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팔미트산이 피지세포에서 염증성 사이토카인의 합성과 피지 생성에 미치는 영향

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최 종 원의 의학박사 학위논문을 인준함

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The effect of palmitic acid on the production of proinflammatory cytokines and sebum in sebocytes

by

Chong Won Choi, M.D.

(Directed by Sang Woong Youn, M.D., Ph.D)

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Approved by thesis committee:

Professor _____ **Chairman**

Professor _____ **Vice Chairman**

Professor _____

Professor _____

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Abstract

The effect of palmitic acid on the production of proinflammatory cytokines and sebum in sebocytes

Chong Won Choi

Medicine, Dermatology

The Graduate School

Seoul National University

Acne is a chronic inflammatory disorder of the pilosebaceous gland unit, which is composed of comedones, papules, and pustules. While multiple etiologic factors are involved in the pathophysiology of acne, four basic etiologic factors are well established: follicular hyperproliferation, increased sebum secretion, presence and role of *Propionibacterium acnes* (*P. acnes*), and inflammation.

Although the relationship between the acne and diet has been controversial, recent studies suggested an alleged relationship between the acne and specific diet such as carbohydrates and dairy products.

Among the various ingredients, the insulin-like growth factor-1 and testosterone in the food were reported to be causative factors in acne. Recently, high fat diet was suggested as an aggravating factor in acne pathogenesis.

We performed *in vitro* studies to evaluate the effects of high fatty acid state on the development and aggravation of acne. The SZ95 sebocytes were supplemented with palmitic acid to mimic the increased level of serum free fatty acids. The effects of palmitic acid on the lipid production were analyzed by evaluating the intracellular lipids. In addition, the secretion of proinflammatory cytokines including IL-6 and IL-8 were also analyzed by using RT-PCR and ELISA.

The SZ95 sebocytes supplemented with palmitic acid showed increased cytoplasmic lipid accumulation and intracellular triglycerides normalized to the protein contents. In addition, the results of RT-PCR and ELISA showed increased mRNA expression and secretion of IL-6 and IL-8. The Western blot revealed that the changes in SZ95 sebocytes after palmitic acid treatment were regulated by NF-κB and MAPK signaling pathway.

Based on the results, we suggest that the palmitic acid can initiate or aggravate the acne in patients with high serum free fatty acids induced by high fat diet. These results also support the previous epidemiologic studies which presented a positive association with diet and acne.

Keywords: Acne, Sebocyte, Inflammation, Lipid, Proinflammatory cytokine

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List of Abbreviations and Symbols

ACTH: adrenocorticotropic hormone

Akt: protein kinase B

α-MSH: alpha-Melanocyte-stimulating hormone

BCA: bicinchoninic acid

cDNA: complementary DNA

CRH: corticotropin-releasing hormone

DHT: dihydrotestosterone

DMEM: Dulbecco's modified Eagle medium

DMSO: dimethyl sulfoxide

DNA: deoxyribonucleic acid

ELISA: enzyme-linked immunosorbent assay

FBS: fetal bovine serum

FoxO1: forkhead box O1

IGF-1: Insulin-like growth factor-1

IL-1: interleukin-1

IL-6: interleukin-6

IL-8: interleukin-8

JNK: c-Jun N-terminal kinases

MAPK: Mitogen-activated protein kinases

MC-5R: Melanocortin 5 receptor

MCP-1: monocyte chemoattractant protein-1

mRNA: messenger ribonucleic acid

mTORC1: mechanistic target of rapamycin complex 1

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells

PBS: phosphate-buffered saline

PI3K/Akt: phosphoinositol-3-kinase -protein kinase B

POMC: pro-opiomelanocortin

PPAR: peroxisome proliferator-activated receptors

P. acnes: Propionibacterium acnes

SD: standard deviation

SDS: sodium dodecyl sulfate

RT-PCR: real time - polymerase chain reaction

TLR2: toll-like receptor 2

TLR4: toll-like receptor 4

TNF-α: tumor necrosis factor alpha

Introduction

Sebocytes and sebaceous gland

Sebocytes are components of epithelial cells and compose the sebaceous glands. The major function of sebocyte is to synthesize and accumulate the sebum in the lipid droplet.[1, 2] The fully differentiated sebocytes release their cytoplasm and the released sebum reaches the skin surface. The human sebum contains a variety of lipids including triglycerides, diglycerides, free fatty acids, wax esters, squalene, and cholesterol.[2, 3] The role of sebum in human is not fully understood. The secreted sebum covers the skin surface and plays roles in the formation and function of skin lipid barrier.[1, 4] Moreover, peptides and lipids with antimicrobial activity exist in the sebum and they participate in the biological barrier in the skin.[1, 5-7]

In the sebaceous glands, sebocytes produce the sebum, accumulate them in their cytoplasm, and release the sebum into excretory ducts.[2] In the mature sebaceous glands, three different populations of sebocytes exist. The outermost narrow area, called peripheral zone, consists of small and actively proliferating sebocytes that are in contact with the basal membrane, comprising about 40% of sebaceous glands.[2, 8, 9] The maturation zone, inner area of outermost peripheral zone, consists of enlarged sebocytes accumulating lipids, also comprising about 40% of sebaceous glands.[2, 8, 9] As the sebocytes move from the outermost zone to the center of the gland, they stop to proliferate and begin to differentiate and make lipid droplets. In center of the gland, the fully differentiated sebocytes, which are bearing lipid droplets, compose

the necrosis zone.[2, 8, 9] In the necrosis zone, the sebocytes disintegrate the cell membranes and release their cytoplasmic contents into the excretory duct, which is called as holocrine secretion.[2, 8, 9]

It is important to know how the activity of sebocytes is controlled for controlling the sebum production and managing the disease associated sebaceous glands such as acne. Multiple factors were known to control the production of sebum in the sebaceous glands. Among them, the effects of sex hormones, growth hormones, and neuroendocrine mediators have been established.[1, 2] Androgens stimulate the sebum production in the sebaceous glands.[1, 2, 9-11] Other sex hormones like testosterone and 5α-dihydrotestosterone increase the proliferation of sebocytes.[1, 2, 9-11] The growth hormone and insulin-like growth factor-1 (IGF-1) were also reported to increase lipid synthesis in human sebocyte cell line.[12] Recently, the regulation of sebaceous gland activity by neuroendocrine regulators was reported. In sebocytes, the existence of POMC peptides, such as α-MSH, adrenocorticotropic hormone (ACTH), β-endorphin and their receptors was reported.[1, 13-16] The role of ACTH and α-MSH in the stimulation of differentiation via melanocortin 5 receptor (MC-5R) was also reported.[1, 16] In addition, the receptors for the neuroendocrine regulators of hypothalamic-pituitary axis were found in the human sebocytes.[17]

