약학석사학위논문

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2017년 8월

서울대학교 대학원

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ABSTRACT

The Function of AP-1 and NR1D1 as Coregulator of LXR

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Liver X receptor (LXR) α and β are nuclear receptors that regulate genes controlling lipid metabolism in numerous tissues. LXRs have been suggested as a potential therapeutic target to treat various pathological disorders that are driven by lipid metabolism. Thus numerous efforts to find ligands and modulators that have the ability to regulate LXR activity are being made to treat diseases and have been highlighted. Therefore, we aimed to identify the modulators and molecular mechanisms which regulate LXR-mediated lipid metabolism in certain tissues. We found two cases of regulating the activity of LXR in skin and liver. First, in skin, Raffinose, an oligosaccharide, activated LXR transcriptional activity and induced genes involved in LXR-mediated lipid metabolism, water transport and keratinocyte differentiation through in vitro and in vivo study. However, Raffinose

is not a ligand of LXRs. To elucidate the mechanism, we focused on the correlation between LXRs and AP-1 family that plays a critical role in keratinocyte survival and differentiation. Raffinose and TO901317 induced the expression of JunD and Fra1, and increased DNA binding of c-Jun on the AP-1 response element in the promoter of involucrin and loricrin. Interestingly, LXRs also bound to AP-1 sites after treatment with Raffinose and TO901317. JunD and Fra1 enhanced transcriptional function of LXR and increased expression of genes involved in LXR-mediated lipid metabolism. Therefore, the results of the first case suggest that Raffinose inducess AP-1 family, c-Jun, JunD and Fra1, which leads to the induction of LXR signaling and stimulation of keratinocyte differentiation. Secondly, in liver, we showed that nuclear receptor subfamily 1 , group D, member1 (NR1D1) upregulated genes of LXRα and SREBP1-c and induced transcriptional acitivity of LXRα and LXRβ. In vitro screening for finding NR1D1 ligand showed that Compound1 antagonizes transcriptional activity of NR1D1. This Compound1 suppressed transcriptional function of LXR as well as protein levels of LXR and LXR-mediated lipogenic genes. Taken together, our results suggest that as transactivating coregulators of LXR, AP-1 family and NR1D1 regulate lipid metabolism in skin and liver. Our observations may help applying on treating a variety of diseases.

keywords : LXR, AP-1, NR1D1, Lipid metabolism, Keratinocyte differentiation

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I . INTRODUCTION

The Liver X receptor (LXR) is a member of the nuclear receptor family and is comprised of two isoforms LXRα and LXRβ. LXRα is predominantly expressed in metabolically active tissue such as liver, kidney, intestine, adipose tissue and macrophagy. LXRβ is ubiquitously expressed in almost all tissues and organs. (Zhao *et al.*, 2010) LXRs as ligand-dependant transcription factors are activated by endogeous oxysterol, oxidized cholesterol metabolites. (Bełtowski, 2008)

LXRs play an important role in maintaining homeostasis in the body. Activated LXRs mainly regulate lipid and cholesterol metabolism, but are also known to be involved in glucose homeostasis, differentiation and inflammation in various tissues. (Figure 1, Zelcer *et al.*, 2006)

LXRs are highlighted as potential therapeutic targets in a variety of diseases such as atherosclerosis, cancer, autoimmune diseases and skin diseases. (Figure 2, Hong et al., 2014; Komati *et al.*, 2017) LXRs stimulate reverse cholesterol transport, cholesterol conversion to bile acid, and intestinal cholesterol absorption. Together, LXRs exhibit anti-atherosclerotic effects. (Baranowski, 2008) LXRs also suppress proliferation of cancer cells and angiogenesis of endothelial cells, which leads to suppression of tumor growth in breast cancer, colorectal cancer, hepatocarcinoma and melanoma. (Lin *et al.*, 2015)

LXRs also repress Th-17 cell differentiation and relieve autoimmune diseases such as multiple sclerosis and rheumatoid arthritis. (Cui *et al.*, 2011) In skin diseases such as atopic dermatitis and contact dermatitis, LXRs have a critical role in inflammation and epidermal barrier function. LXRs attenuate inflammation by suppressing cytokine mediated expression of proinflammatory genes such as IL-1β and MMPs by inhibiting NF-κB signaling. LXRs also enhance epidermal barrier function by stimulating differentiation, lipid synthesis and transport, water transport and lamellar body formation through expression of the relevant genes such as AP-1, Aquaporin3, ABCA1, ABCG1, GAPT-3, and β-glucosidase. (Figure 3, Schmuth *et al.*, 2014)

However, although activated LXRs are known to be beneficial for a variety of diseases, there are also controversial sides. For non alcoholic fatty liver disease (NAFLD), LXRs increase accumulation of triglyceride in liver by inducing hepatic lipogenesis, which exacerbates nonalcoholic steatohepatitis. (Ducheix *et al.*, 2013) Because of this, the LXR inverse agonists have been suggested to alleviate NAFLD by inhibiting fibrosis and lipid accumulation. (Griffet et al., 2015) Therefore, in order to use LXRs as therapeutic targets for the treatment of various pathological diseases, understanding LXR signaling for therapeutic strategies is essential.

Activator protein 1 (AP-1) is a transcription factor that regulates cell differentiation, proliferation, and apoptosis in response to external stimuli such as cytokines, growth factors, oncogenes, tumor promoters

and chemical carcinogens. (Figure 4, Hess *et al.*, 2004) AP-1 is controlled via post-translational modification, DNA binding dimer composition and interact with various cellular factors. (Vesely *et al.*, 2009) In relation to AP-1 and LXRs, it has been reported that AP-1 cis regulatory motif is enriched in LXRβ-RXRα binding site and AP-1 directly interact with LXR in keratinocyte. (Shen *et al.*, 2011) However, it is unknown how the interaction between AP-1 and LXR affects LXR mediated lipid metabolism in keratinocyte. In the first part of this study, we demonstrated that AP-1 regulated by Raffinose could affect the keratinocyte differentiation and lipid synthesis in association with LXRs.

Nuclear receptor subfamily 1 group D member 1 (NR1D1) known as Rev-erbα is a nuclear receptor which is highly expressed in metabolic tissues including skeletal muscle, brown and white adipose, brain, liver and kidney. (Figure 5, Yin *et al.*, 2010) In general, NR1D1 acts as a transcriptional repressor that recruits cerepressors such as NcoR1 and SMRT and suppress expression of target genes by endogenous ligand such as heme. (Everett *et al.*, 2014) Regarding lipid metabolism, NR1D1 inhibits ApoCⅢ, a component of HDL and VLDL, that regulates triglyceride levels and lipoprotein lipase activity in liver. NR1D1 also modulates bile acid metabolism either directly or indirectly. (Duez *et al.*, 2009; Le Martelot *et al.*, 2009; Ramakrishnan et al., 2006) However, the correlation between NR1D1 and LXR, the main regulator of lipid metabolism, is not known in liver.

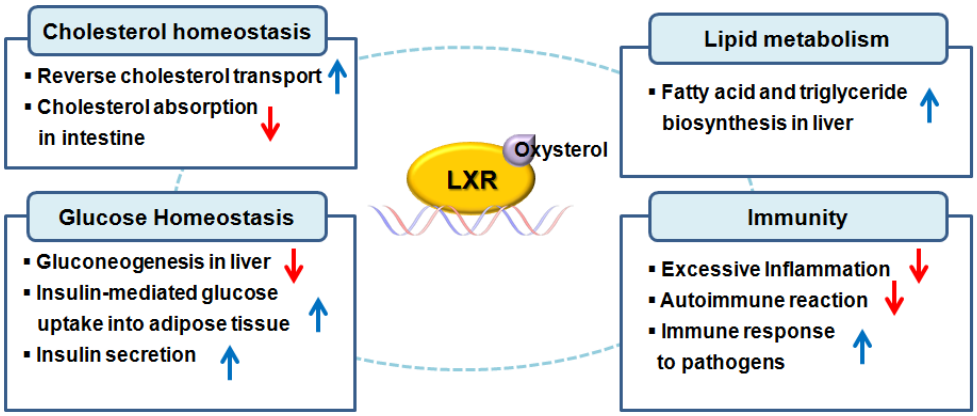


Figure 1. Biological roles of LXRs in the body.

Schematic illustration of LXRs and its biological roles. LXRs are activated by endogenous ligand such as oxysterol and oxidized cholesterol metabolites and maintain homeostasis of body by regulating various metabolisms and the immunity system.

