

Video Article

Saturated Fatty Acids Induce Ceramide-associated Macrophage Cell Death

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URL: <https://www.jove.com/video/56535>

DOI: [doi:10.3791/56535](https://doi.org/10.3791/56535)

Keywords: Immunology, Issue 128, Bone marrow-derived macrophages, BSA (bovine serum albumin), saturated, fatty acid, palmitic acid, stearic acid, ceramide, cell death

Date Published: 10/31/2017

Citation: Zhang, Y., Hao, J., Sun, Y., Li, B. Saturated Fatty Acids Induce Ceramide-associated Macrophage Cell Death. *J. Vis. Exp.* (128), e56535, doi:10.3791/56535 (2017).

Abstract

Macrophages highly express epidermal fatty acid-binding protein and adipose fatty acid-binding protein. They actively uptake saturated and unsaturated fatty acids, which might play a critical role in regulating their immune functions. Numerous studies have shown that various fatty acids, saturated or unsaturated, may possess different impacts on cell growth and function. However, the approaches used for fatty acid preparation vary, which may lead to non-physiological results. Serum albumin, a natural carrier for fatty acids in mammalian peripheral blood, is recommended for forming a conjugate complex with the sodium salt of fatty acids to study fatty acid function in mammalian cells, thus minimizing the toxicity of fatty acid soap. Thus, a simple, relatively quick heating and sonicating method is developed and presented here for BSA-fatty acid conjugate formation. We describe a protocol using saturated fatty acids, especially stearic acids to induce severe cell death in mouse bone-marrow derived macrophages. We further demonstrate that saturated fatty acid-induced cell death is positively associated with accumulated cellular ceramide levels. This method can be extended for studies of the impact of fatty acid on other mammalian cells.

Video Link

The video component of this article can be found at <https://www.jove.com/video/56535/>

Introduction

Fatty acids play a critical role in energy metabolism and in the synthesis of membrane phospholipids in different kinds of cells. Fatty acids have a low aqueous solubility. Appropriate preparation of fatty acid is of critical importance for studying the biological functions of fatty acids in mammalian cells. When fatty acids are prepared with ethanol, many fatty acids may show their toxic soap (detergent) effect on the cell membrane, even at relatively low concentrations¹. As a natural, major transporter for free fatty acids in the serum, serum albumin is considered a good carrier for fatty acid delivery *in vitro* for fatty acid function assays^{2,3,4}. However, the details of the preparation of fatty acid and serum albumin conjugate are usually not available even though many research papers using fatty acids have been published.

Macrophages highly express epidermal fatty acid-binding protein and adipose fatty acid-binding protein^{5,6,7,8}. They actively uptake saturated and unsaturated fatty acids which may regulate their immune functions. To study the impact of fatty acids on macrophages and other cells, different methods of fatty acid preparation were applied^{1,7,9}. Using appropriately prepared fatty acid/serum albumin conjugates to investigate the impact of fatty acids on macrophage function is of critical importance in obtaining biologically meaningful data. Studies on the impact of fatty acids on macrophage function can provide basic knowledge and potential therapeutic targets relating to fatty acid metabolism in macrophage-involved diseases.

Protocol

The protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of University of Louisville.

1. Mouse Bone Marrow Derived Macrophages (BMDMs)

1. Euthanize a 6- to 8-week-old, healthy wild type mouse using CO₂. Pin it down to a foam board and spray it with 70% ethanol until it is soaked. Remove the tibia/femur bones with forceps and scissors. Put them in a petri dish with 5 mL 1x phosphate-buffered saline (PBS). Perform all procedures under sterile conditions.
2. Cut open both ends of the tibia/femur bone with a scissor in a sterile tissue culture hood. Flush the bone with a 25 G needle with PBS + 2% fetal bovine serum (FBS) into a 15 mL tube.
3. Centrifuge the bone marrow cells at 500 x g, 4 °C for 5 min.
4. Resuspend the cell pellet in 1 mL red blood cell lysis buffer to lyse red blood cells (RBC) for less than 1 min. Dilute immediately with 9 mL 1x PBS. Centrifuge again as in step 1.3. Decant the supernatants.