Acne

Acne is one of most prevalent skin disorder of adolescents.[18, 19] Although acne

is a self-limited and benign condition, it can cause physical and emotional burden. Acne is a chronic inflammatory disorder of the pilosebaceous gland unit, which is comprised of comedones, papules, and pustules. While multiple etiologic factors are involved in the pathophysiology of acne, four basic etiologic factors are well established.[18, 20] These four key etiologic factors are follicular hyperproliferation, increased sebum secretion, presence and role of *Propionibacterium acnes (P. acnes)*, and inflammation.[18] The basic etiologic factors are related to one another and influenced by other innate or adaptive immune systems and hormonal systems.[18]

By following the processes of initiation and development of acne lesions, the role of etiologic factors in the acne pathogenesis can be easily understood. In the beginning of an acne lesion, the formation of microcomedones, which is the dilation of the hair follicle and the precursor of acne lesions, occurs.[21] The hyperproliferative and hyperkeratotic infundibular epidermis can cause plugs in the follicular opening, and the plugs lead to accumulation and retention of keratin, sebum and bacteria in the follicle.[18] The inducing factors for the follicular hyperproliferation are not well known, but the effects of interleukin-1 (IL-1), androgen, and *P. acnes* were suggested.[18, 22] In previous studies, the role of IL-1 in the follicular keratinocyte hyperproliferation and the formation of microcomedones was well established.[23, 24] IL-1α, when added to human follicular keratinocytes, induced microcomedone formation with hyperproliferation of follicular keratinocytes, and the IL-1 receptor antagonist showed inhibition of microcomedone formation.[23, 24] In addition to cytokines, androgens also play a role in the development of microcomedones.[18, 25] The expressions of enzymes that can produce dihydrotestosterone (DHT), a

potent androgen, were increased in the follicular keratinocytes of acne patients and the increased DHT can induce the proliferation of follicular keratinocytes.[22, 26] In addition to cytokines and hormones, other factors were reported to control the proliferation of follicular keratinocytes. Recently, the regulation of follicular keratinocyte proliferation by the essential fatty acid such as linoleic acid was reported. A decreased level of linoleic acid was reported in the acne patients and the low level of linoleic acid was related to the hyperproliferation of follicular keratinocytes.[27]

In the pilosebaceous gland unit, three different kinds of microorganisms exist: anaerobic *P. acnes*, aerobic staphylococci, and lipophilic yeasts.[21] It is well established that *P. acnes* plays an important role in the development of acne. In a previous study, adolescent acne patients had higher *P. acnes* density compared to the normal control[28]. At the opening of the sebaceous glands and ducts, the sebum secretion creates a lipid-rich and anaerobic environment where *P. acnes* can flourish. The increased *P. acnes* play a role in the formation of comedones and generation of inflammation in the acne lesions. The lipase from *P. acnes* hydrolyzes the triglyceride from the sebum into glycerol and free fatty acids. The free fatty acid from the triglyceride can induce the formation of comedones and initiate inflammatory responses in the acne lesions.[29, 30] In addition, *P. acnes* in the acne lesions can stimulate the migration of inflammatory cells from the blood and increase the level of proinflammatory cytokines.[31, 32] The toll-like receptor 2 (TLR2) on polymorphonuclear cells and monocytes located around the sebaceous gland unit can recognize *P. acnes* and these cells release the proinflammatory cytokines such as IL-1α, IL-6, and TNF-α.[33, 34]

It is well known that both follicular hyperproliferation and increased sebum production play initiating roles in the pathogenesis of acne.[21, 35] The acne patients produced increased amount of sebum, and the amount of sebum on the skin surface showed positive correlations with the number of various types of acne lesions in the acne patients.[18, 35, 36] Among the various components of sebum lipids, triglycerides and lipoperoxides were reported to have pathogenic roles in the acne.[18, 37-39] The lipoperoxides in the sebum can increase the sebum production via activating the peroxisome proliferator-activated receptors (PPAR) pathway.[18, 38, 39] In addition, the increased sebum production can potentiate the role of *P. acnes* in the pathogenesis of acne: *P. acnes* in the pilosebaceous gland unit breaks down the triglycerides in the sebum into the free fatty acids, and the newly formed free fatty acids then promote the formation of microcomedones and initiate the inflammatory processes in the pilosebaceous gland unit.[18, 37]

It is also important to know which mechanisms give rise to the increased sebum production in the acne patients. The increased sebum production in the acne patients were reported to be associated with corticotropin-releasing hormone (CRH), androgens, and estrogens.[18, 21, 40-42] In the skin, keratinocytes and sebocytes have the receptors for CRH, and the CRHs can increase the lipid synthesis and sex hormone production in the sebocytes.[42, 43] In the acne patients, the receptors for CRHs were unregulated in the sebaceous glands.[42, 43] As to androgens, previous studies reported that the increased sebum production in the acne patients was associated with the high level of blood androgens, increased activity of type I 5α reductases, and increased sensitivity of sebaceous glands to circulating

androgens.[44-46] In the development of acne, the proliferation and differentiation of sebocytes are required, and the androgens can control the sebocyte proliferation and promote the differentiation.[18, 21] In addition, the increased activation of PPARs, which is a key regulating transcriptional factor in the lipogenesis and the sebum production, is also mediated by the androgens.[47]

Acne and diet

The relationship between acne and diet has been controversial. Before 1960s, foods including sweets, chocolate, and fats were claimed to be associated with acne, but the association with acne and specific food was expelled in 1970s.[48] However, recent studies suggested new alleged relationships between acne and specific diet such as carbohydrates and dairy products.[49-51] These studies found that the severity of acne was significantly associated with the consumption of dairy products or carbohydrates. With regards to the dairy products, retrospective and prospective studies found significant associations between the severity of acne and the amount of milk consumption, especially skim milk.[49, 52] They suggested that the consumption of milk can increase the IGF-1 in the blood and that sex hormone precursor in dairy products can act as testosterone or DHT.[50, 53] The aggravation of acne after consumption of milk can be explained by the effects of IGF-1 or sex hormones on the sebum production. In addition to dairy products, new findings about the association of carbohydrates consumption and the severity of acne were reported.[54, 55] In a study performed in Papua New Guinea, the westernized food

with high glycemic load diets played roles in the development of acne.[55] It was postulated that the hyperinsulinemia induced by westernized diet could change the endocrinologic status and increase the development of acne.[54, 55] Recently reported studies also supported the relationship between the glycemic load and the severity of acne.[56-58] These studies showed an improvement in the severity of acne after having low glycemic food diets. It was suggested that the glycemic loads in the food can change the insulin sensitivity and modify the hormonal status such as IGF-1 and androgens. In addition, the low glycemic load diet can also alter the composition of sebum: the low glycemic load diet increased the ratio of saturated fatty acids to monounsaturated fatty acids, and the ratio showed negative correlation with the number of acne lesions.[51]