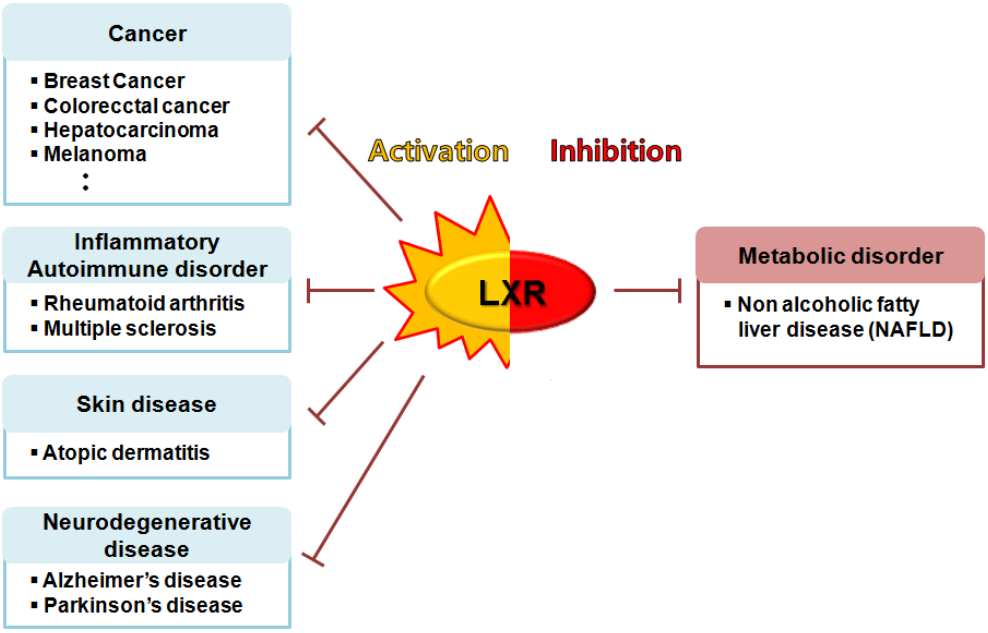


Figure 2. The various pathological conditions related to Liver X Receptors (LXRs).

The scheme of the various pathological disorders which have been suggested to have a potential of being treated through modulating LXR activity. (Komati et al., 2017)

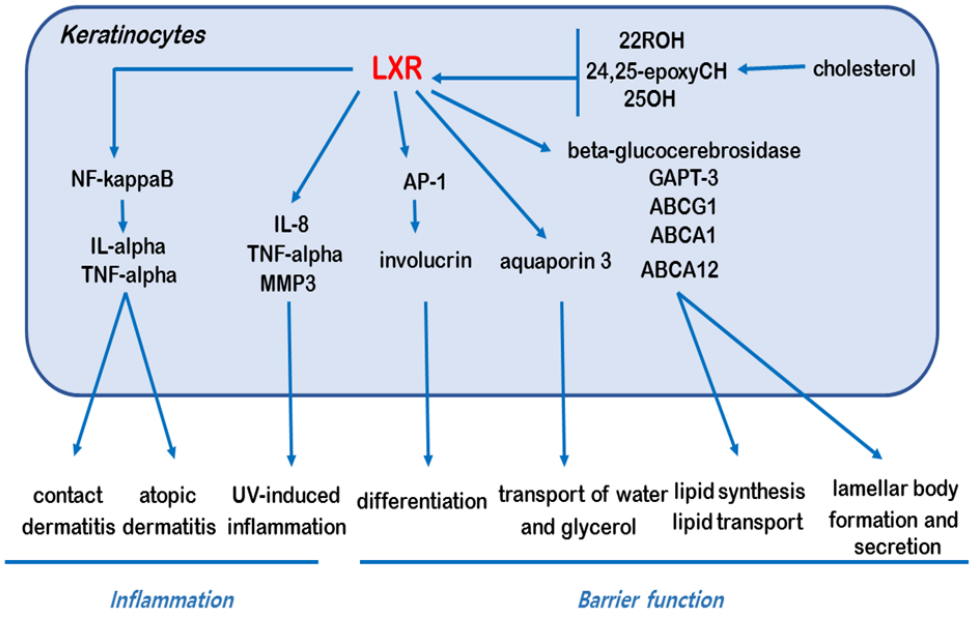


Figure 3. The functions of LXRs in epidermal homeostasis and inflammation in keratinocyte.

The scheme of the role of LXRs in epidermal homeostasis and inflammation. Activation of LXRs regulate expression of key genes involved inflammation and epidermal homeostasis, which leads to alleviation UV-induced skin inflammation, irritant and allergic contact dermatitis, and improvement of atopic dermatitis.via downregulation of various inflammatory mediators like cytokines and MMPs and enhancement of epidermal barrier functions such as keratinocyte differentiation, lipid metabolism, and lamellar body formation and secretion. (Schmuth et al., 2014)

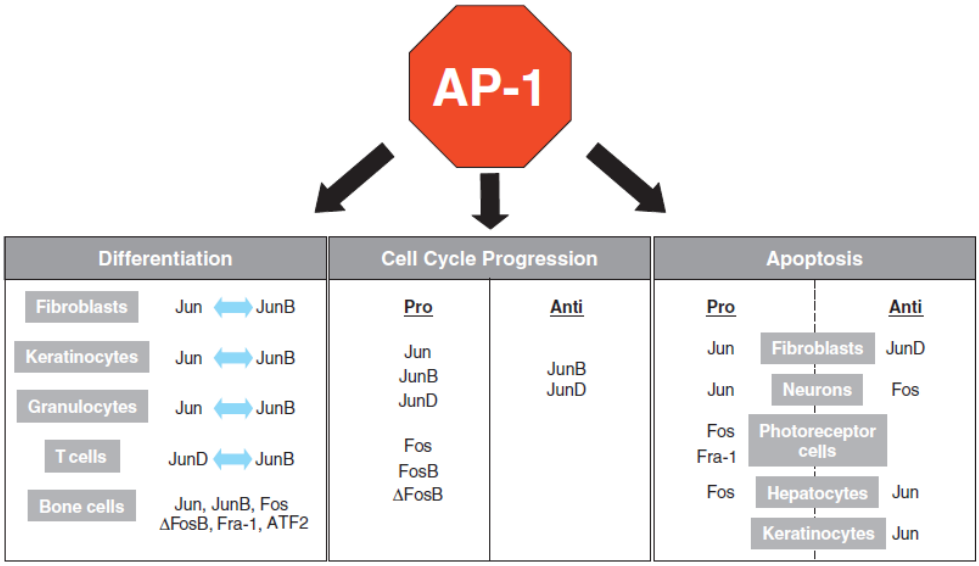


Figure 4. Roles of AP-1 subunits in various cellular processes.

The scheme of the roles of AP-1 subunits in various cellular processes. In various cell types, AP-1 members affect cellular processes such as differentiation, cell cycle regulation or apoptosis, depending on the function of individual subunits. The double-headed arrow indicates antagonistic task for AP-1 members. (Hess et al., 2004)

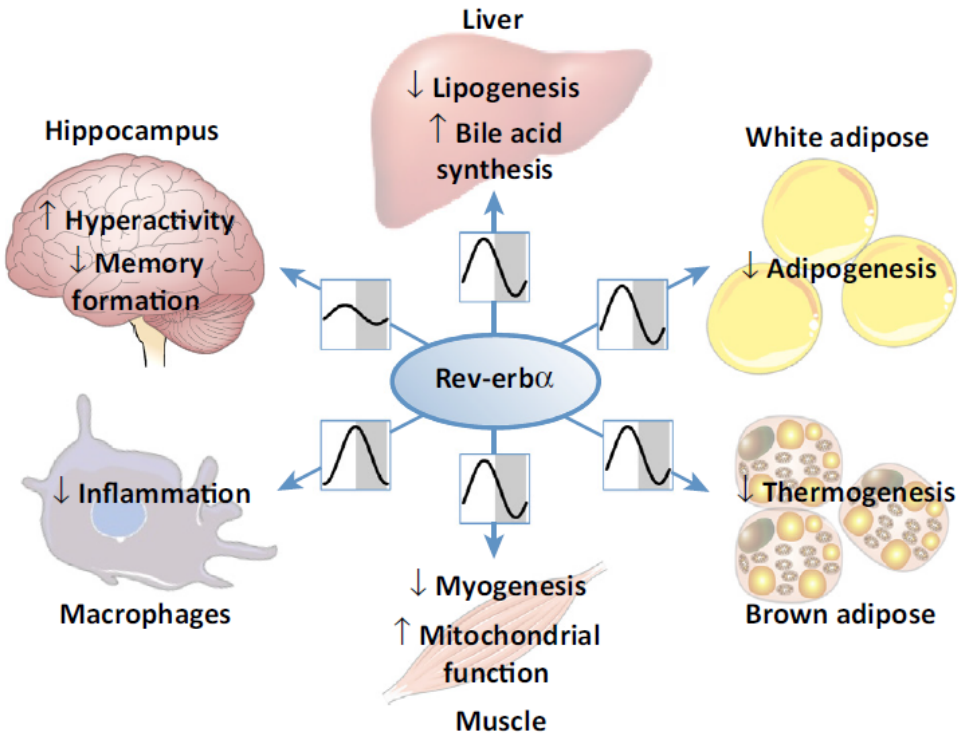


Figure 5. The physiological roles of NR1D1 in the various tissues.