5. Resuspend the cell pellet in 10 mL 1x PBS, filter the cell suspension through a sterile 40 μ m nylon mesh into another 15 mL tube to remove cell debris/clumps. Centrifuge again as in 1.3. Decant the supernatants.
6. Resuspend the cell pellet in 15 mL Roswell Park Memorial Institute medium (RPMI) 1640 with 5% FBS and 10 μ g/mL gentamicin, plate the cells in a 100-mm tissue culture dish at 37 °C incubator for 60 min. Gently swirl the dish, take out the floating cells, and count them.
7. Plate 6×10^6 cells in a 100-mm dish with 12 mL macrophage differentiating media (RPMI 1640 with 5% FBS and 10 μ g/mL gentamicin, mixed with 30% L929 conditioned media¹⁰) with 10 ng/mL recombinant mouse macrophage colony-stimulating factor (M-CSF) for 2 days.
8. After culturing for 2 days in a 37 °C incubator with 5% CO₂, feed each dish with 6 mL fresh macrophage differentiating media with 10 ng/mL M-CSF.
9. On day 5, take 8 mL of the old media out without disturbing the cells and add 10 mL fresh macrophage differentiating media with 10 ng/mL M-CSF.
10. **On day 7, harvest the bone marrow-derived macrophages (BMDMs) using a cell lifter.**
NOTE: Attached macrophages can also be dissociated with 5 mL 1 mM EDTA in 1x PBS for 5 to 10 min after removing the float cells and washing plate twice with 5 mL 1x PBS. Centrifuge the cells as in step 1.3., resuspend them in fresh macrophage differentiating media, and count them with a hemocytometer.
 1. Confirm the BMDMs' phenotype by flow cytometry as described⁵. Prepare the derived mouse macrophages in a certain concentration for the following studies.

2. BSA-fatty Acid Conjugate Preparation

1. Prepare 2 mM BSA in sterile PBS. For example, weigh 6.65 g BSA, add 30 mL 1x PBS to dissolve, then add more PBS until 50 mL, scaling components to make 2 mM BSA in 1x PBS.
2. Prepare 5 mM individual sodium salt of fatty acids in 2 mM BSA. Heat to 37 °C or higher, if necessary, and sonicate the mixture of 2 mM BSA and sodium salt of fatty acids until a clear solution is obtained.
NOTE: Compared to unsaturated fatty acids, saturated fatty acids need a higher temperature and more sonication time for dissolving.
3. Filter the fatty-acid-BSA solution through a 0.22 μ m filter, aliquot it into 1.5 mL sterile microcentrifuge tubes, and store them at 4 °C for short term use or at -20 °C for long term storage. No precipitation should be observed after storage at 4 °C in the refrigerator.

3. Saturated Fatty Acids Induce Cell Death of Mouse Bone Marrow Derived Macrophages

1. Plate the BMDMs in a 24-well tissue culture plate with 0.4×10^6 /mL in macrophage differentiating media.
2. Treat the cells with the 0.4 mM palmitic acid and stearic acid for 16 to 24 h at a 37 °C incubator with 5% CO₂. Use BSA as the negative control.
3. Harvest the cells with a cell lifter after treatment and spin down the cells at 500 x g, 4 °C for 5 min.
4. Stain the cells with Annexin V-Alexa 488 and 7-aminoactinomycin D (7-AAD) in 100 μ L annexin V binding buffer for 15 min at room temperature.
5. Dilute the sample with 200 μ L of annexin-binding buffer, mix gently, then keep the samples on ice after the incubation period.
6. Analyze the stained cells as soon as possible by flow cytometry⁵. Annexin V-Alexa 488 is detected in FITC channel and 7-AAD is detected in PerCP/PerCP-Cy5.5 channel.

4. Intracellular Ceramide Staining

1. After fatty acid treatment, harvest the cells as in step 3.3. Then fix the cells in 4% paraformaldehyde for 30 min.
2. Wash the sample with 1 mL 1x PBS, spin down as step in 3.3, and decant the supernatant.
3. Stain the cells with anti-ceramide primary antibody (1:200 dilution) in 100 μ L 1x permeabilization buffer for 30 min.
4. Wash and spin down the cells again as in step 4.2.
5. Stain the cells with anti-mouse IgM secondary antibody (1:500 dilution) in 100 μ L 1x permeabilization buffer for 30 min.
6. Wash and spin down the cells again as in step 4.2. Add 250 μ L 1x PBS and analyze the stained cells by flow cytometry⁵.
NOTE: See the detailed reagent list in the Table of equipment and reagents.