Recent studies suggested that over-nutrition induced by inappropriate diet has been alleged to be the cause of metabolic dysfunction and chronic low grade inflammation.[59, 60] High fat diet can induce the hypertrophy in the adipose tissue. The hypertrophied adipose tissue releases free fatty acids into the circulating blood.[61, 62] These free fatty acids in the circulating blood, especially saturated fatty acids such as palmitic acids, can trigger inflammatory responses in various types of cells and tissues via the TLR2 or TLR4 signaling pathways.[59, 63, 64]

The objectives of this study

To date, few studies were reported about the role of free fatty acids on the sebum production. In addition, there were no studies about the role of free fatty acid on the

production of proinflammatory cytokines. We performed *in vitro* studies using sebocytes to evaluate the effects of over-nutrition induced by inappropriate diet on the development and aggravation of acne. In this study, to mimic the increased level of serum free fatty acids in the subjects with over-nutrition induced by inappropriate diet, we supplemented SZ95 sebocytes with palmitic acid and evaluated the effects of palmitic acid on the lipid production and the induction of expression and secretion of proinflammatory cytokines.

Materials and Methods

Cell culture and treatment

The SZ95 sebocytes, an immortalized human sebocyte cell line, was used in the experiments.[11] The SZ95 sebocytes maintain the major morphologic, phenotypic, and functional characteristics of normal human sebocytes.[11] The SZ95 sebocytes were cultured in Dulbecco's modified Eagle medium (DMEM)/F-12 (3:1), antibiotics (100U/ml penicillin and 100mg/ml streptomycin), 10% fetal bovine serum (FBS; Gibco BRL, Rockville, MD), and 5 ng/ml recombinant human epidermal growth factor (Invitrogen, Grand Island, NY) at 37℃ in a 5% CO_2 incubator.

Thiozolyl Blue [3-(4,5-Dimethylthiazol-2-yl) -2,5-Diphenyl Tetrazolium Bromide] (MTT) Assay

MTT assay was performed to determine the viability and activity of SZ95 sebocytes after palmitic acid treatment. The SZ95 sebocytes were seeded, incubated for 48 hours, and then treated with various doses of palmitic acids in 2% FBS for 24 hours or 36 hours. As a control, the SZ95 sebocyte was treated with vehicle (70% ethanol). After 24 hours or 36 hours of palmitic acid treatment, 200 μL of MTT solution (5 mg/mL) was added to each well and incubated for 4 hours at 37℃ in the dark. After removing the supernatant, 2 mL of dimethyl sulfoxide (DMSO) was

added to dissolve formazan crystals with gentle shaking for 5 min at room temperature. Then the absorbance of the dissolved formazan crystals in DMSO was measured using spectrophotometer at 540 nm and the results were expressed as fold changes of control.

Crystal violet staining

Crystal violet staining was used to determine the effects of palmitic acid treatment on the number of SZ95 sebocytes. The SZ95 sebocytes were seeded, incubated for 48 hours, and then treated with various doses of palmitic acid in 2% FBS for 24 hours or 36 hours. At the end of the treatment period, the SZ95 sebocytes were washed with PBS and fixed with 10% formaldehyde for 15 mins. Then the fixed SZ95 sebocytes were stained with crystal violet solution (0.1% crystal violet in 10% ethanol) for 15 mins. After staining with crystal violet, the stained SZ95 sebocytes were rinsed with PBS and all water was removed from SZ95 sebocytes. For determining the number of SZ95 sebocytes, 1 mL of 2% sodium dodecyl sulfate (SDS) was added. To help all crystal violet elucidated, crystal violet-stained cells within 2% SDS were placed for 30 mins. Then the absorbance of the extracted crystal violet dye in 2% SDS was measured using spectrophotometer at 570 nm and the results were expressed as fold changes of control.

Oil red O staining

Oil red O staining was used to measure the quantitative level of neutral lipids in the

SZ95 sebocytes.[65] The SZ95 sebocytes were seeded on 6-well culture plates, incubated for 48 hours, and then treated with various doses of palmitic acid in 2% FBS for 36 hours. As a control, the SZ95 sebocyte was treated with vehicle (70% ethanol). At the end of the treatment period, the SZ95 sebocytes were washed with phosphate-buffered saline (PBS) and fixed with 10% formaldehyde for 15 mins. Then the fixed SZ95 sebocytes were stained for 15 min with filtered oil red O solution (0.5% oil red O in Propylene glycol). After staining with oil red O solution, the stained SZ95 sebocytes were rinsed with PBS. After rinsing, for determining the extent of neutral lipids in the SZ95 sebocytes, 1 mL of 100% isopropanol was added. A gentle pipetting was used to help the extraction of oil red O dye from the stained SZ95 sebocytes. Then the absorbance of the extracted oil red O dye in isopropanol was evaluated using spectrophotometer at 518 nm and the results were expressed as fold changes of control.

Determination of triglyceride contents

ELISA for triglyceride was used to measure the amount of triglycerides in the SZ95 sebocytes. The SZ95 sebocytes were seeded on 100π culture dishes, incubated for 48 hours, and then treated with various doses of palmitic acid in 2% FBS for 36 hours. The SZ95 sebocytes were washed with PBS. After harvesting using scrapers, the cells were lysed with lipid extraction buffer (0.9% sodium chloride and 1% Triton X100 in distilled water). Then a fluorescent enzymatic method with commercially available determination kits (BCS, Seoul, Korea) was used to

determine the quantitative amount of triglycerides. The amount of triglycerides was normalized to the protein content. Proteins were determined by bicinchoninic acid (BCA) assay method. The results were expressed as fold changes of control.

Determination of cytokines

Enzyme-linked immunosorbent assay (ELISA; Duoset kit; R&D, Minneapolis, MN) was used to measure the amounts of IL-6 and IL-8 released to the culture media following palmitic acid treatment. The SZ95 sebocytes were seeded on 6-well culture plates, incubated for 48 hours, and then treated with various doses of palmitic acid in 2% FBS for 12 hours or 24 hours. As a control, the SZ95 sebocyte was treated with vehicle (70% ethanol). At the end of the treatment period, the media was harvested and the measurements of IL-6 and IL-8 in the culture media were performed according to the manufacturer's instructions.