The scheme of NR1D1 circadian expression patterns and its physiological roles in various tissues. NR1D1 regulates crucial metabolic, inflammatory, and behavioral functions in a tissue-specific manner. (Everett et al., 2014)

Ⅱ. PURPOSE of the STUDY

LXRs have been suggested as therapeutic targets for treating a variety of pathological disorders. Therapeutic strategy through regulation of LXRs activity has been developed consistently. However. the roles of LXRs depend on specific tissues and their mechanisms are varied. Thus, the application of therapeutic strategy targeting LXRs to a variety of pathological conditions requires the identification of modulators and regulatory mechanisms that regulate LXRs in specific tissues. In skin, LXRs are important regulators of epidermal barrier enhancement. Through nuclear receptor activity screening, we found that Raffinose activates LXRs in keratinocyte. Raffinose has been reported as a potential cosmetic ingredient by promoting lamellar formation. However, the molecular mechanism of this effect by Raffinose is unknown. In liver, LXRs play a role in maintaining metabolism homeostasis as cholesterol and glucose sensors. NR1D1 is also known as an integrator involved in lipid metabolism, cell proliferation and inflammation. However, there is no known correlation between NR1D1 and LXRs in liver lipid metabolism.

In this study, we aimed to find the modulators of LXRs and identify theirs effect on lipid metabolism.

Ⅲ. MATERIALS and METHODS

1. Cell lines and cell treatment

HaCaT and CV1 cells were maintained under 5% CO2 at 37℃ in Dulbecco's modified Eagle's medium (DMEM; SH30243.01; Hyclone; Pennsylvania, USA) supplemented with 10% fetal bovine serum (FBS; SH30919.03; Hyclone) and 1% penicillin and streptomycin (P/S; 15140; Gibco; New Hampshire, USA). And HepG2 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS, Welgene) and 1% P/S.

D-(+)-Raffinose (R0250) was purchased from Sigma-Aldrich (St Louis, MO), the synthetic LXR agonist TO901317 (CAS 293754-55-9) and synthetic NR1D1 agonist GSK4112 (CAS 1216744-19-2) was from Cayman Chemical (Michigan, USA). The 50 compounds for NR1D1 activity screening were provided by Dr. Young-Ger Suh (College of Medicine Seoul National University, Seoul, Korea).

2. Reporter gene assay

HaCaT, CV1 and HepG2 cells were seeded in a 24well plate. pCMV-mJunD was provided from Dr. Hongduk Yun (College of Medicine Seoul National University, Seoul, Korea). Cells were transfected with a DNA mixture containing luciferase reporter promoter, expression vectors as indicated and β-galactosidase

expression vector using X-tremeGene HP DNA transfection reagent (Roche). Transfection was performed according to manufacturer's protocol. To harvest cell lysate, 200 μl of luciferase cell culture lysis 5X reagent (E1531; Promega) was used. The luciferase reporter promoter activity was normalized by β-galactosidase.

3. Fluorescence resonance energy transfer (FRET)assay

To analyze binding affinity of Raffinose to LXRα and LXRβ, FRET assays were performed using LanthaScreen TR-FRET LXRα and LXRβ coactivator assays (PV4655, PV4658; Thermo Scientific, Waltham, MA). Briefly, various concentrations of Raffinose were incubated in a solution containing the ligand binding domain of LXR and fluorescein-tagged co-activator peptide. After incubation for 30 min at room temperature, the mixtures were analyzed on a microplate reader with background emission at 490 nm, and binding signal at 520 nm after excitation at 340 nm. The ratio of emission signals at 520 nm and 490 nm were plotted against the log of the compound concentrations and fitted to a sigmoidal dose-response curve using Graphpad prism 5.0 (GraphPad Software, San Diego, CA).

4. Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed as described previously (Lee et al., 2012). Nuclear lysates were sonicated, and the lysates were immunoprecipitated using anti-LXR (PA1-332, Thermo Scientific), anti-c-Jun (9165S ; Cell signaling technology, Massachusetts, USA)

and normal IgG antibodies for overnight at 4℃. DNA was prepared by phenol-chloroform extraction and amplified by PCR using specific primer corresponding to the AP-1 binding site on the Involucrin and Loricrin promoters The primer sequence used are in the table below (Table 1).

5. Animal, tissue preparation and Immunohistochemistry in skin *in vivo* model

Adult hairless mice (Skh:HR-1) 8 weeks of age (SLC Japan, Tokyo, Janpan) were applied topically twice a day for 4days with 5% Raffinose dissolved in a mixture of PEG400 and PEG 3350 at weight ratio of 3:2 (0.1ml applied to $2cm^2$ area) which generated by Dr. Dae-Duk Kim. Control hairless mice were treated with vehicle alone. At the end of treatment, the dorsal skin was collected and the epidermis was isolated. Skin samples were fixed overnight in 4% formaldehyde and embedded in paraffiin. Immunohistochemistry was examined with antibodies against Filaggrin (sc-66192, Santa Cruz Biotechnology, Santa Cruz, CA), Involucrin (924401, BioLegend, San Diego, CA), AQP3 (ab125219, Abcam, Cambridge, UK), LXR (PP-PPZ0412-10, Perseus Proteomics), SCD1 (sc-14719, Santa Cruz biotechnology), and ChREBP (NB400-135, Novus Biologicals, Littleton, CO).

6. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated using EASY-BLUE™ Total RNA Extraction Kit (iNtRON Biotechnology; Gyeonggi-do, Korea) according to the manufacturer's instructions. cDNA was synthesized from total RNA in a reaction mixture containing random hexamer (48190-011; Invitrogen) and M-MLV reverse transcriptase (28025-013; Invitrogen). qRT-PCR was performed using SYBR Green PCR mix (4367659; Applied Biosystems; New Hampshire, USA). The resulting ΔCt values were normalized with GAPDH. The primer sequence used are in the table below (Table 1).

7. Western blot assay

Cells were washed twice with cold PBS and then harvested with a lysis buffer (10 mM Tris Cl, 100 mM NaCl, 1 mM EGTA, 10% glycerol, 0.5% Triton X-100, 30 mM sodium pyrophosphate, 1 mM sodium fluoride, 1 mM sodium orthovanadate, 5 mM glycerol 2-phosphate) supplemented with a protease inhibitor cocktail (11.836.153.001; Roche Diagnostics; Basel, Switzerland), using a cell scraper. After 30 minutes of incubation on ice, cell lysates were centrifuged at 14,000 rpm for 10 minutes. Supernatant was collected for the protein quantification through BCA Protein Assay Kit (23225; Pierce; New Hampshire, USA). Protein samples were made and loaded onto a 7%, 8.5%, 9% or 10% gel. After SDS-polyacrylamide gel electrophoresis, proteins were transferred onto a polyvinylidene

difluoride (PVDF) membrane via dry-transfer method. Blocking was done in 5% non-fat dry milk in PBS with 0.1% Tween-20 (PBST) for 1 hour under room temperature. Then membranes were incubated in specific antibodies against LXRα (ab41902, Abcam), LXRβ (14278-1-AP; Proteintech Group, Chicago, IL), Involucrin (I9018, Sigma-Aldrich), JunD (sc-74; Santa Cruz Biotechnology), Fra1 (sc-28310; Santa Cruz Biotechnology), ABCG1 (ab52617, Abcam), ChREBP (NB400-135; Novus Biologicals, Littleton, CO), SREBP1-c (sc-8284; Santa Cruz Biotechnology), FASn (3189S; Cell signaling technology), NR1D1 (14506-1-AP; Proteintech Group) and α-tubulin (05-829, Millipore, Billerica, MA) in 5% non-fat dry milk in PBST overnight in 4℃. Membranes were washed with PBST and incubated in secondary antibodies for 1 hour under room temperature. Amersham ECL solution (RPN2106; GE Healthcare; Pennsylvania, USA) was used for detection after washing with PBST for remove antibodies.

8. Statistical analyses

Statistical analyses were calculated using GraphPad Prism (GraphPad Software; California, USA). Significance of the data from cell counting assay was determined by two-way ANOVA test. All other comparisons were made using Mann-Whitney U test. $P < 0.05$ denotes statistical significance.