Representative Results

Obesity increases free fatty acid concentrations in serum. As professional phagocytes, macrophages actively take up fatty acids to maintain host homeostasis. During these processes, overloaded lipids may induce macrophage cell death. To this end, we cultured BMDMs *in vitro* with obese levels of dietary fatty acids and measured macrophage cell death using flow cytometric staining. Compared to the BSA control, saturated fatty acids, in particular stearic acids, induced significant cell death of BMDMs. Dead cells were shown as double positive populations stained by Annexin V and 7-AAD (**Figure 1a-b**). Of note, unsaturated fatty acids did not induce significant macrophage cell death⁵. The data suggest the toxic effect of increased levels of free saturated fatty acids *in vivo*.

Similarly, using a macrophage cell line whose viability was susceptible to the treatment of either palmitic acids or stearic acids⁵, we found that macrophages' susceptibility to palmitic acid-induced cell death was also affected by the number of cells in the culture (**Figure 2a**). Simply put, under the same cultural conditions, the more macrophages in the wells, the less cell death induced by the fatty acids. Moreover, palmitic acid-induced cell death was positively associated with accumulated levels of cellular ceramides (**Figure 2b**). When macrophages were cultured in a high-density condition, medium nutrients were depleted faster for energy metabolism, these cells were less susceptible to palmitic acid-induced cell death compared to macrophages cultured in a low-density condition. In contrast, when cells were exposed to a nutrient-enriched condition (e.g., obesity), excess fatty acids can be metabolized to produce toxic ceramides, thereby leading to fatty acid-induced cell death. Thus, cell cultural conditions may bring variation to the results of saturated fatty acid-induced cell death.

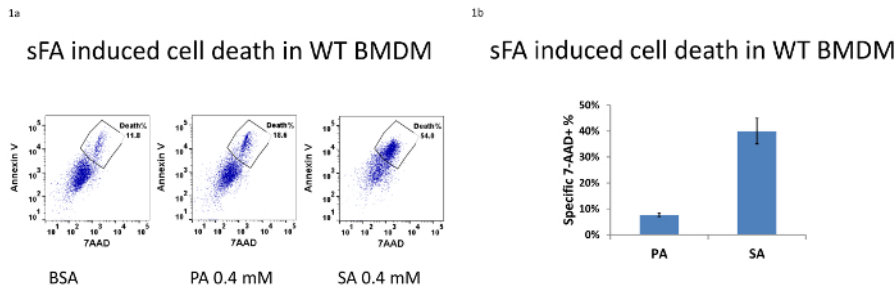


Figure 1: High concentration of fatty acids, especially stearic acids, induce macrophage cell death. BMDMs (day 7) were plated as 0.4×10^6 /mL in fresh macrophage differentiating media. After cell attachment for 0.5 - 1 h, BMDMs were treated with the designated concentration of each fatty acid for 24 h. Media alone and BSA were used as negative controls. After treatment, cells were lifted and harvested, then spun down at 500 g for 5 min at 4 °C. Cells were stained with 7-AAD and Annexin V Alexa 488 in 100 μ L annexin-binding buffer for 15 min. Then add another 200 μ L annexin-binding buffer to each sample before flow cytometry analysis. **(A)** Demonstration of flow cytometry data of cells induced by fatty acids. **(B)** Summary of the cell death induced by fatty acids. Error bar represents sample SD. The cell death induced by palmitic acid (PA) and particularly stearic acid (SA) is statistically significant compared to BSA control's. [Please click here to view a larger version of this figure.](#)