Western blot analysis

To determine the changes in the levels of phosphorylation in signaling pathway after palmitic acid treatments, Western blot analysis was performed. The SZ95 sebocytes were seeded on 100π culture dishes, incubated for 48 hours, and then treated with various doses of palmitic acid in 2% FBS for 0 min and 30 min. As a control, the SZ95 sebocyte was treated with vehicle (70% ethanol). The SZ95 sebocytes were washed with PBS. After harvesting using scrapers, the cells were lysed with RIPA buffer (Millipore, Billerica, MA) containing protease inhibitors and

phosphatase inhibitors. Equal amounts of protein were loaded and were run on SDS-polyacrylamide gels. Then the proteins on the gels were transferred onto nitrocellulose membranes, and the membranes were incubated with various primary antibodies. Then the membranes were incubated with secondary antibody conjugated with peroxidase activity, and the chemiluminescence was used to visualize the specific proteins. The primary antibodies used in Western blot analysis were as follows: phospho-IκB (p- IκB) (1:1000, ab12135, Abcam), IκB (1:2000, ab7217, Abcam), phosphor-JNK (p-JNK) (1:1000, 3270, Cell Signaling), JNK (1:1000, 9165S, Cell Signaling), phospho-Akt (1:1000, 4058S, Cell Signaling), Akt (1:1000, 9272, Cell Signaling), and ß-actin (1:1000, SC47778, Santa Cruz).

Real-time PCR (RT-PCR)

RT-PCR was used to evaluate the gene expressions after palmitic acid treatments. The SZ95 sebocytes were seeded on 6-well culture plates, incubated for 48 hours, and then treated with various doses of palmitic acid in 2% FBS for 12 hours or 24 hours. As a control, the SZ95 sebocyte was treated with vehicle (70% ethanol). At the end of the treatment period, Trizol method (Life Technologies, Rockville, MD) was used to prepare total RNA from SZ95 sebocytes. One μg of total RNA was reverse-transcribed to complementary DNA using the First Strand cDNA Synthesis Kit (MBI Fermentas, Vilnius, Lithuania) according to the manufacturer's instructions. RT-PCR was performed to quantitatively evaluate the level of mRNA expression of each gene by using 7500 Real-time PCR System (Applied Biosystems, Foster City,

CA) with SYBR Premix Ex Taq (Takara Bio, Shiga, Japan) according to the manufacturer's instructions. The RT-PCR condition was 50℃ for 2 minutes, 95℃ for 2 minutes, followed by 40 cycles at 95℃ for 15 seconds, and 60℃ for 1 minute. The expression level of each gene was presented as fold changes normalized by the level of 36B4. The primers used in RT-PCR were listed below.

Statistical analysis

All the data were presented as the mean ± SD. Significance was analyzed by the Kruskal-Wallis test. Differences were considered statistically significant when $P<0.05$.

Primer sequences

	Forward primer	Reverse primer
36B4	TGGGCTCCAAGCAGATGC	GGCTTCGCTGGCTCCCAC
IL-6	CTCCTTCTCCACAAGCGCC	GCCGAAGAGCCCTCAGGC
IL-8	CTCTTGGCAGCCTTCCTG	TTGGGGTCCAGACAGAGC

Results

The effect of palmitic acid on the proliferation and the lipid production

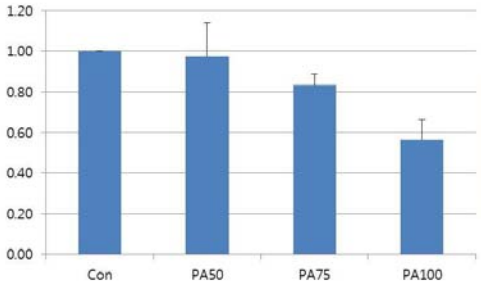
To determine the effects of palmitic acid on the cell number and viability of SZ95 sebocytes, MTT assay and crystal violet assay were performed. The results of MTT assay showed that the viability of SZ95 sebocytes were above a fold change of 0.80 in the concentrations of 50 μM (a fold change of 0.97 ± 0.16 in 24 hour and 1.08 ± 0.04 in 36 hour) and 75 μM (a fold change of 0.83 ± 0.05 in 24 hour and 0.89 ± 0.14 in 36 hour) compared to that of control treated with vehicle only (Figure 1a and 1b). However, the viability of SZ95 sebocytes treated with 100 μM of palmitic acid was less than a fold change of 0.80 (Figure 1a and 1b). The results of crystal violet showed that the changes in the cell number of the SZ95 sebocytes after palmitic acid treatment were within 20% of the cell number of the control treated with vehicle only. The results of crystal violet assay showed that the cell numbers of SZ95 sebocytes after palmitic acid treatment were as follows: in the concentrations of 50 μM (a fold change of 1.19 ± 0.03 in 24 hour and 1.15 ± 0.08 in 36 hour), 75 μM (a fold change of 1.12 ± 0.07 in 24 hour and 1.10 ± 0.05 in 36 hour), and 100 μM (a fold change of 1.10 ± 0.07 in 24 hour and 1.08 ± 0.07 in 36 hour) (Figure 1c and 1d).

Oil red O staining and ELISA for triglyceride were performed to investigate the role of palmitic acid in the lipid production of the SZ95 sebocytes. When SZ95 sebocytes were treated with palmitic acid, a significant increase in cytoplasmic lipid

accumulation in SZ95 sebocytes was detected. As shown in Figure 2a, the results of Oil red O staining showed that treatment of palmitic acid increased the neutral lipids in SZ95 sebocytes (a fold change of 1.22 ± 0.04, 1.24 ±0.07, and 1.29 ± 0.03 in 50 μM, 75 μM, and 100 μM, P=0.047). The levels of cytoplasmic lipids were further quantified by measuring triglyceride using ELISA (Figure 2b). The treatment of palmitic acid on SZ95 sebocytes also increased the intracellular triglycerides normalized to the protein contents in a dose-dependent manner (a fold change of 1.28 ± 0.25, 1.81 ±0.57, and 2.19 ± 0.78 in 50 μM, 75 μM, and 100 μM, P=0.042).

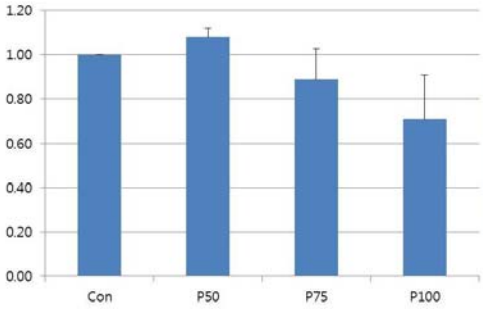
Figure 1. The results of MTT assay and crystal violet assay

(a)



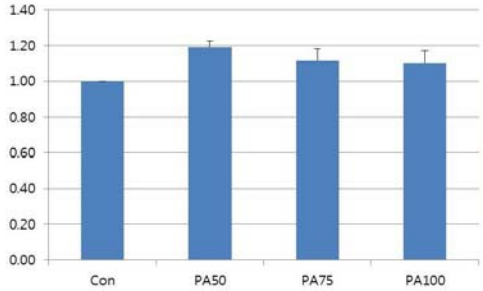
Con	PA 50	PA 75	PA 100
1.00 ± 0.00	0.97 ± 0.16	0.83 ± 0.05	0.56 ± 0.10

(b)