Table 1. Primer sequences of qRT-PCR and ChIP assay

Gene		primer sequences	
LXRα	Sense Antisense	5′-AGTGTCGGCTTCGCAAAT-3′ 5′-AGAAGCATCACCTCGATCG-3′	qRT-PCR
LXRβ	Sense Antisense	5′-GAGTCACAGTCACAGTCGCAG-3′ 5′-TCTCTAGCAGCATGATCTCGATA-3′	qRT-PCR
ChREBP	Sense Antisense	5′-ACAGCAACAAGACCGAGAAC-3′ 5′-TGAAGGACTCAAACAGAGGC-3′	qRT-PCR
SCD1	Sense Antisense	5′-ACTGGTGATGTTCCAGAGGA-3′ 5′-GTTTCCATCTCCGGTTCTTT-3′	qRT-PCR
ABCA1	Sense Antisense	5′-GGTGATGTTTCTGACCAATGTGA-3′ 5′-TGTCCTCATACCAGTTGAGAGAC-3′	qRT-PCR
ABCG1	Sense Antisense	5′-ACACCATCCCCACGTACCTA-3′ 5′-GATGACCCCTTCGAACCCA-3′	qRT-PCR
Fillagrin	Sense Antisense	5′-GGGAAGTTATCTTTTCCTGTC-3′ 5′-GATGTGCTAGCCCTGATGTTG-3′	qRT-PCR
Involucrin	Sense Antisense	5′-ACCCATCAGGAGCAAATGAAA-3′ 5′-GCTCGACAGGCACCTTCTGGCA-3′	qRT-PCR
Loricrin	Sense Antisense	5′-AGACCCAGCAGAAGCAGGCG-3′ 5′-AGCAGAACTAGATGCAGCCG-3′	qRT-PCR
AQP3	Sense Antisense	5′-GACAGAAGGAGCTGGTGTCC-3′ 5′-ATGAGGATGCCCAGAGTGAC-3′	qRT-PCR
GAPDH	Sense Antisense	5′-GTGCAGTGCCAGCCTCGTCC-3′ 5′-CAGGCGCCCAATACGGCCAA-3′	qRT-PCR
Involucrin promoter	Sense Antisense	5′-CTCCCTTGCATACACACACA-3′ 5′-GGTTAGCAGGGGCTCAGTAT-3′	ChIP
Loricrin promoter	Sense Antisense	5′-GCCCATCTCAAGAATGCCAA-3′ 5′-GAAGATGCTGGCAATGTGAG-3′	ChIP

Ⅳ. RESULTS

1. Raffinose activates transcriptional function of LXR, but is not a ligand of LXR

The Raffinose family of oligosaccharides (RFOs) are α-galactosyl derivatives of sucrose that abundantly exist in the plant kingdom and found in a large variety of seeds (Figure 6A). Among the RFOs, the trisaccharide Raffinose has been reported as an effective cosmetic ingredient with moisturizing function for dry skin. (Sakurai *et al.*, 2005) To investigate the effect of Raffinose on LXR transcriptional activity, we used LXR response element (LXRE) reporter gene assay in HaCaT cells. The Raffinose treatment induced LXR-mediated reporter activity (Figure 6B). We next examined whether Raffinose could bind directly to LXRα and LXRβ using TR-FRET Coactivator assay. Unlike LXR agonist TO901317, Raffinose did not induce a signal from the ligand-dependent coactivator recruitment to LXRα or LXRβ (Figure 6C). These results suggested that Raffinose is an activator of LXRα and LXRβ.

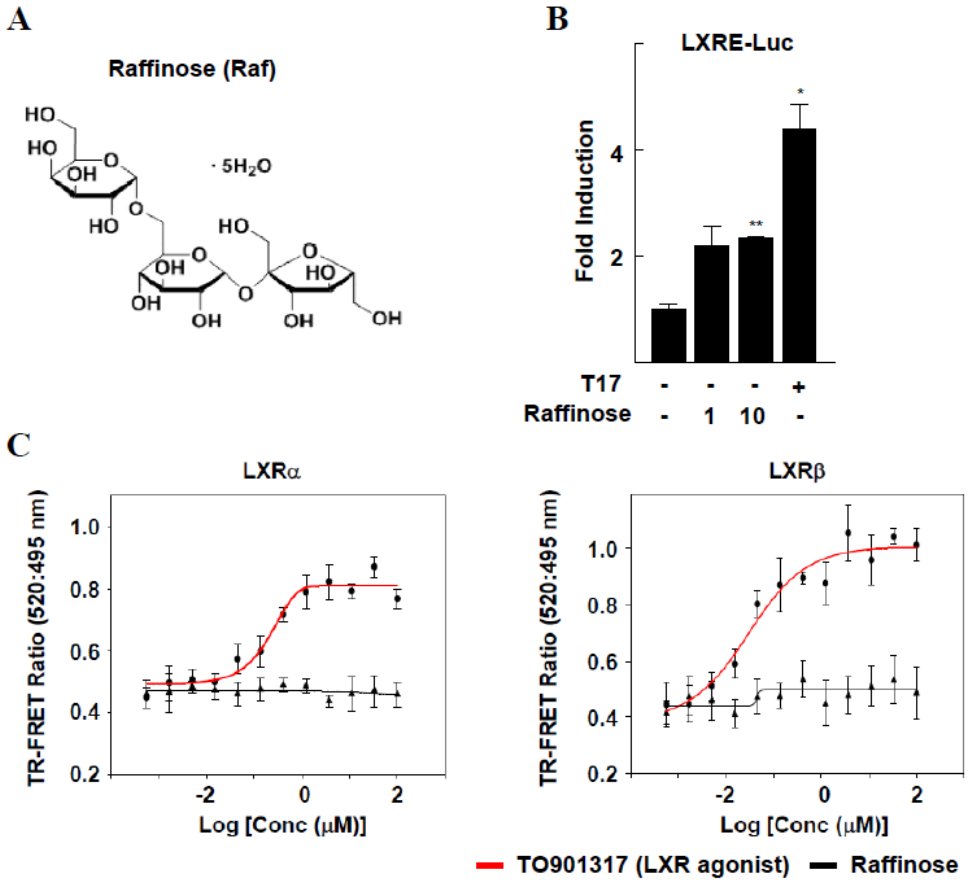

A
Raffinose (Raf)
· 5H2O
B
LXRE-Luc
Fold Induction
T17
Raffinose
C
LXRα
LXRβ
TR-FRET Ratio (520:495 nm)
Log [Conc (μM)]
TO901317 (LXR agonist)
Raffinose

Figure 6. Raffinose activates transcriptional activity of LXRα, but does not directly bind to LXRs

(A) Chemical Structure of Raffinose (Raf)

(B) CV1 cells were transcfected with LXRE-Luc and expression vector for LXRα, and then treated with Raffinose or TO901317 (T17) for 24h. Data represent the means ±SEM (n=3). *p < 0.05 compared with vehicle control.

(C) TR-FRET assay was performed using Lanthascreen LXRα or LXRβ co-activator assay kit (Invitrogen, Madison, WI). Y-axis represents ratio of fluorescence intensity at 520 nm (signal) and at 495 nm (background). X-axis represents log scale of raffinose (black line) or TO901317 (red line) concentration. TO901317 was used as positive control. Data represents mean ± SD (n=4). The EC50 value of TO901317 was calculated as 30 nM and 29 nM for LXRα and LXRβ, respectively. (Data from HJ Oh and YH Han)

2. Raffinose stimulates lipid metabolism and epidermal differentiation in the skin of Hairless mouse

In order to investigate the effects of Raffinose on the gene expression involved in lipid metabolism and epidermal differentiation, we applied Raffinose in an ointment to the skin of hairless mice (Figure 7A). Raffinose increased expression of genes involved in LXR-downstream lipogenic genes such as liver X receptor, stearoyl-CoA desaturase (SCD1) and carbohydrate-responsive element-binding protein (ChREBP); keratinocyte differentiation, fillagrin and involucrin; a gene involved in water transport Aquaporin 3 (Figure 7B). These data indicate that Raffinose induces the expression of LXR-downstreeam lipogenic genes and stimulates epidermal differentiation.

