Activation of NLRP3 Inflammasome by Palmitic Acid in Human Sebocytes

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Background: Sebocytes are the main cells involved in the pathogenesis of acne by producing lipids and inflammatory cytokines. Although palmitic acid (PA) has been suggested to induce an inflammatory reaction, its effect on sebocytes remains to be elucidated.

Objective: In the present study, we investigated whether PA promotes inflammasome-mediated inflammation of sebocytes both in vivo and in vitro.

Methods: We intradermally injected PA into the mice ears. And, we treated cultured human sebocytes with PA. Inflammasome-mediated inflammation was verified by immunohistochemistry, Western blot and ELISA.

Results: PA-treated mice developed an inflammatory response associated with increased interleukin (IL)-1β expression in the sebaceous glands. When PA was added to cultured human sebocytes, caspase-1 activation and IL-1β secretion were significantly enhanced. In addition, NLRP3 knockdown attenuated IL-1β production by sebocytes stimulated with PA. PA-mediated inflammasome activation required reactive oxygen species.

Conclusion: These findings indicate that PA activates the NLRP3 inflammasome before induction of an inflammatory response in sebocytes. Thus, PA may play a role in the inflammation of acne.

Keywords: Inflammasomes, NLRP3, Palmitic acid, Sebocyte

INTRODUCTION

Acne is one of the most common skin diseases, and pathogenic factors include increased sebum production, changes in follicular growth and differentiation, Propionibacterium acnes colonization, and inflammation. Several reports have shown that P. acnes induce interleukin (IL)-1β production by triggering activation of the NLRP3 inflammasome, providing evidence for a potential role of inflammasome-related innate immunity in acne pathogenesis.

Palmitic acid (PA), one of the most abundant saturated free fatty acids (FFAs) in plasma, promotes chronic inflammatory responses by inducing the synthesis of inflammatory cytokines and is emerging as an important contributor to the pathogenesis of type 2 diabetes and other metabolic disorders. Much evidence suggests a role of the inflammasome in the onset of FFA-induced inflammation, and PA also has been proposed to play an important role in NLRP3 inflammasome-mediated inflammatory responses.

Increased sebum excretion and alterations in certain lipid components are major features of acne pathogenesis. Triglycerides, the most abundant sebum components, are converted to FFAs by bacterial hydrolases found on the skin surface. PA is one of the principal FFAs, and its level increases significantly...
in acne lesions\textsuperscript{11,12}. PA might be involved in acne inflammation by triggering the production of proinflammatory cytokines in keratinocytes\textsuperscript{13}. Together with keratinocytes, sebocytes may act as immune-active cells and can recognize pathogens. In addition, sebocytes are known to synthesize considerable amounts of FFAs\textsuperscript{9,11}. However, there are little researches on the inflammatory reaction of sebocytes induced by FFAs. In the present study, we investigated whether PA stimulates IL-1\(\beta\)-mediated inflammatory responses through the inflammasome in sebocytes.

**MATERIALS AND METHODS**

**Reagents and antibodies**
Sodium palmitate, N-acetyl-L-cysteine (NAC), and fatty acid-free bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St Louis, MO, USA). We used the following specific antibodies for Western blotting and immunohistochemical analysis: anti-IL-1\(\beta\) and -AIM2 (Abcam, Cambridge, MA, USA); anti-caspase-1 (Cell Signaling Technology, Danvers, MA, USA); anti-NLRP3 (Adipogen, San Diego, CA, USA); and anti-\(\beta\)-actin (Santa Cruz Biotechnologies, Santa Cruz, CA, USA).

**Palmitic acid–bovine serum albumin conjugation**
To enhance the solubility of PA in cell-based assays, we used BSA as a carrier and stabilizing agent for insoluble PA. Sodium palmitate was dissolved in 150 mM NaCl at 70°C and then conjugated with fatty acid-free BSA in 150 mM NaCl, at a 3:1 molar ratio.

**Animal test**
Seven-week-old C57BL/6 mice (Orient Bio, Seongnam, Korea) were used in all experiments. Mice were kept under the pathogen-free condition in the animal facility of Chungnam National University School of Medicine, at 24°C to 25°C, provided with food and water ad libitum. All animal experiments were approved by Chungnam National University Institutional Animal Care and Use Committee (CNU-00639).

A 20-\(\mu\)l aliquot of 100 \(\mu\)M PA was intradermally injected into the central portion of one ear and an equal volume of PBS was injected into the other ear (control). The increase in ear thickness was measured using microcalipers (Mitutoyo, Kawasaki, Japan) 24 hours after PA injection. Six female mice were used for this study. Twenty four hours after injection, mice were euthanized by cervical dislocation. For histological examination, the ear was fixed in 10% (v/v) formaldehyde, embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

**Immunohistochemistry**
The paraffin-embedded sections were de-waxed, rehydrated, and incubated overnight at 4°C with the anti-IL-1\(\beta\) antibody. After washing, sections were incubated with peroxidase-conjugated secondary antibody (Dako, Carpinteria, CA, USA) and visualized with Chemmate envision detection kit (Dako).