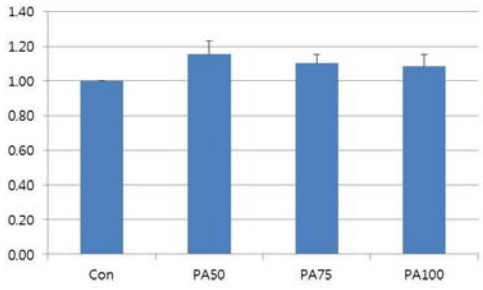
Con	PA 50	PA 75	PA 100
1.00 ± 0.00	1.08 ± 0.04	0.89 ± 0.14	0.71 ± 0.20

(c)



Con	PA 50	PA 75	PA 100
1.00 ± 0.00	1.19 ± 0.03	1.12 ± 0.07	1.10 ± 0.07

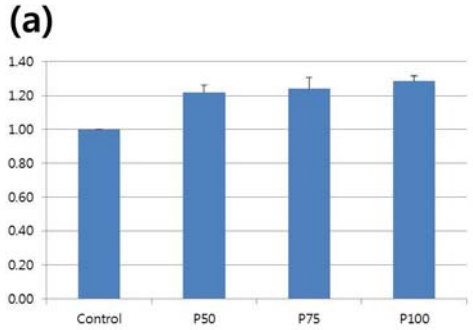
(d)



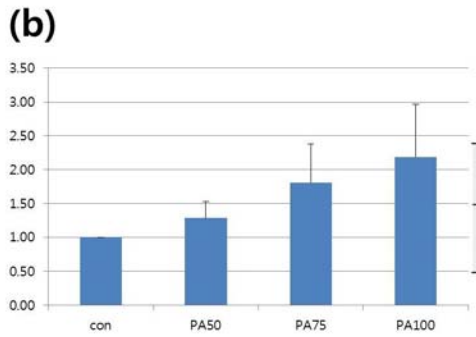
Con	PA 50	PA 75	PA 100
1.00 ± 0.00	1.15 ± 0.08	1.10 ± 0.05	1.08 ± 0.07

After incubating SZ95 sebocytes for 48 hours and treating with various doses of palmitic acid in 2% FBS for 24 or 36 hours, MTT assay and crystal violet assay were performed. As a control, the SZ95 sebocytes were treated with vehicle only (70% ethanol). The viability of SZ95 sebocytes measured by MTT assay was above a fold change of 0.80 in the concentrations of 50 μM and 75 μM (Figure 1a and 1b). In contrast to the results of MTT assay, the changes in the cell numbers of the SZ95 sebocytes after palmitic acid treatment measured by crystal violet assay were within 20% compared to that of control treated with vehicle only (Figure 1c and 1d).

Figure 2. The results of oil red O staining and ELISA for triglyceride



Con	PA 50	PA 75	PA 100
1.00 ± 0.00	1.22 ± 0.04	1.24 ± 0.07	1.29 ± 0.03



Con	PA 50	PA 75	PA 100
1.00 ± 0.00	1.28 ± 0.25	1.81 ± 0.57	2.19 ± 0.78

To investigate the role of palmitic acid in the lipid production of SZ95 sebocytes, the intracellular lipid and triglycerides were evaluated by using oil red O staining and ELISA for triglyceride. SZ95 sebocytes were incubated for 48 hours and then were treated with various doses of palmitic acid in 2% FBS for 36 hours. Oil red O staining was used to measure the amount of intracellular neutral lipids. ELISA for triglyceride was also performed for measuring the amount of triglycerides in the SZ95 sebocytes. The treatment of palmitic acid resulted in the increased amount of the neutral lipids in SZ95 sebocytes measured by oil red O staining (a fold change of 1.22 ± 0.04, 1.24 ±0.07, and 1.29 ± 0.03 in 50 μM, 75 μM, and 100 μM compared to

control, P=0.047, Figure 2a). The treatment of palmitic acid also increased the amount of intracellular triglycerides normalized to the protein content in SZ95 sebocytes in a dose-dependent manner (a fold change of 1.28 ± 0.25, 1.81 ±0.57, and 2.19 ± 0.78 in 50 μM, 75 μM, and 100 μM compared to control, P=0.042, Figure 2b).

Palmitic acid induced the production and the secretion of IL-6 and IL-8 in SZ95 sebocytes

RT-PCR and ELISA were performed to determine the effects of palmitic acid on the production and the secretion of the cytokines in SZ95 sebocytes. After palmitic acid treatment for 12 hours and 24 hours, we found the statistically significant changes in the level of IL-6 and IL-8 mRNA expression (Figure 3a and 3b). The expression of IL-6 after 12 hours of palmitic acid treatment was increased by a fold of 2.33 ± 0.61, 3.22 ± 0.21, and 4.16 ± 0.52 in 50 μM, 75 μM, and 100 μM, respectively (P=0.016). After 24 hours of palmitic acid treatment, increased expressions by a fold of 2.17 ± 0.15, 3.39 ± 0.23, and 4.87 ± 1.08 was found in 50 μM, 75 μM, and 100 μM were found, respectively (P=0.016). The IL-8 expression in 12 hours of palmitic acid treatment was also increased by a fold of 5.05 ± 0.42, 8.11 ± 2.01, and 10.72 ± 3.80 (P=0.022). Moreover, compared to control, we also found the increased expression of mRNA expression of IL-8 by a fold of 1.54 ± 0.27, 2.64 ± 0.52, and 3.74 ± 0.97 in 50 μM, 75 μM, and 100 μM, respectively, after 24 hours of palmitic acid treatment (P=0.026).

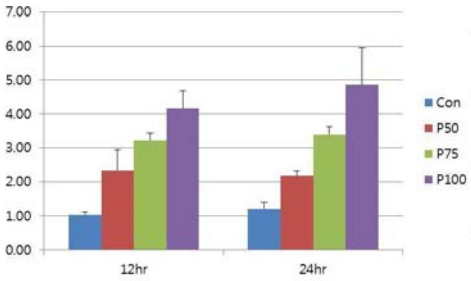
The cytokines from culture media were evaluated by ELISA assay. The secretion of IL-6 and IL-8 was increased in SZ95 sebocytes after palmitic acid treatment in a statistically significant manner (Figure 4a and 4b). The secreted IL-6 after 12 hours of palmitic acid treatment was 126.12 ± 3.65 pg/mL, 231.18 ± 28.05 pg/mL, 259.68 ± 12.12 pg/mL, and 228.43 ± 52.33 pg/mL in control, 50 μM, 75 μM, and 100 μM, respectively, and the changes after palmitic acid treatment were statistically

significant (P=0.038). The secreted IL-6 after 24hours of palmitic acid treatment also showed statistically significant changes (P=0.019). The secreted level of IL-6 after 24 hours of palmitic acid treatment were 163.55 ± 16.65 pg/mL, 389.62 ± 63.78 pg/mL, 508.72 ± 30.31 pg/mL, and 615.61 ± 112.29pg/mL in control, 50 μM, 75 μM, and 100 μM, respectively.

