Effects of topical application of Phospholipid derivatives on the secretion of sebum on the skin of the fuzzy rats

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Summary

The fuzzy rat that expresses hypersecretion of sebum and hyperplastic sebaceous glands is a genetic mutant for the study of many pharmacological aspects especially human acne. Through this model, we examined the effects of several phospholipids on the secretion of sebum after topical application. The phospholipid derivatives were phosphatidylcholine (PC), hydrogenated phosphatidylcholine (HPC), phosphati dylserine (PS) and hydrogenated phosphatidylserine(HPS). All agents were dissolved into the vehicle (1,3-Butanediol, ethanol and water) at 0.5% weight volume and applied on the dorsal area of the fuzzy rat. To observe histological changes, the skin biopsies were stained with Oil Red O and the size and morphology of sebaceous gland was observed under microscope. Topical treatment with PC and/or HPC showed a marked decrease in sebum excretion. Especially hydrogenated PC (HPC) appeared to have more predominant sebosuppressive function than any other treatment. The other agents such as PS and HPS showed a marginal effect on sebum secretion. With the sebosuppressive activity, HPC and PC seem to have a good potential application on acne treatment. In order to obtain more insights into possible mechanisms behind the above observations, effects of each phospholipid on the expression of peroxisome proliferator-activated receptor (PPAR) genes were investigated. Recently, it has been demonstrated that

expression and activation of PPAR subtypes appear to modulate the accumulation of cytoplasmic fat droplets that characterizes the sebocyte differentiation(1). It was also previously suggested that PPARγ antagonist would seem possible to interfere sebum production without side effects (2). In this study we examined the diverse effects of the tested phospholipids on the expression of several PPAR genes based on reverse transcription-polymerase chain reaction (RT-PCR) from the topically treated skin of fuzzy rats. The results and possible implications are discussed.

Introduction

Phospholipids are indispensable for live organisms and play an essential role for both health and nutrition because of their diverse functions in cells. Phospholipids play an important role in maintaining membrane integrity as well as in cell signaling. The major phospholipids in cells are phosphatidylcholine, phophatidylethanolamine, phosphatidylserine and sphingomyelin(3). Phospholipids have a "polar head group" such as choline, serine, attached to a backbone (i.e.glycerol) through a phosphate group and hydrophobic tails of two fatty acid chains. Saturated (e.g. palmitic acid, stearic acid) or unsaturated(e.g. oleic acid, linoleic acid) form can be existed in the fatty acid chains. Based upon their properties, they could be used as a topical regimen for skin to help maintain and repair plasma membranes(4). In addition to the beneficial effects such as smoothing of the skin, protection of skin and increase of skin moisture, it has also been suggested that phosphatidylcholine is highly efficient in the treatment of mild to moderate acne. Skins with acne showed lower levels of linoleic acid in the sebum than that of normal skin (5). This phenomenon led to hypothesize that the essential fatty acid deficiency in the cells of the follicular epithelium disturbed the keratinization which could be responsible for comedogenesis (6,7). However, there have been no accepted mechanisms that relate the production of excessive comedogenic lipids(sebum) due to the deficiency of phospholipid to the pathogenicity of acne until now. There are no known function of Phosphatidylserine(PS) on skin yet. Recently, however, PS has showed some beneficial effect on skin barrier function as well as on UV protection(unpublished data).

The aim of the present study was objectively to evaluate the effects of topically applied various phospholipid derivatives synthesized in our own laboratory such as phosphatidylserine(PS), hydrogenated phosphatidylserine(HPS), phosphatidylcholine(PC) and hydrogenated phosphatidylcholine(HPC) on the sebosuppressive function. PS and PC were extracted from soybean and then hydrogenated with hydrogen gas (H_2 gas) to make all fatty acid chains to saturated forms.