**Cell culture**
Sebocytes were cultured as described previously\textsuperscript{14}. Cells were maintained in Sebomed\textsuperscript{TM} medium (Biochrom, Berlin, Germany) supplemented with 10% fetal bovine serum (FBS) and 5 ng/ml recombinant human epidermal growth factor (rhEGF; Thermo Fisher Scientific, Waltham, MA, USA). Sebocytes at the 3rd–7th passage were used for all experiments.

**Cytotoxicity test**
For the cytotoxicity test, cells were treated with various doses of PA for 24 hours. Then the medium was replaced with a fresh medium containing 0.5 mg/ml 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) solution and cells were incubated for an additional 2 hours. Finally, the formazan crystal was dissolved with DMSO. Cell viability was determined by measuring optical density at 570 nm using an ELISA reader.

**Real-time reverse transcription-polymerase chain reaction (RT-PCR)**
Two micrograms of total RNA were reverse transcribed and used for PCR amplification. The primer sequences are as follows: IL-1\(\beta\) forward 5’-ACGAATCTCCGACCACCACTA-3’ and reverse 5’-TCCATGGCCACAACAAACTGA-3’. SYBR Green Real-Time PCR Master Mix (Thermo Fisher Scientific) was used for quantitative real-time RT-PCR.

**Enzyme-linked immunosorbsent assay (ELISA)**
To measure the IL-1\(\beta\) level, supernatants were collected and frozen prior to ELISA. IL-1\(\beta\) was detected using ELISA kits from MyBioSource (San Diego, CA, USA), according to the manufacturer’s instruction.
Western blotting
Cells were lysed in Pro-prep™ solution (Intron, Deajeon, Korea). Samples were run on sodium dodecyl sulfate-polyacrylamide gels, transferred to nitrocellulose membranes, and incubated with appropriate primary antibodies. The blots were then incubated with peroxidase-conjugated secondary antibodies and visualized by an enhanced chemiluminescence method (Intron).

Knockdown of gene expression
For knockdown experiments, we used recombinant adenoviruses expressing a microRNA (miR) targeting caspase-1, NLRP3, ASC, and AIM2. For adenoviral transduction, sebocytes were incubated with the adenovirus at a multiplicity of infection of 10 overnight. Cells were replenished with fresh medium and incubated for 2 days further.

Reactive oxygen species detection
We measured mitochondrial reactive oxygen species (ROS) levels using the MitoSOX™ system (Thermo Fisher Scientific). Sebocytes were treated with PA in the presence or absence of the ROS inhibitor NAC and were then loaded with 5 μM MitoSOX™ for 30 minutes at 37°C. Mean fluorescence intensities were determined using a FACS Canto II flow cytometer (BD Biosciences, San Jose, CA, USA).

Statistical analysis
Data were evaluated statistically by one-way ANOVA or Student’s t-test using SPSS software ver. 22.0 (IBM Corp., Armonk, NY, USA). Statistical significance was set at \( p<0.05 \).

RESULTS

Induction of inflammation by palmitic acid in mouse ears
We first injected PA intradermally into mouse ears to determine whether PA induces the inflammatory reaction in vivo. Significant cutaneous erythema and swelling were observed in the test but not control ears (Fig. 1A). Moreover, PA-treated ears exhibited significant increases in ear thickness compared to controls (Fig. 1B). Histologically, PA induced a significant increase in the dermal infiltration of inflammatory cells (Fig. 1C).

To investigate the effect of PA on IL-1β production in vitro,
we treated human primary sebocytes with PA. PA at various concentrations up to 100 μM exerted no cytotoxic effect on cultured sebocytes (data not shown). IL-1β processing requires two signals: induction of pro-IL-1β and the autocatalytic cleavage of pro-caspase-1 to caspase-1. In sebocytes, PA induced the expression of pro-IL-1β in a dose-dependent manner, together with the increase of mature IL-1β in supernatants (Fig. 2A). These data suggest that PA provides the primary signal triggering the processing of IL-1β by the inflammasome, as also found in previous reports. We next investigated the expression of IL-1β mRNA using real-time PCR and found that PA enhanced IL-1β mRNA levels (Fig. 2B). Similarly, PA significantly increased the IL-1β secretion in a dose-dependent manner (Fig. 2C).