As to the secretion of IL-8 in the culture media from SZ95 sebocytes, the secreted IL-8 in 12 hours were 86.52 ± 9.24 pg/mL, 126.78 ± 13.95 pg/mL, 158.41 ± 32.98 pg/mL, and 142.88 ± 31.12 pg/mL. We found a statistically significant changes in the secreted IL-8 in control, 50 μM, and 75 μM (P=0.039). The secreted IL-8 after 24 hours of palmitic acid treatment were 140.12 ± 10.35 pg/mL, 261.47 ± 30.23 pg/mL, 384.17 ± 75.56 pg/mL, and 501.29 ± 140.05 pg/mL in control, 50 μM, 75 μM, and 100 μM, respectively, and these results were statistically significant (P= 0.022).

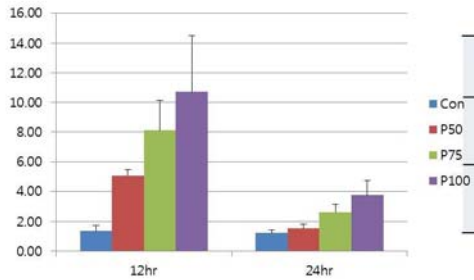
Figure 3. The results of mRNA expression for IL-6 and IL-8

(a)



IL-6	Con	PA 50	PA 75	PA 100
12hr	1.04 ± 0.07	2.33 ± 0.61	3.22 ± 0.21	4.16 ± 0.52
24hr	1.21 ± 0.19	2.17 ± 0.15	3.39 ± 0.23	4.87 ± 1.08

(b)

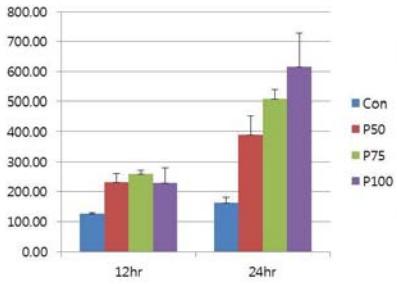


IL-8	Con	PA 50	PA 75	PA 100
12hr	1.35 ± 0.38	5.05 ± 0.42	8.11 ± 2.01	10.72 ± 3.80
24hr	1.22 ± 0.20	1.54 ± 0.27	2.64 ± 0.52	3.74 ± 0.97

RT-PCR was used to evaluate the gene expressions after palmitic acid treatments. The SZ95 sebocytes were incubated for 48 hours, and then treated with various doses of palmitic acid in 2% FBS for 12 hours or 24 hours. As a control, the SZ95 sebocytes were treated with vehicle only (70% ethanol). At the end of the treatment period, total RNA was reverse-transcribed to complementary DNA. RT-PCR was performed to evaluate the level of mRNA expression of each gene. The expression level of each gene was presented as a fold change normalized by the level of 36B4. The results of RT-PCR showed increased expressions of IL-6 and IL-8 after 12 hours and 24 hours after palmitic acid treatment (Figure 3a and 3b). Significant changes in mRNA expression were seen in IL-6 (P=0.016 at 12 hour and P=0.016 at 24 hour) and IL-8 (P=0.022 at 12 hour and P=0.026 at 24 hour).

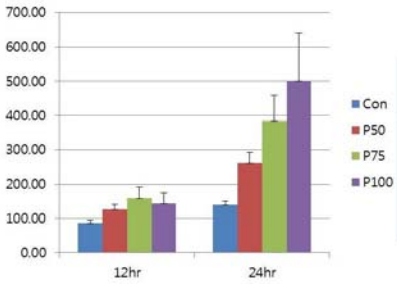
Figure 4. The results of ELISA for secreted IL-6 and IL-8

(a)



IL-6	Con	PA 50	PA 75	PA 100
12hr	126.12 ± 3.65	231.18 ± 28.05	259.68 ± 12.12	228.43 ± 52.33
24hr	163.55 ± 16.65	389.62 ± 63.78	508.72 ± 30.31	615.61 ± 112.29

(b)



IL-8	Con	P50	P75	P100
12hr	86.52 ± 9.24	126.78 ± 13.95	158.41 ± 32.98	142.88 ± 31.12
24hr	140.12 ± 10.35	261.47 ± 30.23	384.17 ± 75.56	501.29 ± 140.05

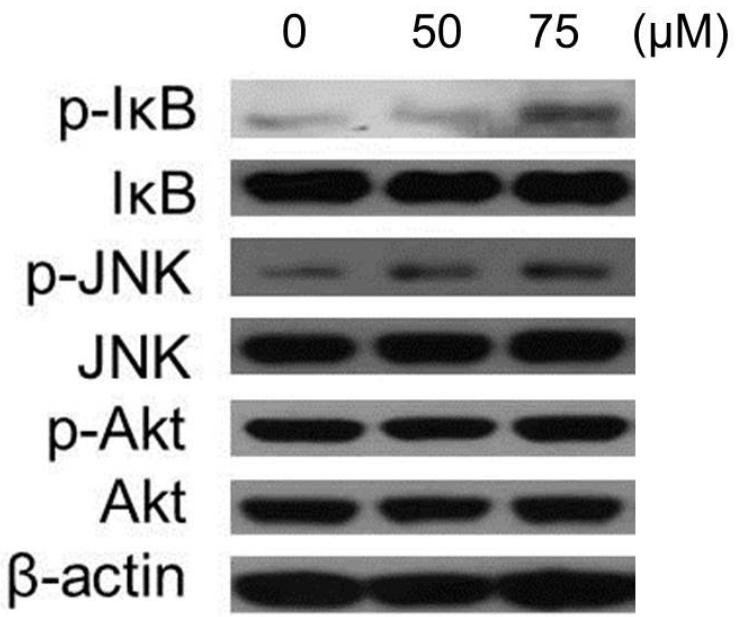
IL-6 and IL-8 released to the culture media after palmitic acid treatment was measured by using ELISA assay. The SZ95 sebocytes were seeded on 6-well culture plates, incubated 48 hours, and then treated with various doses of palmitic acids in 2% FBS for 12 hours or 24 hours. As a control, the SZ95 sebocyte was treated with vehicle only (70% ethanol). At the end of the treatment period, the media was harvested and the levels of IL-6 and IL-8 in the culture media were measured. The ELISA results showed that the secretion of IL-6 and IL-8 was increased by palmitic acid treatment (Figure 4a and 4b). The changes in secreted IL-6 in culture media were statistically significant in control, 50 μM, and 75 μM, and 100 μM ($P=0.038$ at 12 hour and $P=0.019$ at 24 hour, respectively). As to the secreted IL-8 at 12 hours of

palmitic acid treatment, statistically significant changes were found in control, 50 μM, and 75 μM (P=0.039). The secreted IL-8 at 24 hours of palmitic acid treatment showed statistically significant changes in control, 50 μM, and 75 μM, and 100 μM (P= 0.022).