Acne is a disease unique to humans. Although, the precise mechanisms of acne are not known yet, it is generally accepted that there are several factors involved in development of acne. The overproduction of sebum, abnormal desquamation of follicular corneocytes and proliferation of *Propionibacterium acnes* are the major factors involved in the development of acne. *Proprionibacterium acnes* produce enzymes that hydrolyze the sebum into free fatty acids which are believed to induce inflammatory response. As supposed, the complexicity of the disease in moderate to severe cases may require multiple approaches to treatment. Not only the anti-bacterial treatments but also sebostatic agents that can reduce the overproduction of sebum are used concurrently for the acne patients. Sebum excretion is predominantly greater in patients with cystic acne. Therefore attempts to reduce the sebum excretion are a logical approach for the acne treatment. Peroxisome proliferator-activated receptor(PPAR) were discovered as a subfamily of "orphan receptors" within the nonsteroid receptor family of nuclear hormone receptors(8). PPAR subtypes(α,γ and δ) were investigated in skin because of their known role in regulating lipid metabolism. It was recently demonstrated that expression and activation of PPAR plays a unique role in the differentiation of sebocytes that are specialized epithelial cells, giving rise to the lipid-rich holocrine secretion, sebum(9). As the most relevant positive regulator of sebum production, it was previously suggested to develop PPARγ antagonist to reduce sebum formation(2). For this reason, we examined the effect of tested phospholipids on the expression of PPARγ gene as well to elucidate the biochemical mechanism that involved partially in the regulation of sebaceous gland.

Materials and Methods

Materials: PhosphatidylSerine(PS), Hydrogenated PhosphatidylSerine(HPS), PhosphatidylCholine(PC) and Hydrogenated PhosphatidylCholine(HPC) were prepared at Doosan Biotech (Yongin, Korea). PS/HPS 90 consisted of PS/HPS 90% and PC 10%. Whereas, PS/HPS 40 was composed of PS/HPS 40% and 60%

PC at the rest.

Animal model: We used six to eight week old male and female fuzzy rats(Charles River Laboratories, USA) in this study. Fuzzy rat strain also known as the WF/Pm-"fz"rat which "WF" designation refers to the fuzzy mutation originating from an inbred Wistar Furth colony. This strain that expresses hypersecreted sebum and formation of hyperplastic sebaceous glands has been used for many dermatological researches especially for acne.

Treatments and tissue preparation: All chemicals were dissolved into the vehicle (1,3-butanediol, ethanol and water) at 0.5% weight per volume. Animals were treated twice a day with the agents on the dorsal area for 2 months and control animals were treated with vehicle only. After 2months the applied rats were photographed at the backs and skin tissue samples were collected for histological examinations or RNA extraction.

Oil Red O Staining: Applied tissue samples were collected and fixed by 10% formalin in phosphate buffer for 1hr. The sections were then stained with 0.3% filtered Oil Red O solution in a mixture of isopropanol/water (60/40, vol/vol) for 15min at room temperature, then washed twice with water for 15min each and visualized.

RNA extraction: Total RNA was extracted from the full thickness skin tissue by adding Trizol reagent (InVitrogen) and subsequent homogenization. The homogenized tissues were extracted with chloroform, followed by isopropanol precipitation at -20°C .The yield of RNA was quantified by measuring the optical density at 260nm .

Reverse transcription-polymerase chain reaction (RT-PCR): Total RNA (2μg from each sample) was used for first-strand cDNA synthesis using oligo-dT and reverse transcriptase from Bioneer (Korea) following the manufacturer's instruction. An aliquot (5 μl) of the resultant cDNA was added to the PCR reaction mixture. 10mM Tris-HCl, pH 9.0, 50mM KCl, 1.5mM MgCl2, 0.2mM dNTPs, 0.625 U Taq polymerase and 0.5 μM each primer set. The primer sequences used for PPARγ had been published previously. So the primers consisted of PPARγ-up 5'-ATAAAGTCCTTCCCGCTGACCAAAGCC-3', and PPARγ-down 5'-