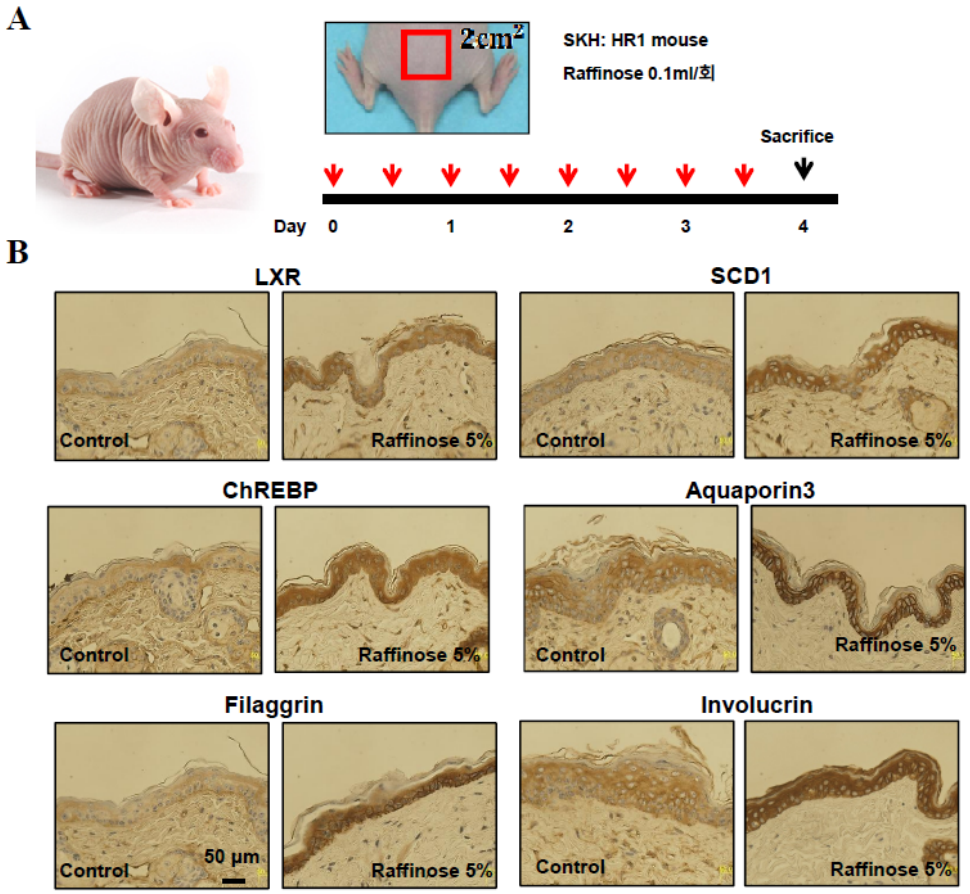


Figure 7. Raffinose increases expression of genes involved in lipid metabolism and keratinocyte differentiation in epidermis of hairless mice

(A) Schemes for animal experiments in this study. Seven-week-old hairless mice were treated topically to dorsal twice a day for 4 days. (B) Immunohistochemical staining for LXR, SCD1, ChREBP, AQP3, Filaggrin, and Involucrin in control and 5% raffinose-applied mouse epidermis. 100x magnification.

3. Raffinose induce transcription of genes involved in lipid metabolism and keratinocyte differentiation in HaCaT cell

We also tested the effects of Raffinose on lipid metabolism and keratinocyte differentiation in HaCaT cell. Consistent with the results obtained from the in vivo studies, Raffinose upregulated the transcription of genes involved in LXR-downstream lipogenic genes such as LXRs, SCD1 and ChREBP; genes involved in lipid transport such as the ABC family transporters, ABCA1 and ABCG1; a gene involved in water transporter, AQP3, and keratinocyte differentiation, Fillagrin, Involucrin and Loricrin (Figure 8). These results of in vitro studies also demonstrated that Raffinose regulates genes involved in lipid metabolism and keratinocyte differentiation.

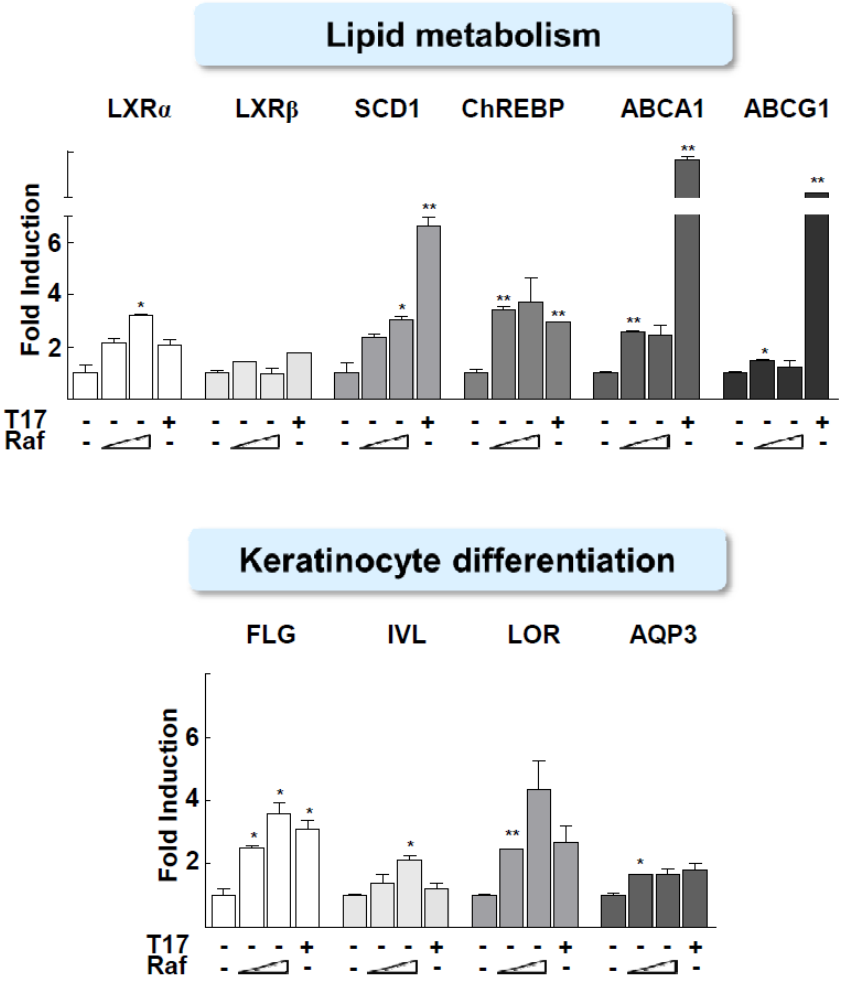


Figure 8. Raffinose induces transcription of genes involved in lipid metabolism and keratinocyte differentiation

HaCaT cells were treated with vehicle, 1 μM or 10 μM Raffinose or 1 μM TO901317 for 24h. Total RNA isolated from cells was used to analyze mRNA expression using qRT-PCR. Data represent the means ±SEM (n=3). *$p < 0.05$ compared with vehicle control.

4. AP-1 family are regulated by Raffinose and involved in keratinocyte differentiation

Activator protein 1 (AP-1) family is a transcription factor which has a critical role in keratinocyte survival and differentiation. (Angel *et al.*, 2001) It has been reported that AP-1 can form a cross talk with nuclear receptors and regulate transcription of their target genes. (Altucci *et al.*, 2001) We hypothesized that LXR-mediated lipid metabolism and keratinocyte differentiation by Raffinose could be regulated by AP-1 family. To investigate the involvement of AP-1 family in the effects of Raffinose, we examined the expression of JunD and Fra1 which are AP-1 family components and known as important regulators in an early stage of keratinocyte differentiation. Western blot analysis showed that JunD and Fra1 were upregulated by Raffinose treatment in HaCaT cell (Figure 9A). Chromatin immunoprecipitation (ChIP) assay showed that Raffinose and TO901317 induced DNA binding of c-Jun in the AP-1 motif present in the upstream promoters of Involucrin and Loricrin. LXRs also bound to the AP-1 motif by Raffinose or TO901317, which suggested that Raffinose could induce a cross-talk between LXRs and AP-1 (Figure 9B). These results indicate that Raffinose regulate expression and action of AP-1 family, which leads to stimulation of lipid metabolism and keratinocyte differentiation.

