-only Data Supplement) although lysosomal rupture was already observed at this time point (Figure 4A).

To confirm whether increased crystallization enhances IL-1β release, J774-hIL1B cells were exposed to low temperature (32°C). Intracellular crystallization in PA- or SA-treated cells was increased by exposure to low temperature (Figure VIIA in the online-only Data Supplement). Similarly, IL-1β release in response to PA and SA was clearly enhanced under exposure to low temperature (Figure VIIIB and VIIIC in the online-only Data Supplement). However, the exposure to low temperature did not affect IL-1β release in response to other inflammasome stimuli such as ATP and nanosilica (Figure VIIID in the online-only Data Supplement).

To further confirm that the polarization structures are indeed crystalline, we performed an x-ray diffraction analysis (at 100 K at the beamline 17A of Photon Factory, Japan). The isolated single cells treated with PA are found to show sharp diffraction spots (Figure VIIIE in the online-only Data Supplement), confirming the presence of a well-ordered crystal lattice. These results strengthen the evidence that SFAs cause intracellular crystallization.

**SFA Crystals Induce NLRP3 Inflammasome Activation in Macrophages**

To investigate whether SFA-derived crystallization activated the NLRP3 inflammasome, we prepared PA- or SA-derived crystals (PA-C or SA-C) by evaporation of FFA solution in ethanol and examined their effects in lipopolysaccharide-primed primary macrophages. PA-C or SA-C induced IL-1β release, whereas OA-derived oil droplets failed to induce IL-1β release (Figure 5A and 5B). PA-C also induced IL-1α release like cholesterol crystals (Figure IXA and IXB in the online-only Data Supplement). In addition, PA-C induced the expression of cytokines and chemokines, including Il1b, Tnfa, Il6, Cxcl1, and Cxcl2 (Figure IXC through IXG in the online-only Data Supplement). Similar to SFAs, caspase-1 activation was apparently detected in PA-C– or SA-C–treated cells (Figure 5C and 5D). To further investigate the role of the NLRP3 inflammasome, primary macrophages were isolated from wild-type and NLRP3−/− mice and then stimulated with PA-C, SA-C, or OA-derived oil droplets. Indeed, the IL-1β release induced by PA-C and SA-C was markedly inhibited in NLRP3−/− macrophages (Figure 5E). Furthermore, PA-C–induced inflammasome activation was partially but significantly inhibited by cytochalasin D, bafilomycin A1, and CA-074 Me (Figure 5F). These results suggest that SFA crystals are phagocytosed and cause lysosomal dysfunction, leading to NLRP3 inflammasome activation.
SFA Crystals Provoke IL-1β–Mediated Inflammatory Responses In Vivo

To further clarify the role of FA crystals in inflammation, we investigated whether excess SFAs cause crystal formation and induce subsequent inflammatory responses in vivo. To address this issue, we used EP emulsion because intravenous injection of EP emulsion increases plasma palmitic acid levels.

When added in lipopolysaccharide-primed primary macrophages, EP emulsion induced IL-1β release in a LPL-dependent manner and crystal formation, whereas ethyl oleate emulsion failed to induce these effects (Figure XA through XC in the online-only Data Supplement). Then, we intravenously injected EP emulsion in mice and found deposition of crystals in red pulp of the spleen (Figure 6A). EP emulsion increased IL-1β expression and promoted the neutrophil infiltration into the spleen (Figure 6B through 6D; Figure XIA

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### Figure 4. Saturated fatty acids cause intracellular crystal formation.

- **A and B**, Murine primary macrophages were treated with palmitic acid (PA), stearic acid (SA), and oleic acid (OA) for 24 h and then analyzed using polarized light microscopy. Representative images of crystal formation were shown. **C and D**, Quantitative analysis of crystal formation was performed (n=3). **E and F**, J774-hIL1B cells were treated with PA, SA, and OA for 24 h and then analyzed using polarized light microscopy. Quantitative analysis of crystal formation was performed (n=3). Data are expressed as the mean±SEM. *P<0.05, **P<0.01, ***P<0.005 as determined by 1-way ANOVA with Tukey-Kramer test. Data are representative of 2 independent experiments.

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Moreover, the increased expression of neutrophil markers in EP emulsion–injected mice was attenuated in IL-1β−/− mice (Figure XIC and XID in the online-only Data Supplement). The crystal-like objects and the elevated expression of neutrophil markers were also observed in the pericardial adipose tissue of EP emulsion–injected mice (Figure XIIA through XIIF in the online-only Data Supplement).