GCGGTCTCCACTGAGAATAATGACAGC-3'. Primers for β-actin, which was used as a control, were purchased from Bioneer(Korea) and their sequences were up 5'-CATGCCATCCTGCGTCTGGAC-3', down 5'- TACTCCTGCTTGCTGATCCACATCTGC-3'. The reaction mixtures were placed in a thermal cycler (PERKIN-Elmer/Cetus, Norwalk, Conn.). After an initial denaturing at 94°C for 3min, samples were treated as follows in appropriate cycles ; 94 °C for 30 sec, 60°C for 1min and 72°C for 1min. The numbers of PCR cycles chosen were as follows: 28 cycles for β-actin and 32 cycles for PPARγ. PCR products were visualized on 1.2% agarose gels containing 0.5 μM ethidium bromide. For relative quantitation, the signal intensity of each lane was standardized to that of housekeeping gene, β-actin.

All experiments were performed in duplicate.

3. Results and discussion

3.1. Effect of phospholipids on sebosuppressive function in fuzzy rat

The genetic mutant fuzzy rat which exhibits hypersecretion of sebum was used to investigate the topical effects of various phospholipids. After the 2 months of topical application of each phospholipid, the results were observed and taken photographed shown in Figure 1. HPC showed the strongest sebosuppressive activity that was followed by PC. PS/HPS 40 had a marginal sebosuppressive activity. However, PS/HPS 90 that contained 90% PS/HPS did not suppress the sebum secretion in this experimental condition. This rather intriguing observation could be attributed to the PC portion from the PS 40 sample preparation underlying the importance of PC in preventing sebum secretion. The hydrogenation of phospholipids seemed to change not only the physical properties of the phospholipids but also biological activities. HPC consistently showed better sebosppressive activity than that of the parental PC although the difference was not so big. Over the 2 months period of topical application, however, PC showed faster suppressive activity than HPC then gradually surpassed by HPC.

This was rather unexpected observation since the hydrogenation of PC that turned all the unsaturated fatty

acid compositions into the saturated ones may have had negative effect on the sebosuppressive activity of PC.

In fact, more than 80% of total fatty acid composition of soy-PC is unsaturated fatty acids, among which linoleic acid is the most abundant class at 67%.

3.2 Effect of phospholipids on sebaceous gland

It is known that there is a good correlation between the size of sebaceous gland and the amount of sebum secreted. To assess the effect of applied phospholipids on the sebaceous gland, skin sections were stained with Oil Red O. Through the histological observation, the size of sebaceous glands of the fuzzy rats treated with PC and HPC decreased in comparison to those which were applied with PS, HPS and vehicle-treated animals as we expected (Fig.2). The sebaceous follicles of PC and HPC have also shrunk significantly in size. The result was consistent with the previously known observation that the size of sebaceous gland correlated with the severity of seborrhea.

3.3 mRNA expression of PPARγ following 2 months of PC and HPC treatments in fuzzy rat

Peroxisome proliferator-activated receptor γ (PPARγ) is a member of ligand-dependent transcription factors. Arachidonic acid and linoleic acid are known ligands and activators of PPARγ. PPARγ has recently been found to specifically affect sebaceous cell growth and differentiation and highly expressed in adipose tissue. During sebocyte differentiation PPARγ expression is increased indicating a unique role in stimulating sebocyte lipogenesis. Forced expression of PPARγ in fibroblasts turns them into adipocytes. To investigate the possible signal pathway involved in the regulation of sebum production, we next examined PPARγ mRNA expression from the biopsies skin sections (Fig. 3). The tested agents were only PC and HPC due to its inhibitory effect on seborrhea of animal test. The RT-PCR analysis revealed an intriguing result. that showed the expression of PPARγ mRNA was oppositely regulated by PC and HPC. PPARγ mRNA

expression was markedly suppressed by HPC treatment. On the other hand, significant stimulation of PPARγ mRNA expression by the treatment of PC was observed. In fact, higher PPARγ mRNA expression from the skin of fuzzy rat treated with PC was unexpected because the topical application of PC on fuzzy rats clearly reduced the secretion of sebum. This result strongly indicates that PC and HPC have the same sebosuppresive effects but the intracellular mechanisms involved in lipogenesis must have distinct pathways.