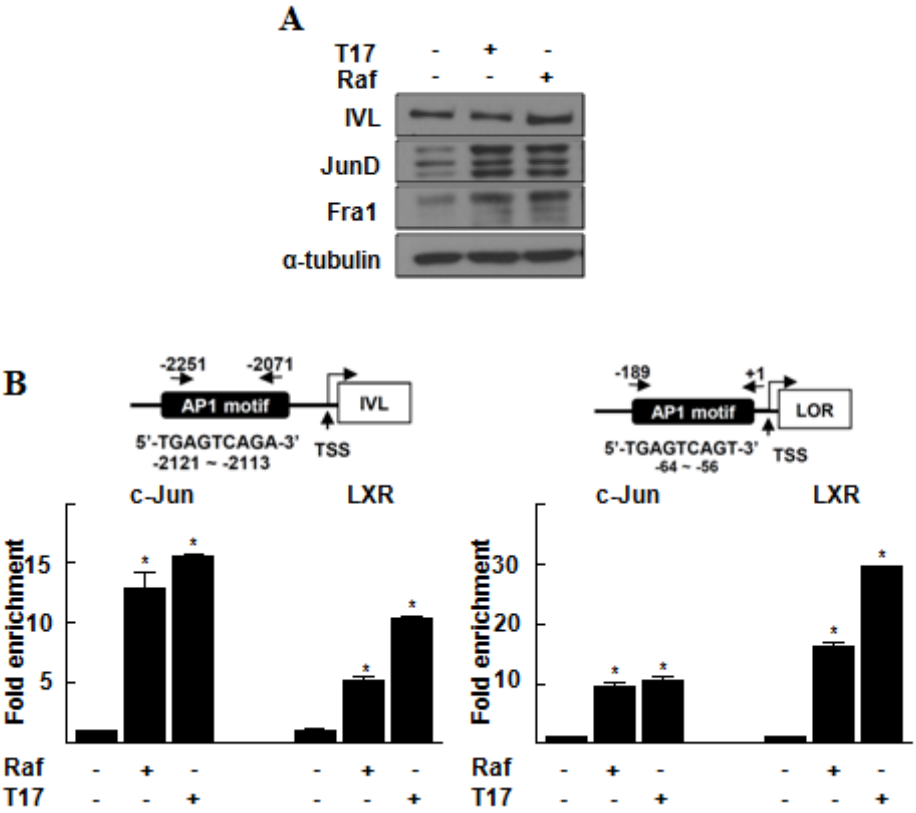

A
T17
Raf
IVL
JunD
Fra1
α-tubulin
B
-2251
-2071
AP1 motif
IVL
5'-TGAGTCAGA-3'
-2121 ~ -2113
TSS
-189
+1
AP1 motif
LOR
5'-TGAGTCAGT-3'
-64 ~ -56
TSS
c-Jun
LXR
Fold enrichment
Raf
T17

Figure 9. Raffinose induces expression of JunD and Fra1 as well as DNA binding of c-Jun in AP-1 motif present in the promoters of Involucrin and Loricrin

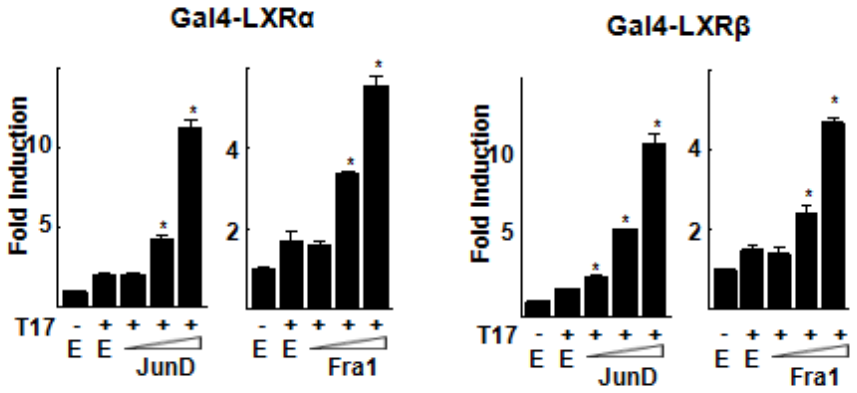
HaCaT cells were treated with vehicle, 1 μM Raffinose or 1 μM TO901317 for 24h. (A) The expression levels of involucrin, JunD and Fra1 were measured by western blotting.

(B) Schematic representation of primers used for ChIP assay (upper). ChIP assay was performed with specific antibodies against c-Jun and LXR (lower). Data represent the means ±SEM (n=3). *$p < 0.05$ compared with vehicle control.

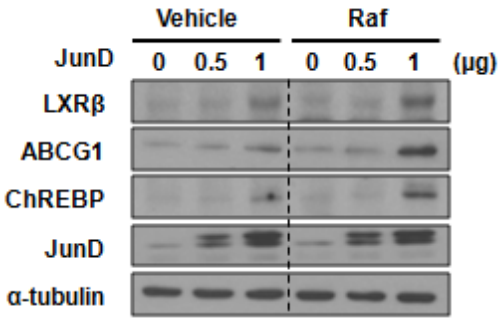
5. JunD and Fra1 enhances LXR signaling

It has been reported that about 80% of the LXRβ/RXRα binding sites in keratinocyte contain AP-1 motifs in their vicinity. (Shen *et al.*, 2011) We tested whether AP-1 could affect transcriptional activity of LXRs and expression of LXR downstream genes. We conducted reporter gene assay in CV1 cells. JunD and Fra1 dose-dependantly upregulated the TO901317-induced transcriptional activity of LXRα and LXRβ (Figure 10A). Overexpression of JunD upregulated expression of LXRβ and its downstream genes such as ABCG1 and ChREBP. Raffinose further enhanced these expressions (Figure 10B). These findings demonstrate that AP-1 family such as JunD and Fra1 induced by Raffinose or TO901317 enhance the transcriptional activity of LXR, which stimulates lipid metabolism and keratinocyte differentiation (Figure 10C).

A


Gal4-LXRα
Gal4-LXRβ
Fold Induction
10
5
4
2
T17
E E
JunD
Fra1

B


Vehicle
Raf
JunD
0 0.5 1 0 0.5 1 (μg)
LXRβ
ABCG1
ChREBP
JunD
α-tubulin

C

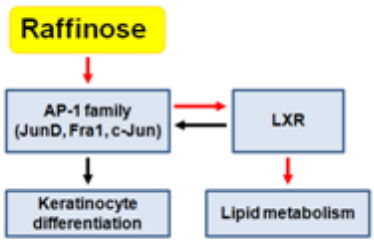

Raffinose
AP-1 family
(JunD, Fra1, c-Jun)
LXR
Keratinocyte
differentiation
Lipid metabolism

Figure 10. JunD and Fra1 enhance transcriptional function of LXR and expression of LXR and its downstream target genes

(A) CV1 cells were transfected with Gal4-tk-Luc, pGal4-LXRα or pGal4-LXRβ, together with empty vector (E), or 10, 50, or 100 ng expression vector for JunD or Fra1. After transfection, the cells were treated with 0.1 μM TO901317 (T17) for 24 h. The β-galactosidase activity was used to normalize luciferase activity. Data represent the means ± SEM (n=3). *P< 0.05 compared with T17 treatment with empty vector.

(B) HaCaT cells were transfected with the indicated amount of expression vector encoding human JunD for 24 h, and treated with vehicle or 1 μ M raffinose for 24 h. Expression of proteins was analyzed by western blotting.

(C) Schematic representation of the proposed molecular mechanism of raffinose action in the lipid metabolism and keratinocyte differentiation.

6. NR1D1 regulates expression and transcriptional function of LXRs

To identify the role of NR1D1 on LXR signaling, we examined whether NR1D1 affects expression and transcriptional activity of LXRs. When NR1D1 was introduced exogenously to HepG2 cells, expression of LXRα and SREBP1-c was upregulated. NR1D1 agonist, GSK4112, also increased their expressions (Figure 11A and B). A reporter gene assay showed NR1D1 induced transcriptional acitivity of both LXRα and LXRβ in a dose dependent manner (Figure 11C). These results suggested that NR1D1 regulates LXR signaling.

7. In vitro Screening, the Compound1 antagonized transcriptional function of NR1D1

Through the effect of NR1D1 on LXR signaling, we applied a new compound that regulates function of NR1D1 on LXR signaling. We first used Rev-erb response element (Rev-erbRE) reporter to find compounds which regulate transcriptional activity of NR1D1 in CV1 cells. Total of 50 compounds modified from the NR1D1 agonist SR9009 were used in the in vitro screening. Among the 50 compounds, we found that one compound antagonized transcriptional activity of NR1D1. This compound was named Compound1 (Figure 12). We confirmed NR1D1 antagonizing effect of Compound1 through activation of Gal4-DBD-NR1D1 on Gal4-tk-Luc reporter in CV1 cells (Figure 13A and B).