Finally, we investigated whether fatty acid crystals provoke neutrophil infiltration in an IL-1β–dependent manner. Intraperitoneal injection of crystals, including cholesterol and monosodium urate, induces NLRP3 inflammasome-driven IL-1β release and acute peritoneal inflammation, accompanied by infiltration of neutrophils. Using this acute inflammation model, we examined whether SFA-derived crystals exerted inflammatory potency in vivo. Flow cytometry analysis clearly showed that peritoneal infiltration of neutrophils (Ly-6G+CD45R−) was significantly increased after injection of PA-C or Ch-C, although there was no significant difference in the total number of peritoneal cells (Figure 6E through 6G). The mature IL-1β was detected in the peritoneal lavage fluid of both PA-C– and Ch-C–injected mice (Figure XIII in the online-only Data Supplement). Additionally, resident macrophages completely disappeared from the peritoneal lavage fluid at this time point (data not shown). These findings are consistent with a previous study describing the monosodium urate crystal–induced murine peritoneal model of acute gout. The infiltration of neutrophils was significantly

![Figure 5](http://ahajournals.org)
Figure 6. Saturated fatty acid crystals provoke IL-1β (interleukin-1β)–mediated peritoneal inflammation in vivo. A through D, Wild-type (WT) mice were injected intravenously with vehicle, ethyl palmitate (EP) emulsion, or ethyl oleate (EO) emulsion every 2 h. After 4 h, crystallization and neutrophil recruitment to the spleen were analyzed. A, The crystallization in the spleen was analyzed using polarized light microscopy. B, The IL-1β levels in the spleen tissues were determined by ELISA. C, The spleen sections were immunohistochemically stained for Gr-1. D, The expression levels of Ly6g and Cxcr2 were determined by real-time polymerase chain reaction. E through G, WT mice were injected intraperitoneally with vehicle (PBS), palmitic acid–derived crystals (PA-C; 2 mg/mouse) or cholesterol crystals (Ch-C; 2 mg/mouse; n=7 for each). After 6 h, the peritoneal lavage cells were harvested and analyzed by flow cytometry. E, Representative plots of neutrophils (CD45+Ly6G+CD45R−). F and G, The number of total live cells (F) and neutrophils (G) in the peritoneal lavage was quantified. H and I, WT and IL-1β−/− mice were injected intraperitoneally with PA-C (2 mg/mouse; n=9–7 for each). The number of total live cells (H) and neutrophils (I) in the peritoneal lavage was quantified. *P<0.05, **P<0.005 as determined by Mann–Whitney U test or Kruskal–Wallis test.
decreased in PA-C–injected IL-1β−/− mice (Figure 6H and 6I). These findings indicate that SFA-derived crystals trigger the IL-1β–dependent inflammatory responses in vivo.

Discussion

The major findings of this study are as follows: (1) PA and SA induced the NLRP3 inflammasome activation and subsequent IL-1β release in macrophages, which were inhibited in the presence of OA; (2) the inhibitory effect of OA on the NLRP3 inflammasome activation was accompanied by accumulation of triglycerides; (3) the inhibition of FA acyl-CoA synthesis failed to inhibit but rather promoted PA-induced IL-1β release with increased intracellular FFAs; (4) SFAs caused lysosomal dysfunction and intracellular crystallization; and (5) SFA-derived crystals induced NLRP3 inflammasome activation in vitro and provoked acute inflammatory responses in an IL-1β–dependent manner in vivo. The results of this study clearly indicate that excess SFAs cause intracellular crystallization and subsequent lysosomal dysfunction, leading to the activation of the NLRP3 inflammasome. These findings clarify the novel molecular mechanism by which SFAs activate the NLRP3 inflammasome.

Increasing evidence indicates that inflammatory responses induced by SFAs play a central role in the development of metabolic diseases. However, the precise mechanism underlying the SFA-induced inflammatory responses has not been fully understood. In the present study, we demonstrated that PA and SA caused intracellular crystallization and subsequent lysosomal dysfunction, thereby leading to NLRP3 inflammasome activation and subsequent IL-1β release in macrophages. Furthermore, the SFA-induced intracellular crystallization and NLRP3 inflammasome activation were significantly inhibited in the presence of OA. These findings suggest the importance of the imbalance between SFAs and USFAs in lipotoxic dysfunction. In this regard, Salvadó et al.21 reported that the excess of SFAs in the skeletal muscle triggers endoplasmic reticulum stress, which is restored by USFAs. Although it is currently unknown how imbalanced FAs causes lipotoxicity, the conversion of FFAs to triglycerides is thought to be responsible for the alleviation of lipotoxicity. In fact, overexpression of diacylglycerol O-acyltransferase 2, a major enzyme of triglyceride synthesis, in macrophages reduces adipose inflammation and insulin resistance induced by a high-fat diet.24 Consistently, OA not only increased intracellular levels of triglycerides but also inhibited inflammasome activation in PA-treated cells. Thus, we assume that the inhibitory effect of OA on the inflammasome activation is mediated through, at least in part, the conversion of FFAs to triglyceride.