We next explored whether PA activates caspase-1. Caspase-1 is an effector molecule of the inflammasome that induces IL-1β production. PA increased the expression of IL-1β mRNA and protein, as well as the cleavage of pro-caspase-1 to caspase-1 (Fig. 2D). To further investigate the role of caspase-1 in IL-1β secretion, we pretreated the sebocytes with a specific caspase-1 inhibitor (Z-YVAD) and found that it significantly inhibited the IL-1β secretion induced by PA (Fig. 2E). Moreover, we knocked down the expression of caspase-1 using a recombinant adenovirus expressing miR specific for caspase-1 (miR-Casp1), and found that this significantly reduced the IL-1β secretion induced by PA (Fig. 2F).

**Fig. 2.** Palmitic acid (PA) increases interleukin (IL)-1β secretion via caspase-1 (Casp1) activation in human sebocytes. (A) The levels of pro-IL-1β protein in cell extracts (CE) and mature IL-1β protein (mIL-1β) in supernatants (SN) were determined by Western blotting. (B) Quantitative RT-PCR analysis of IL-1β mRNA expression after PA stimulation. (C) IL-1β levels in SN after addition of various levels of PA were assessed by ELISA. (D) Pro-caspase 1 (Pro-Casp1) protein and the active Casp1 p20 subunit were analyzed by Western blotting. (E) Sebocytes were pretreated with specific Casp1 inhibitor (Z-YVAD) and next stimulated with PA. IL-1β levels in SN were assessed by ELISA. (F) Expression of Casp1 was knockeddown by a recombinant adenovirus expressing miR specific for Casp1 (miR-Casp1). A recombinant adenovirus expressing scrambled miR (miR-Scr) was used as negative control. (G) Sebocytes were transduced with adenoviruses expressing miR-Casp1 and miR-Scr, then treated with PA. The levels of pro-IL-1β and mIL-1β were measured by Western blotting. (H) SN IL-1β levels were also measured by ELISA. Values are presented as mean ± standard deviation. ns: no significant difference. Data were compared using Student’s t-tests (n = 5; *p < 0.05, **p < 0.01, ***p < 0.001).
maturation by cleavage of the precursor form\textsuperscript{15,16}. PA induced secretion of the mature active form of caspase-1 (p20) (Fig. 2D). And pretreatment with a caspase-1 inhibitor (Z-YVAD) significantly inhibited PA-induced IL-1β secretion by sebocytes (Fig. 2E). We used a recombinant adenovirus expressing a microRNA specific for caspase-1 (miR-Casp1) to confirm the functional role played by this enzyme in IL-1β activation. When miR-Casp1 was expressed, caspase-1 (p20) was markedly decreased (Fig. 2F). Knockdown of caspase 1 significantly reduced PA-induced IL-1β secretion (Fig. 2G, H). These data suggest that PA-mediated induction of caspase-1 played a significant role in IL-1β secretion by sebocytes.

**Palmitic acid enhances IL-1β secretion via the NLRP3 inflammasome in human sebocytes**

ASC is an adaptor protein of the inflammasome complex which recruits pro-caspase-1 and activates the effector caspase\textsuperscript{17}. To further confirm the inflammasome activation by PA, we suppressed gene expression using a recombinant adenovirus expressing miR specific for ASC (miR-ASC) (Fig. 3A). ASC knockdown attenuated PA-mediated IL-1β release (Fig. 3B, C), indicating that ASC sensed the PA level before induction of IL-1β secretion. We next explored which inflammasome (NLRP3 or AIM2) was involved. We generated recombinant adenoviruses expressing miR-NLRP3 and miR-AIM2 (Fig. 3A, D). miR-NLRP3 significantly reduced caspase-1 activation and consequently PA-induced release of IL-1β (Fig. 3B, C) whereas miR-AIM2-transfected sebocytes were not affected (Fig. 3E, F).

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**Fig. 3.** Induction of interleukin (IL)-1β by palmitic acid (PA) was NLRP3-dependent. (A) Expressions of ASC and NLRP3 were knocked down by the recombinant adenoviruses expressing miR specific for caspase-1 (miR-Casp1) and for NLRP3 (miR-NLRP3). A recombinant adenovirus expressing scrambled miR (miR-Scr) was used as negative control. (B) Adenoviruses expressing miR-ASC and miR-NLRP3 were transduced into sebocytes subsequently stimulated with 100 μM PA for 24 hours, and supernatants (SN) and cell extracts (CE) were analyzed by Western blotting. (C) SN IL-1β levels were also assayed by ELISA. (D) Expression of AIM2 was knocked down by the recombinant adenoviruses expressing miR specific for AIM2 (miR-AIM2). (E) Adenoviruses expressing miR-AIM2 was transduced into sebocytes subsequently stimulated with 100 μM PA for 24 hours, and SN and CE were analyzed by Western blotting. (F) SN IL-1β levels after transduction of the recombinant adenoviruses expressing miR-AIM2 and PA treatment were also assayed by ELISA. Values are presented as mean ± standard deviation. ns: no significant difference. Data were compared using Student’s t-tests (n=5; **p<0.01, ***p<0.001).