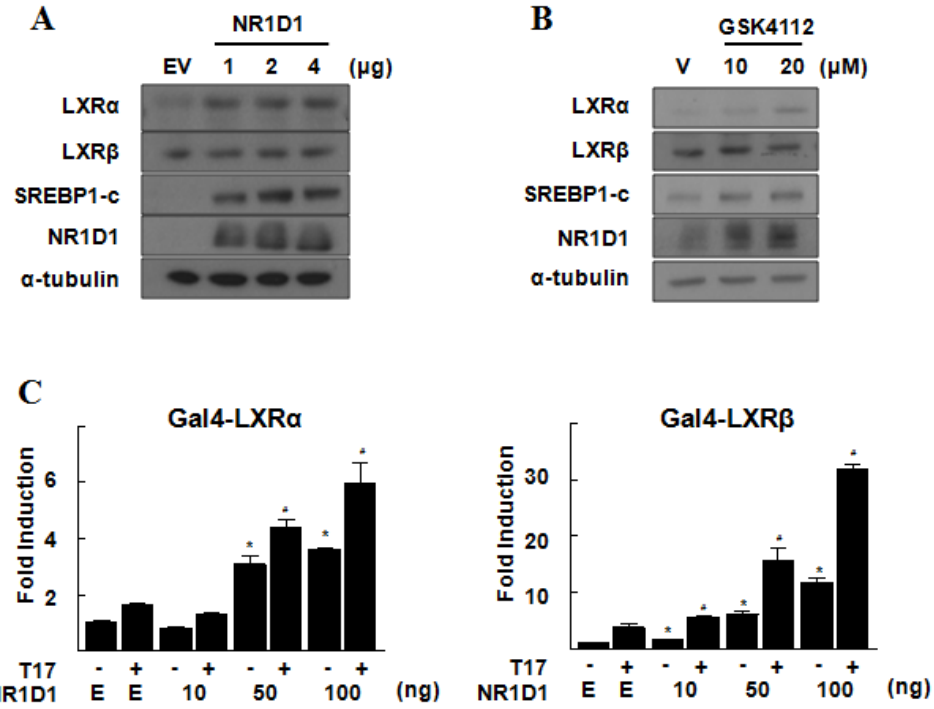

A
NR1D1
EV 1 2 4 (μg)
LXRα
LXRβ
SREBP1-c
NR1D1
α-tubulin
B
GSK4112
V 10 20 (μM)
LXRα
LXRβ
SREBP1-c
NR1D1
α-tubulin
C
Gal4-LXRα
Fold Induction
6
4
2
T17
NR1D1
- + - + - + - +
E E 10 50 100 (ng)
Gal4-LXRβ
Fold Induction
30
20
10
T17
NR1D1
- + - + - + - +
E E 10 50 100 (ng)

Figure 11. NR1D1 induces expression of LXRα and its downstream target genes as well as transcriptional function of LXRα and LXRβ

(A) HepG2 cells were transfected with the indicated amount of expression vector encoding human NR1D1 for 24h. The expression of proteins were analyzed by western blotting.

(B) HepG2 cells were treated with 10 μM or 20 μM GSK4112 for 24h. The expression of proteins were analyzed by western blotting.

(C) CV1 cells were transfected with Gal4-tk-Luc, pGal4-LXRα or pGal4-LXRβ, together with empty vector (E), or 10, 50, or 100 ng expression vector for NR1D1. After transfection, the cells were treated with 0.1 μM TO901317 (T17) for 24 h. The β-galactosidase activity was used to normalize luciferase activity. Data represent the means ± SEM (n=3). *P< 0.05 compared with vehicle or T17 treatment with empty vector.

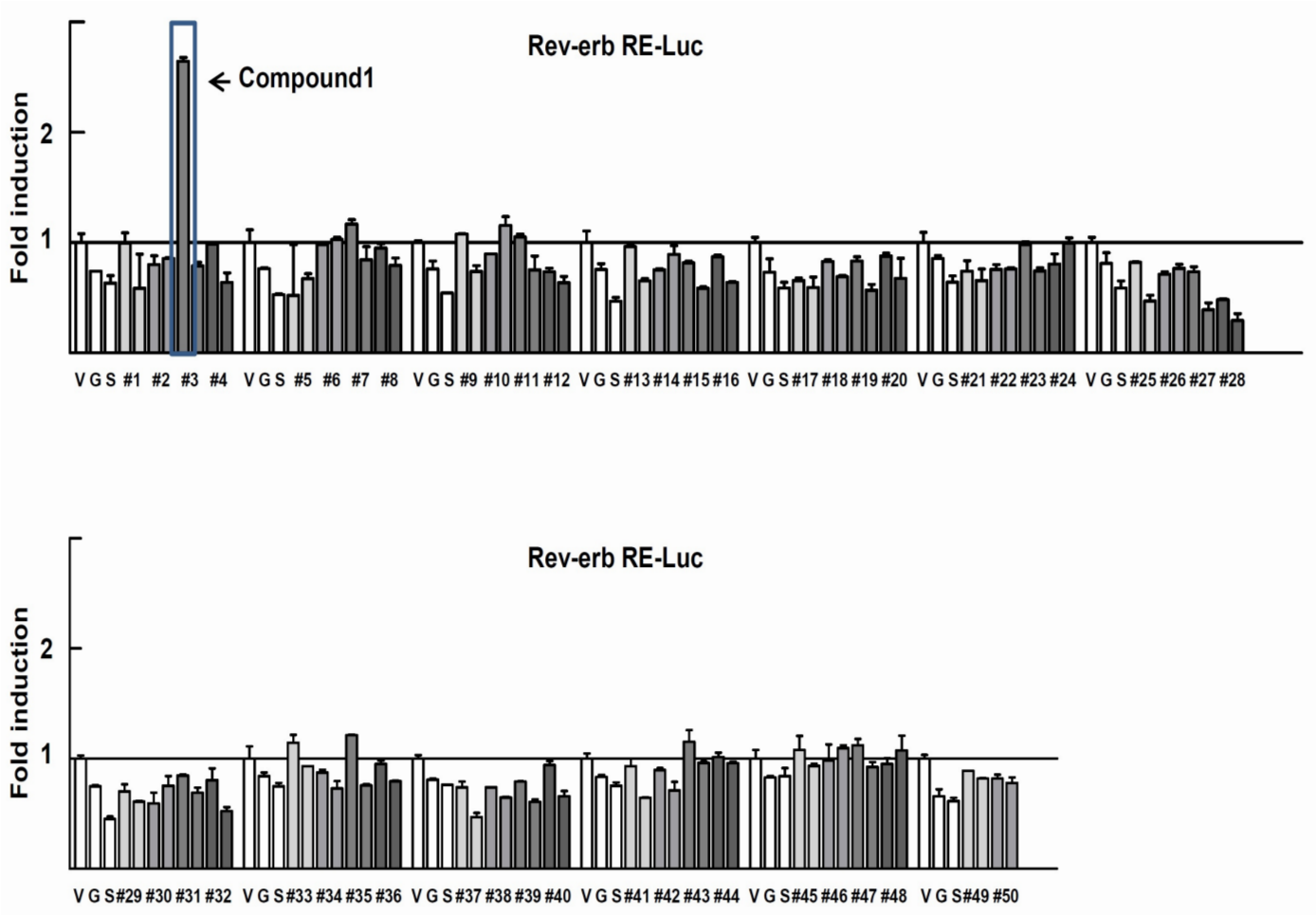

Rev-erb RE-Luc
Compound1
Fold induction
2
1
V G S #1 #2 #3 #4 V G S #5 #6 #7 #8 V G S #9 #10 #11 #12 V G S #13 #14 #15 #16 V G S #17 #18 #19 #20 V G S #21 #22 #23 #24 V G S #25 #26 #27 #28
Rev-erb RE-Luc
Fold induction
2
1
V G S #29 #30 #31 #32 V G S #33 #34 #35 #36 V G S #37 #38 #39 #40 V G S #41 #42 #43 #44 V G S #45 #46 #47 #48 V G S #49 #50

Figure 12. Analysis of NR1D1 activity for SR9009 modified compounds

CV1 cells were transfected with Rev-erb response element (Rev-erbRE) luciferase reporter and expression vector encoding human NR1D1 together. After 4h of transfection, the cells were treated with vehicle(V), 10 μM GSK4112(G), 10 μM SR9009(S), or 1 μM or 10 μM compounds for 24h. The β-galactosidase activity was used to normalize luciferase activity.