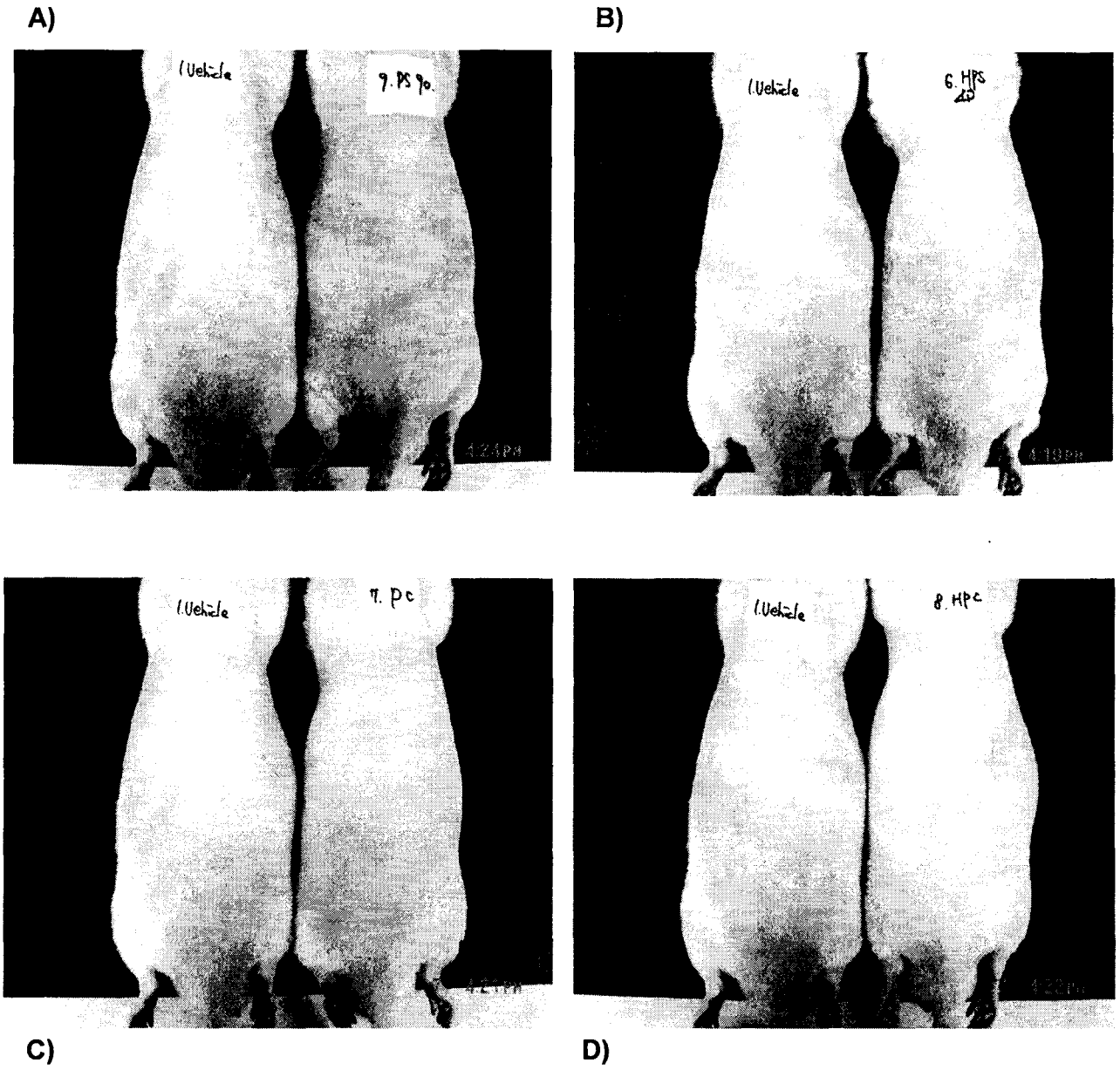

Fig.1 Effect of the phospholipids (PS, HPS, PC and HPC) on the sebum production of fuzzy rat. Reductions of the secretion of sebum were observed in the group treated topically at the dorsal with PC (C) and HPC (D). In