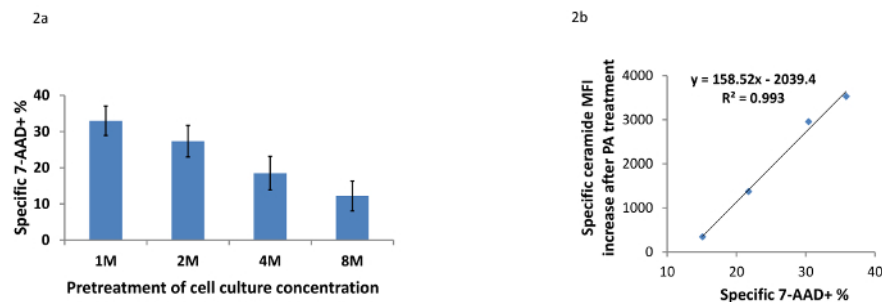


Figure 2: The availability of media nutrients impacts macrophages' susceptibility to palmitic acid induced cell death. **(A)** Cell nutrition status impacts its tolerance to saturated fatty acid-induced stress. **(B)** Cell susceptibility to saturated fatty-acid induced cell death is highly correlated with accumulated cellular levels of ceramide. The media nutrient gradient was achieved by culturing the wild type macrophage cell line at 1M, 2M, 4M and 8M ($M=1 \times 10^6$) in 6-mm petri dishes with 6 mL RPMI 1640 with 5% FBS and 10 μ g/mL gentamicin for 18 hr. The media color is more yellow due to more nutrient depletion when a greater number of cells are cultured in the dishes. More than 90% of the cells are still highly viable. The attached viable cells were lifted by pipetting after sitting in PBS for 5 min and re-cultured immediately at 0.2×10^6 /mL in fresh media in 24-well plates. Cells were treated immediately with 0.4 mM palmitic acid for 18 h using BSA as the control for each source (nutrient gradient) of cells. Then all treated cells were harvested and stained with 7-AAD for cell death or stained with intracellular ceramide. Spontaneous cell death (7-AAD+) percentages were less than 8.6%. Treatment specific cell death was estimated as the % difference in 7-AAD+ between treatment and its own BSA control **(a)**. The treatment specific ceramide level was estimated as the mean fluorescence intensity (MFI) difference between the treatment and its own BSA control **(b)**. This experiment was repeated, and representative data are presented. Error bar represents sample SD. [Please click here to view a larger version of this figure.](#)

Discussion

The proper preparation of fatty acid solution is of critical importance to study the biological function of fatty acids. The neutralization of fatty acid increases its solubility in aqueous solution. However, sodium salts of fatty acids, especially saturated fatty acids, are still of low solubility in water or PBS as we observed. One method is to use 95 - 100% ethanol to help dissolve fatty acid¹. Using this method, higher toxicity to cells may be observed even at lower concentrations for less toxic unsaturated fatty acids. Another way to prepare fatty acid solution is to utilize methyl- β -cyclodextrin as a delivery system so that fatty acids have optimal conditions, remaining in solution in their monomeric form^{9,11}. Fatty acids prepared with such a method appeared to have normal function, but conducting the method is complicated. The impact of methyl- β -cyclodextrin on cell growth is that high concentration could lead to cell death¹². For saturated stearic and palmitic acids, a high temperature (60 °C) is used to help with solubilization⁹.

Since serum albumin is a major fatty acid-binding protein in extracellular fluid, which aids in transporting fatty acid in the lymphatic and vascular systems, it is favored as the natural carrier for the functional study of fatty acids. To avoid the low solubility of fatty acid salt in aqueous solution, sonication will greatly facilitate the conjugation of fatty acid with BSA, thus aiding in fatty acid solubilization. It takes much longer (hours) to solubilize the saturated fatty acids than the unsaturated fatty acids (30 min) with same amount of volume and concentration. Although heating and sonication are critical for BSA-fatty acid conjugation, a high temperature (less than 50 °C) is suggested for obtaining clear BSA-PA, BSA-SA conjugate solutions, and a low temperature is strongly recommended for solubilizing unsaturated fatty acids due to their susceptibility to oxidation at high temperature. The 5:2 molar ratio of fatty acid to BSA is considered good compared to the 3:1 or 6:1 molar ratio that was published⁷. This can ensure less totally-free fatty acid in the solution to avoid the toxicity of fatty acid soap. BSA-fatty acids prepared this way appear to be stable solutions (no precipitation, at least) at 4 °C for at least 3 months as shown in their functionality on macrophage cell death. Seal the cap of the tube if prepared fatty acids are not to be used for a long period of time.