Palmitic acid activated NF-κB and JNK signaling pathways in SZ95 sebocytes

Western blot analysis was performed to find which pathway transduced the signals after palmitic acid treatments. The increased phosphorylation of IκB and JNK was found after palmitic acid treatments (Figure 5). The level of phosphorylation in IκB and JNK was increased in a dose-dependent manner: the Western blot analysis showed that the level of phosphorylation with 75 μM of palmitic acid treatment was higher compared to 50 μM both in IκB and JNK. However, the level of phosphorylation of Akt after palmitic acid treatments was not changed: the level of phosphorylation of Akt with 50 and 75 μM of palmitic acid treatment showed no difference compared to control.

Figure 5. The results of Western blots after palmitic acid treatment



To determine the changes in the levels of phosphorylation in signaling pathway after palmitic acid treatments, Western blot analysis was performed. The SZ95 sebocytes were incubated for 48 hours, and then treated with various doses of palmitic acid in 2% FBS for 0 min and 30 min. As a control, the SZ95 sebocytes were treated with vehicle only (70% ethanol). After palmitic acid treatment, an increased phosphorylation of IκB and JNK was found. However, the phosphorylation of Akt was not changed compared to control.

Discussion

Acne is a disease of the adolescent. The prevalence of acne changes according to age: during the adolescence, the prevalence of acne is about 90%, while about 50% of individuals in the second and third decades have acnes.[29] From the differences in the prevalence in different age groups or populations, the association of acne with the environmental factors was suggested.[19, 29] Among the environmental factors, the westernized diet has been pointed to be the main culprit.[19, 29] The association between acne and westernized diet was supported by the epidemiologic studies focused on populations who moved from traditional society to westernized society and westernized diet.[54, 55] These studies showed that the change in diet from traditional diet to westernized diet aggravated or initiated the acne in the population. Among the various food in westernized diet, the refined carbohydrates, milk, dairy products, and saturated fatty acids were suggested to promote the development of acne.[29] Although the exact mechanism of westernized diet in the development or aggravation of acne is not fully understood, recent studies found new mechanisms in the acne pathogenesis. Insulin/IGF-1 signaling pathway which is activated by westernized diet plays a major role in the mechanism of diet that influences the development of acne.[29] IGF-1 enhances the cell proliferation and growth by activating the phosphoinositol-3-kinase (PI3K)-protein kinase B (Akt) signaling cascade.[66] Activation of IGF-1 signaling is reported to enhance the androgen synthesis and conversion of testosterone to DHT, an androgen with high affinity.[67,

68] Insulin and IGF-1 can also activate androgen-dependent signal transduction by downregulating forkhead box O1 (FoxO1), a cosuppressor of androgen receptor.[69] In addition, insulin and IGF-1 also regulate cell cycle and lipogenesis by activating mechanistic target of rapamycin complex 1 (mTORC1), a nutrient-sensitive kinase.[70] By regulating androgen-dependent signal transduction and other signaling pathways, IGF-1 and insulin can initiate or aggravate acne.

As for the association between refined carbohydrates and acne, an increased intake of refined carbohydrates was reported to modulate the sex hormone binding globulin and IGF binding proteins, and these changes changed the bioactivity of free IGF-1 and free androgens in the serum.[58, 71] In addition, refined carbohydrates changed the expression of microRNAs such as microRNA-21 that modulates PI3K/Akt and FoxO1 signaling.[72, 73] Consumption of milk, designed to promote the growth of newborn by anabolic metabolism, also promotes insulin/IGF-1 signaling pathway by providing sufficient amount of amino acids including tryptophan.[74] Moreover, milk can transfer bovine microRNA to human via exosomes, membranous microvesicles containing microRNAs, and these microRNA from cow's milk can be taken up and modify human gene expression.[29, 75, 76] Among the bovine microRNAs in exosomes, bovine microRNA-21 is included and identical to humans'.[29, 76] Lastly, saturated free fatty acids such as palmitic acid were reported to initiate the formation of microcomedone by activating mTORC1.[29]

In this study, to evaluate the effects of palmitic acid on the lipid production and the induction of expression and secretion of proinflammatory cytokines, we supplemented sebocyte cell line with free fatty acid to mimic the increased level of

serum free fatty acids in the obese or the condition of high fat diet. For experiments, SZ95 sebocytes, which maintain the major morphologic, phenotypic, and functional characteristics of normal human sebocytes, were treated with palmitic acid, a major free fatty acid in serum.[11]

The results of oil red O staining and ELISA for triglyceride showed that the amount of cytoplasmic lipid accumulation was increased when SZ95 sebocytes were treated with palmitic acid. Moreover, the amount of cytoplasmic lipid accumulation was increased in a dose dependent manner. These results are in accordance with the previous *ex vivo* study using punch biopsied sebaceous glands.[77] They supplied various radioactive free fatty acids to *ex vivo* sebaceous glands and demonstrated that exogenous free fatty acid was incorporated into wax ester, which is solely synthesized by the sebaceous gland.[77] In addition, most of exogenously treated free fatty acid was used to synthesize triglycerides or polar lipids.[77] Based on the increased amount of cytoplasmic lipid accumulated in SZ95 sebocytes after palmitic acid treatment, we suggest that sebaceous glands can synthesize increased amount of sebum by using high level of serum free fatty acid. Previously, the increased insulin/IGF-1 signaling was pointed out to be the cause of increased sebum secretion after high fat diet or high glucose diet. [29, 58, 71] However, from the results of this study, we suggest a new mechanism of increased sebum secretion after high fat diet or high glucose diet: sebocytes directly uptake the free fatty acids from the blood and use them to synthesize the sebum.