contrast, the topical application of PC or HPS did not reduce the secretion of sebum the fuzzy rats compared to the control group treated only with vehicle.

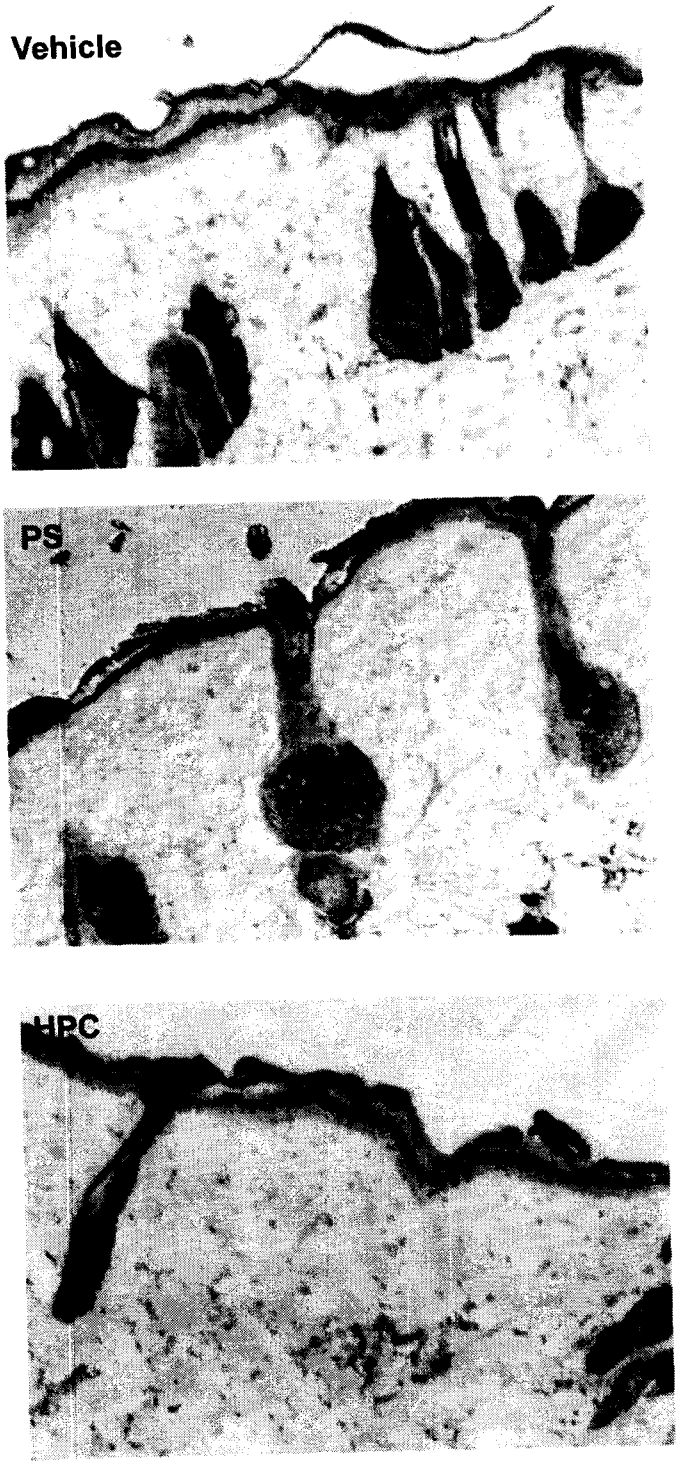

Fig 2. Changes of the size of the sebaceous gland after treatments.