Excess SFAs promote the synthesis of lipid species, such as ceramides and diacylglycerol, which are proposed to induce various biological effects.1,3 In this process, the synthesis of FA acyl-CoA is essential for the production of these lipid species. In particular, ceramide is reported as a danger signal for the NLRP3 inflammasome.16 However, the inhibition of FA acyl-CoA synthesis by triacsin C failed to inhibit PA-induced IL-1β release, indicating little contribution of FA metabolites, including ceramides to the PA-induced inflammasome activation. This is also supported by the findings that PA-induced inflammasome activation was inhibited by neither Elovl6 deficiency nor Scd1 overexpression. In accordance with our data, Camell et al.30 recently reported that macrophage deletion of serine palmitoyltransferase long chain-2, a rate-limiting enzyme involved in the de novo ceramide synthesis pathway, does not alter PA-induced IL-1β release by the NLRP3 inflammasome. However, the elevated intracellular FFA levels by the inhibition of FA acyl-CoA synthesis have been shown to promote apoptosis in SA-treated or very-low-density lipoprotein–treated macrophages.31 Therefore, we have hypothesized that increased FFAs in macrophages are responsible for SFA-induced inflammasome activation and identified intracellular crystallization induced by FFAs as a danger signal for NLRP3 inflammasome activation.

Common pathways implicated in NLRP3 inflammasome activation include lysosomal destabilization and cathepsin leakage into the cytosol, potassium efflux, and generation of mitochondrial ROS. Of these, it is generally accepted that several crystals derived from cholesterol, monosodium urate, and calcium phosphate are phagocytosed by macrophages and cause lysosomal dysfunction, resulting in the NLRP3 inflammasome activation.17,32 Consistently, we observed that lysosomal dysfunction is involved in PA-induced inflammasome activation. Intriguingly, however, the evidence that the phagocytosis inhibitor cytochalasin D failed to prevent SFA-induced inflammasome activation indicates that the crystallization occurs inside the cells by accumulation of FFAs. Intracellular crystallization by lipid species was recently reported by Sheedy et al. They reported that when oxidized low-density lipoprotein was incorporated by macrophages via scavenger receptor CD36, free cholesterol was crystalized in the lysosomes, and it activated NLRP3 inflammasome. Because CD36 also acts as FA transporter, we assume that SFAs are incorporated by macrophages via CD36, at least in part. Another important point to be addressed is that OA inhibited crystallization in SFA-treated macrophages. At physiological temperature, OA is in a liquid state because of its unsaturated bonds, whereas PA and SA are in solid state. In addition, SA has been shown to exhibit higher melting point in the presence of OA than PA. Hence, we postulate that melting temperature of fatty acids influences the crystallization and subsequent NLRP3 inflammasome activation.

We clearly demonstrated that excess SFAs induce crystallization and IL-1β release not only in primary macrophages in vitro but also in mice in vivo. In particular, we detected the crystal deposition and neutrophil infiltration in the red pulp of the spleen. Because macrophages are abundant in the red pulp of the spleen, we postulate that SFAs are incorporated and crystallized in macrophages, leading to IL-1β release and neutrophil infiltration. Consistently, PA-C also induces IL-1β–dependent neutrophil infiltration and inflammation. With respect to Ch-C, Warnatsch et al. recently reported that interaction between Ch-C and neutrophils licenses macrophages for cytokine production during the development of atherosclerosis. However, several limitations of this study should be considered. First, the generation of fatty acid crystals in a disease-relevant setting in vivo is still unclear. In this regard, previous reports described that lipid crystals and elevated FFA levels were detected in the synovial fluid of acute monoarthritis patients, suggesting the link between fatty acid crystals and arthritis. Second, although excess SFA
induce crystallization, the composition of the crystals could not be defined. Thus, further investigations are necessary to understand the precise mechanism and the pathological role of SFA-induced crystallization in the pathogenesis of metabolic diseases.

In conclusion, this study shows that SFA-induced intracellular crystallization is responsible for the mechanism by which SFAs activate the NLRP3 inflammasome in the macrophages. Our results also revealed that SFA crystals provoke acute inflammatory responses in an IL-1β-dependent manner in vivo. Thus, our findings provide novel insights into the pathogenesis of metabolic diseases and suggest a potential novel target for the treatment with metabolic diseases.

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Disclosures

None.

References


**Highlights**

- Saturated fatty acids, such as palmitic acid and stearic acid, induced intracellular crystallization and NLRP3 (nucleotide-binding oligomerization domain, leucine-rich repeat and pyrin domain containing 3) inflammasome activation in macrophages.
- The crystallization and subsequent NLRP3 inflammasome activation were inhibited in the presence of oleic acid.
- Saturated fatty acid–derived crystals induced NLRP3 inflammasome activation in vitro and IL-1β (interleukin 1β)–dependent neutrophil recruitment in vivo.