Obesity has become a global health problem in the world.[78, 79] Obesity commonly accompanies comorbidities such as metabolic diseases and

cardiovascular disease. Recent studies found that various inflammatory mediators from the adipose tissues in the obese patients were associated with the development of comorbidities associated with obesity.[80, 81] In the process of developing the comorbidities, free fatty acids via lipolysis and adipokines, cytokines released from adipose tissues, were known to play an important role.[78, 81] In the obese patients, the level of serum free fatty acid was found to be increased compared to normal individuals due to excessive food intake.[82, 83] Recent studies found an association with the increased level of serum free fatty acids in the obese and the harmful changes in human organs or tissues including liver, endocrine organs, and muscles.[84-86] In addition, *in vitro* studies found that the free fatty acids can change the gene expression in macrophages, monocytes, adipocytes, and synovial fibroblasts.[78, 87-89] However, some nutritional researches found that not all fats have equal effects on health.[90, 91] *In vitro* studies found that different kinds of free fatty acids showed different changes in gene expressions: saturated fatty acids promoted the expression of proinflammatory genes, while some unsaturated fatty acids such as ω-3 fatty acids suppressed the proinflammatory genes expression.[92, 93]

Among the free fatty acid, palmitic acid is the most abundant saturated fatty acid in adipose tissue and in Westernized diet.[94] Previous studies showed that palmitic acid plays an important role in the development of inflammation.[95] Palmitic acid can initiate the inflammatory responses by producing various proinflammatory cytokines including IL-6, IL-8, TNF-α, and monocyte chemoattractant protein-1 (MCP-1) from macrophages.[95, 96] Besides the blood cells, palmitic acid can

induce proinflammatory mediators form various cells including synovial fibroblasts, adipocytes, pancreatic β cells, and myocytes.[59, 78, 97, 98] The effect of palmitic acid in skin cells was also reported.[99] The palmitic acid treatment on the HaCaT keratinocytes induces the production of proinflammatory cytokines and proliferation of HaCaT keratinocytes. The effects of palmitic acid on keratinocytes can contribute to the development of ductal hyperkeratinization of pilosebaceous gland unit and the initiation of inflammation in the acne lesion.

In this study, we supplemented with palmitic acid to mimic the increased levels of serum free fatty acids in the obese or the condition of high fat diet. For experiments, SZ95 sebocytes, which maintain the major morphologic, phenotypic, and functional characteristics of normal human sebocytes, were used.[11] In this study, the treatment of palmitic acid onto SZ95 sebocytes resulted in the induction of expression and secretion of proinflammatory cytokines (IL-6 and IL-8). The changes in expressions and secretions of proinflammatory cytokines were statistically significant and in a dose-dependent manner. These results are in agreement with other previous studies which suggested an association between the palmitic acid and the inflammatory processes.[15, 23, 48, 94] In addition, the increased expression and secretion of IL-6 and IL-8 from SZ95 sebocytes may be an important change in acne, because the level of IL-6 and IL-8 is increased in the acne patients and it has been suggested to have an important role in the formation of acne lesions.[99-101] Based on the effects of palmitic acid on the expression and secretion of cytokines, we can suggest that high fat diet with saturated fatty acid can be initiating and aggravating

factors in pathogenesis of acne and this *in vitro* result supports the epidemiologic studies which advocate the effect of high fat diet in promoting and aggravating acne.

In acne patients, it was reported that insulin/IGF-1 signaling pathway enhances the cell proliferation and growth by activating PI3K/Akt signaling.[29, 66] The activated Akt was known to promote androgen receptor signaling by regulating FoxO1 and mTORC.[29, 102, 103] By the linkage of the two signals, insulin/IGF-1 signaling and androgen receptor signaling by PI3K/Akt and FoxO1/mTORC1, the nutritional status can influence the development and aggravation of acne. In this study, we tested whether palmitic acid treatment on the SZ95 sebocytes can activate the PI3K/Akt signaling pathway. However, the phosphorylation of Akt was not changed after the palmitic acid treatment in the SZ95 sebocytes.

In the regulation of proinflammatory gene expressions, NF-κB is reported to be a major transcriptional factor and NF-κB also controls the gene expressions of various proinflammatory cytokines including IL-1β, IL-6, and TNF-α.[99] The activation of NF-κB signaling is mediated by activation of IκB kinases.[104] The activated IκB kinases phosphorylates IκB proteins and then the phosphorylated IκB is ubiquitinized and degraded.[104, 105] This ubiquitination and degradation of IκB proteins leads to transcriptional activation of NF- κB signaling.[104, 105] In this study, palmitic acid treatment increased the phosphorylation of IκB proteins, and the change in phosphorylation was in a dose-dependent manner. This result is in agreement with the previous studies that suggest a link between the expression of proinflammatory cytokines and palmitic acid-induced inflammation.[99, 106] This

result also suggests another possible mechanism in the inflammation of acne in patients with high free fatty acids induced by inappropriate diet.

In conclusion, the palmitic acid treatment on SZ95 sebocytes increased the lipid accumulation in cytoplasm of SZ95 sebocytes. In addition, the SZ95 sebocytes treated with palmitic acid produced and secreted more proinflammatory cytokines. The changes in SZ95 sebocytes after palmitic acid treatment were regulated by NF-κB and JNK signaling pathway. Based on the results, we can suggest that the palmitic acid can initiate or aggravate the acne in the group of patients with high serum free fatty acids induced by inappropriate diet. These results also support the previous epidemiologic studies which presented a positive association with diet and acne.

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국 문 초 록

여드름은 피지샘에 발생하는 만성 염증성 질환이다. 여러가지 요인이 여드름의 유발 및 악화에 관여하는데, 털집과다각질화, 피지분비 증가, *Propionibacterium acnes*의 집락 형성, 염증 반응이 주요 요인이다. 최근에는 고지방식이도 여드름을 악화시키는 원인이 될 수 있음이 알려져 있으나 고지방식이가 여드름을 악화시키는 기전은 아직 밝혀지지 않았다.

이번 연구에서는 피지샘세포주인 SZ95세포를 이용하여 과도한 음식 섭취 등에 의해 유도된 고지방산 상태가 여드름을 유도하고 악화하는 기전을 밝히기 위한 *in vitro* 실험을 진행하였다. 피지샘세포주인 SZ95세포에 팔미트산을 처리하여 고지방식이 등에 의해 유도된 혈중 고지방산 상태를 유도한 후, SZ95세포에서 지질의 합성의 변화와 염증성 사이토카인의 합성 및 분비의 변화를 확인하였다. SZ95세포에서 지질의 합성의 변화는 oil red O 염색과 중성지방에 대한 ELISA를 이용하여 측정하였다. 피지샘세포주인 SZ95세포에 팔미트산을 처리한 결과 oil red O 염색으로 측정한 SZ95세포의 세포질 내의 지질이 증가하였고, 중성지방에 대한 ELISA 실험의 경우 단백질로 보정한 중성지방

역시 증가하였다. 또한 피지샘세포주인 SZ95세포에 팔미트산 처리는 SZ95세포의 IL-6와 IL-8의 mRNA 발현 및 배양액 내 IL-6와 IL-8을 증가시켰다. Western blot을 이용한 실험을 통하여 팔미트산 처리가 SZ95세포에서 NF- κ B와 AKT 신호전달을 활성화시킴을 확인하였다.

이번 연구의 결과는 고지방식이 등에 의해 유도된 고지방산 상태가 여드름을 유도하거나 악화할 수 있음을 제시하는 연구로서 기존의 고지방 식이 등의 특정 식이가 여드름을 악화시킨다는 임상연구의 가설을 실험적으로 입증한 연구이다.

주요어: 여드름, 피지세포, 염증, 지질, 염증성 사이토카인

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