Oil Red R staining of the skin samples showed marked reduction in the size of sebaceous glands that treated with PC or HPC compared to that of the untreated control. No changes in the size of sebaceous glands that treated with PS or HPS were also observed.

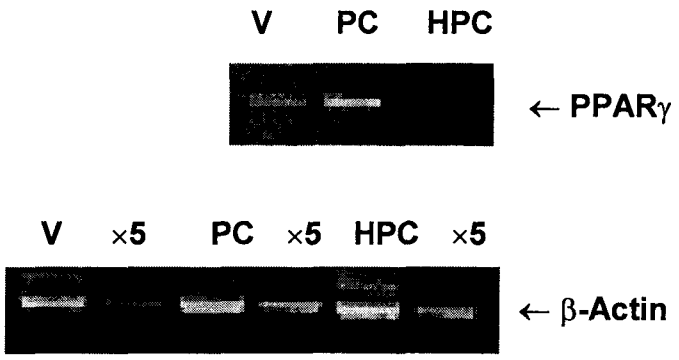

Fig 3. Effects of PC and HPC on PPARγ mRNA expression in fuzzy rat skin tissue analyzed by RT-PCR. mRNA was isolated from PC, HPC and vehicle-treated fuzzy rats and RT-PCR was performed as described in *Materials and Methods*. PPARγ was highly expressed in PC applied rat rather than vehicle(V) –treated rat. Whereas PPARγ mRNA expression from HPC-treated fuzzy rat could only faintly be seen compared to vehicle rat. β-actin mRNA used as internal control and ×5 means the dilution factor.

Discussion

It is generally accepted that the severity of the acne correlates with the degree of the secretion of sebum from the involved lesion. Therefore increased sebum secretion may be a primary cause of the pathogenesis of acne. In this study, we showed that PC and HPC have a significant sebosuppressive effect

when applied topically. However PS and HPS did not have any effect on the secretion of sebum and on the morphological changes of sebaceous glands. Interestingly, the hydrogenation of PC did not weaken its sebosuppressive activity. Instead, the hydrogenation rather rendered it even stronger sebosuppressive activity. At the moment, it was puzzling to address what the molecular mechanisms are behind these observations. Somehow there should be a structure/function differences between PC and HPC. As mentioned previously, PC could be a rich source of linoleic acid that is known to be a ligand/activator of PPARγ. Therefore, hydrogenation of PC abolished the linoleic acid along with other unsaturated fatty acids from the structural moieties of PC, which eventually eliminated the potential positive regulator of PPARγ. Similarities can be found from a case of conjugated linoleic acid(CLA). The unique structural properties of CLA make it quite differently behave from its parental compound, linoleic acid. Although PC and HPC appeared the same inhibitory effect on sebum secretion, the expression of the PPARγ mRNA was regulated oppositely by the two compounds. So it is necessary to further investigate the mechanisms of lipogenesis pathway involved not only PPAR process but also another mechanism in future.

Although there are number of important questions to be addressed in order to get more insight into the differences in the mode of action between PC and HPC in terms of regulating the expression of the PPARγ mRNA, it was clear that both compounds suppress the secretion of sebum in the fuzzy rat model. Currently a clinical study is being carried out using galenic formulations with these compounds and the preliminary results appear to be consistent with that of obtained from the fuzzy rat model system. 13-cis retinoic acid has been known to be the best treatment for acne. It reduces secretion of sebum by up to 90%. However retinoic acid have certain limitations in application because of its significant side effects. Hence, these findings have implications for developing a non-therapeutic topical treatment of the acne vulgaris, the most common skin disorder of adolescents.

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