When a clear solution of BSA-fatty acid in PBS is obtained, consistent results from fatty acid treatment are more likely warranted. However, starving and well-fed macrophages respond quite differently to fatty acid treatment, especially saturated fatty acids. Macrophages with excess

nutrients are more susceptible to saturated fatty acid-induced cell death than starving or starved ones because hungered cells can metabolically detoxify the saturated fatty acids due to their survival and growth request. Such cell death is highly associated with accumulated cellular ceramide levels. Of note, ceramide toxicity occurs only when intracellular ceramide levels reach a certain threshold. To ensure consistent results from fatty acid treated macrophages or any other type of cells, cells should be cultured with fresh media with plentiful nutrients. Any sub-optimal cell culture conditions could result in sub-optimal data.

Overall, these protocols provide much needed details for studying the impact of fatty acids on macrophages by other laboratories. Compared to other methods, this direct approach generates BSA-fatty acid conjugates without using an extra solvent like ethanol. The most challenging step is to prepare accurate, stable BSA-fatty acid solution because sonication generates heat and it is hard to control temperature. Fortunately, unsaturated fatty acids are easy to prepare in a short time and remain stable, while saturated fatty acids appear to stand relatively high temperatures because they are saturated and resistant to oxidation in such a condition. So far, we have not observed any issue or limitation with BSA-fatty acid conjugates prepared this way. Modification of our protocol would be necessary if more sensitive experiments are needed.

Disclosures

The authors declare no financial conflicts of interest.

Acknowledgements

This work was supported partially by the University of Louisville start-up funds and National Cancer Institute (Bethesda, MD) grants R01CA177679, R01CA180986.

References

1. Martins de Lima, T., Cury-Boaventura, M. F., Giannocco, G., Nunes, M. T., & Curi, R. Comparative toxicity of fatty acids on a macrophage cell line (J774). *Clin Sci (Lond)*. **111** (5), 307-317 (2006).
2. Simard, J. R., Zunszain, P. A., Hamilton, J. A., & Curry, S. Location of high and low affinity fatty acid binding sites on human serum albumin revealed by NMR drug-competition analysis. *J Mol Biol*. **361** (2), 336-351 (2006).
3. Penn, A. H., Dubick, M. A., & Torres Filho, I. P. Fatty Acid Saturation of Albumin Used in Resuscitation Fluids Modulates Cell Damage in Shock: In Vitro Results Using a Novel Technique to Measure Fatty Acid Binding Capacity. *Shock*. (2017).
4. Vusse, G. J. Albumin as fatty acid transporter. *Drug Metab Pharmacokinet*. **24** (4), 300-307 (2009).
5. Zhang, Y. *et al.* Adipose Fatty Acid Binding Protein Promotes Saturated Fatty Acid-Induced Macrophage Cell Death through Enhancing Ceramide Production. *J Immunol*. **198** (2), 798-807 (2017).
6. Zhang, Y. *et al.* Epidermal Fatty Acid binding protein promotes skin inflammation induced by high-fat diet. *Immunity*. **42** (5), 953-964 (2015).
7. Wen, H. *et al.* Fatty acid-induced NLRP3-ASC inflammasome activation interferes with insulin signaling. *Nat Immunol*. **12** (5), 408-415 (2011).
8. Zhang, Y. *et al.* Fatty acid-binding protein E-FABP restricts tumor growth by promoting IFN-beta responses in tumor-associated macrophages. *Cancer Res*. **74** (11), 2986-2998 (2014).
9. Ulloa, J. E., Casiano, C. A., & De Leon, M. Palmitic and stearic fatty acids induce caspase-dependent and -independent cell death in nerve growth factor differentiated PC12 cells. *J Neurochem*. **84** (4), 655-668 (2003).
10. Weischenfeldt, J., & Porse, B. Bone Marrow-Derived Macrophages (BMM): Isolation and Applications. *CSH Protoc*. **2008** pdb prot5080 (2008).
11. Dansen, T. B. *et al.* High-affinity binding of very-long-chain fatty acyl-CoA esters to the peroxisomal non-specific lipid-transfer protein (sterol carrier protein-2). *Biochem J*. **339** (Pt 1) 193-199 (1999).
12. Ulloa, J. E. *et al.* Characterization of methyl-beta-cyclodextrin toxicity in NGF-differentiated PC12 cell death. *Neurotoxicology*. **28** (3), 613-621 (2007).