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Therefore, PA induced IL-1β production and secretion via the NLRP3 inflammasome.

**Palmitic acid-induced caspase-1/IL-1β activation requires reactive oxygen species**

We further investigated the mechanism of induction of the NLRP3 inflammasome after PA stimulation. Previous reports have implicated ROS in NLRP3 inflammasome activation by PA\(^5,7\). We explored whether PA increases ROS generation in sebocytes. The MitoSOX™ system showed that PA significantly increased mitochondrial ROS level and this was abrogated by NAC, an inhibitor of ROS generation (Fig. 4A). Western blotting showed that NAC reduced the level of increased caspase-1 (p20) and mIL-1β after PA treatment (Fig. 4B). Additionally, the ELISA showed that the ROS inhibitor significantly blocked PA-induced IL-1β release in sebocytes (Fig. 4C). Together, the data suggest that ROS generation is important for PA-induced NLRP3 inflammasome activation in sebocytes.

**DISCUSSION**

Sebaceous lipids play key roles in the pathophysiology of acne. FFAs, the predominant components of human sebum, induce calcium influx into keratinocytes and abnormal follicular keratinization\(^8\). Sebum lipids also likely trigger acne inflammation. PA significantly decreases hydrogen peroxide production by neutrophils, causing pro-inflammatory mediators to more easily pass to the dermis, in turn aggravating acne inflammation\(^9\). Besides, PA up-regulates IL-1β, IL-6, and tumor necrosis factor-α secretion via NF-κB activation in keratinocytes\(^13\). Although PA is also produced by sebocytes, the effects of PA on sebocytes for acne inflammation have not been fully clarified. In this study, we showed that PA stimulated IL-1β production in sebaceous gland both in vivo and in vitro. An earlier study has reported that human sebocytes contain all elements necessary to form an inflammasome complex\(^1\). We found that PA significantly activated caspase-1 and the NLRP3-ASC inflammasome; such actions were essential for IL-1β production. Caspase-1 activation requires transcription and translation of pro-IL-1β. A previous report has shown that PA alone cannot induce pro-IL-1β production and it is necessary to prime macrophages with various stimuli, including lipopolysaccharides\(^6\). However, other reports have found that PA activates TLR2-mediated pro-inflammatory signaling pathways to create primary signals which, together with PA-mediated pro-IL-1β induction, are sufficient to trigger inflammasome-mediated IL-1β production\(^7\). TLR2 is a major TLR in human sebocytes\(^20\). We also found that PA alone generated sufficient pro-IL-1β to trigger inflammasome-mediated IL-1β release.

The precise mechanism of PA-induced sebocyte inflammasome activation remains unclear. In this study, we have shown that ROS are necessary for NLRP3-mediated caspase-1 activation and subsequent IL-1β release. Oxidative stressors such as ROS may be involved in acne progression\(^21,22\). Moreover, ROS activates the NLRP3 inflammasome by triggering the release of thioredoxin-interacting protein and other important elements associated with acne inflammation\(^2-4\). Therefore, we hypothesize that PA triggers ROS generation, followed by IL-1β release.
via NLRP3 inflammasome activation in sebocytes. Therefore, antioxidants reducing ROS levels may be useful to control acne inflammation.

The source of the PA that acts on sebocytes is not known. Human sebum is a holocrine secretion and sebocytes release their lipids during final differentiation. Therefore, PA may exert an autocrine effect on sebocytes. Another source of PA might be circulating PA which is a major saturated fatty acid in plasma. PA level rises during consumption of high-fat diets and such diets contribute significantly to acne development\(^{23-25}\). Therefore, increased PA due to high-fat diets may have an endocrine effect on the sebaceous glands of acne patients.

Interestingly, it has been shown that unsaturated fatty acids, especially omega-3 fatty acids, inhibit the NLRP3 inflammasome-dependent inflammation induced by saturated fatty acids such as PA\(^6,7,26\). Besides, omega-3 fatty acids such as eicosapentaenoic and docosahexaenoic acid mitigate inflammation in acne patients\(^27,28\). Considering these points, the effect of omega-3 fatty acids on PA-induced inflammasome activation will be an interesting future study.

In conclusion, we demonstrated that PA induces IL-1\(\beta\)-mediated inflammation via the NLRP3 inflammasome in human sebocytes. Together, our data suggest that PA plays significant roles in acne inflammation, and that inhibition of PA-induced inflammasome activation is a possible target for managing acne patients.

**CONFLICTS OF INTEREST**

The authors have nothing to disclose.

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**DATA SHARING STATEMENT**

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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