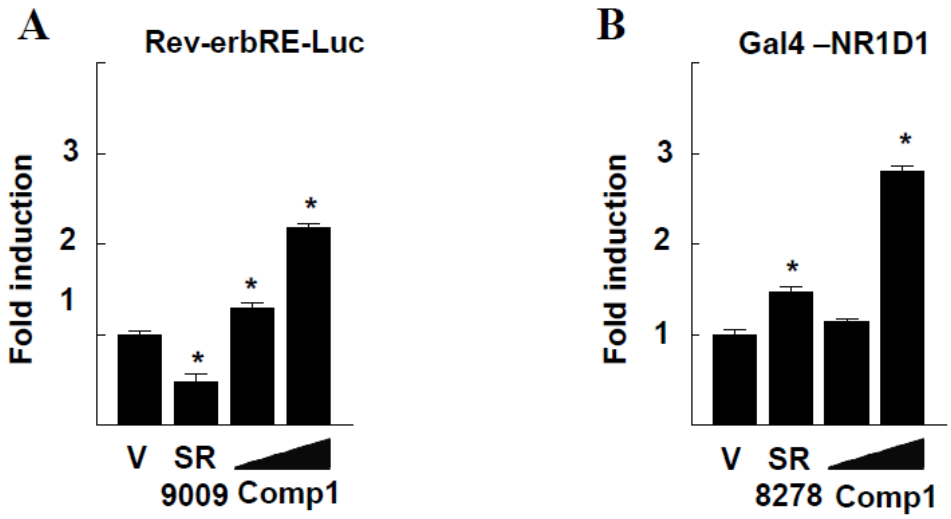


Figure 13. Compound 1 antagonizes transcriptional function of NR1D1

(A) CV1 cells were transfected with Rev-erbRE-Luc and expression vector encoding human NR1D1 together. After 4h of transfection, the cells were treated with vehicle, 10 μM SR9009 or 0.1 μM or 0.5 μM Compound1 for 24h. The β-galactosidase activity was used to normalize luciferase activity. (B) CV1 cells were transfected with Gal4-tk-Luc and pGal4-NR1D1 together After 4h of transfection, the cells were treated with vehicle, 10 μM SR8278 or 0.1 μM or 0.5 μM Compound1 for 24h. The β-galactosidase activity was used to normalize luciferase activity. Data represent the means ± SEM (n=3). *P< 0.05 compared with vehicle control.

8. The Compound1, NR1D1 suppressor, inhibits LXR signaling

After finding the Compound1 which antagonizes transcriptional activity of NR1D1, we examined whether Compound1 could inhibit the function of LXRs in HepG2 cells. Treatment of Compound1 inhibited the TO901317-induced transcriptional activity of both LXRα and LXR β in a dose-dependent manner (Figure 14 A). Western blot analysis showed that Compound1 decreased the basal and TO901317-induced protein levels of LXRs and its downstream target genes (Figure 14B and C). These results suggested that Compound1 which has an antagonizing effect of NR1D1 suppresses lipid metabolism in liver.

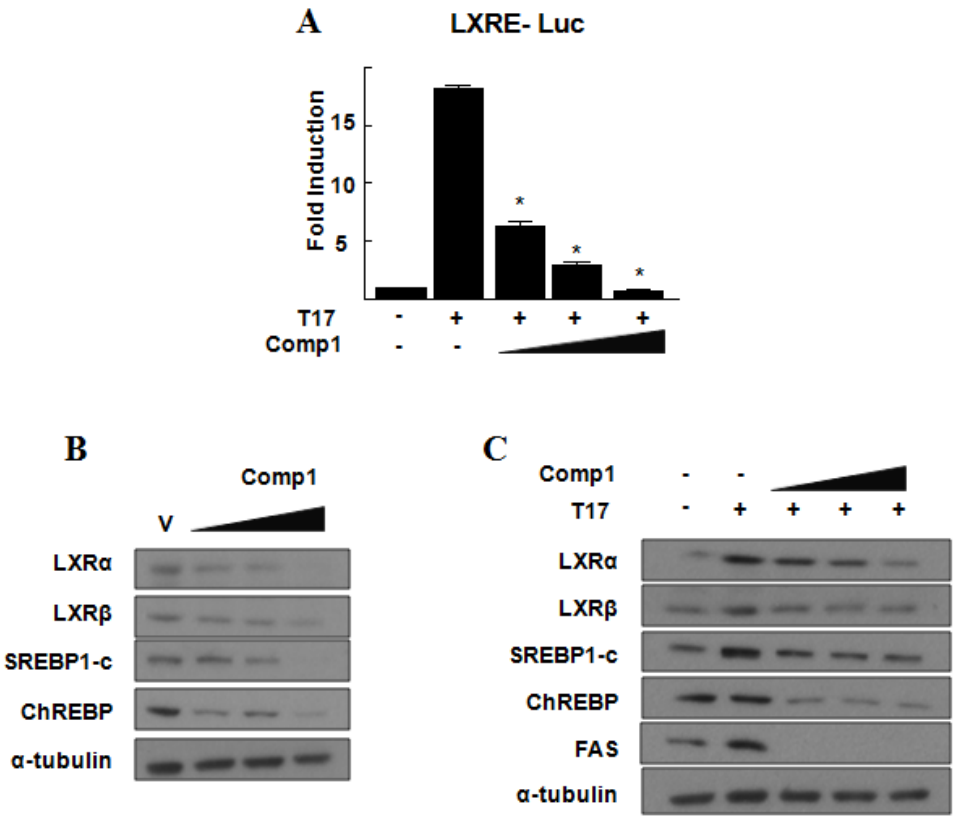

A
LXRE- Luc
Fold Induction
15
10
5
*
*
*
T17
Comp1
B
Comp1
V
LXRα
LXRβ
SREBP1-c
ChREBP
α-tubulin
C
Comp1
T17
LXRα
LXRβ
SREBP1-c
ChREBP
FAS
α-tubulin

Figure 14. Compound1 inhibits LXR-mediated transcriptional activity and expression of genes involved in lipid metabolism

(A) HepG2 cells were transfected with LXRE-Luc. After transfection, the cells were treated with vehicle or 1 μM T0901317, together with vehicle or 0.05, 0.1, or 0.5 μM Compound1 for 24h. The β-galactosidase activity was used to normalize luciferase activity. Data represent the means ± SEM (n=3). *P< 0.05 compared with only T17 treatment.

(B) HepG2 cells were treated with vehicle or 0.05, 0.1, or 0.5 μM Compound1 for 24h. The expression of proteins were measured by western blotting.

(C) HepG2 cells were treated with vehicle or 1 μM TO901317, together with vehicle or 0.05, 0.1, 0.5 μM Compound1 for 24h. The expression of proteins were measured by western blotting.

V. DISCUSSION

LXRs are well known for their role as integrators to regulate the metabolism associated with lipid, cholesterol and glucose homeostasis. LXRs also control a variety of pathways related to development, differentiation, reproduction, immunity and inflammation. (Zelcer *et al.*, 2006) Because of these functions, LXRs have been suggested as potential therapeutic targets for diverse diseases such as lipid disorders, atherosclerosis, chronic inflammation, autoimmunity, cancer and neurodegenerative diseases. (Hong et al., 2014; Komati et al., 2017) Therefore, the insights of modulating LXR signaling are highlighted as targeting strategies for treating diseases in the future. In this study, we sought to identify modulators of LXR signaling and to elucidate the molecular mechanisms involved.

In skin, activation of LXRs by TO901317 and oxysterol enhances epidermal barrier repair by controlling inflammation, differentiation, water transport and lipid metabolism. (Kömüves et al., 2002) Recently, we found that Raffinose, an oligosaccharide ubiquitously presented in plants, activates LXRs transcriptional activity. Raffinose has been proposed as cosmetic ingredients that promote the formation of lamellar structures, which leads to recovery of skin barrier function. (Sakurai *et al.*, 2005) However, the role and molecular mechanism of Raffinose associated with LXR signaling is unknown. Here, we showed the newly discovered function and mechanism of Raffinose

action related to LXR signaling in keratinocyte. Firstly, Raffinose increased mRNA levels of keratinocyte differentiation and lipid metabolism related genes in HaCaT cells. It was also confirmed that the expression of the genes involved in lipid metabolism and keratinocyte differentiation in the epidermis was increased by treatment with Raffinose in Hairless mice. However, these effects of Raffinose did not act as a ligand of LXRs. We focused on activation loops of LXRs and AP-1. AP-1 family as transcription factors regulate expression of genes associated with apoptosis, proliferation, development and differentiation in epidermis. (Hess et al., 2004) Commonly, activity of AP-1 family is regulated by post-translational modification, DNA binding dimer composition and interaction with other proteins. (Vesely *et al.*, 2009) Previous studies have suggested that activation of LXRs by ligands increases the expression and DNA binding of AP-1 on the promoters of Involucrin, leading to keratinocyte differentiation. (Schmuth et al., 2004; Hanley et al., 2000) Raffinose also upregulated the expression of the AP-1 family, particularly JunD and Fra1, and increased the DNA binding of c-Jun to AP-1 motifs present on Involucrin and Loricrin promoter. Interestingly, LXRs also bound to AP-1 motif by treatment of Raffinose. Our related studies showed that this cross-talk was also found in the LXR response element in the ABCA1, ABCG1 and LXRα promoters, indicating that a positive cross-talk between LXRs and AP-1 was involved in action of Raffinose. (Na et al., 2017) Previous bioinformatics analysis of genome-wide cistrome showed that about

80% of LXRβ/RXRα binding sites contain AP-1 motif in their vicinity, which supports the correlation between LXRs and AP-1. (Shen et al., 2011) JunD and Fra1 induced transcriptional activity of LXRs and affected expression of LXR downstream target genes. Overall, this newly identified transactivation mechanism of LXRs and AP-1 by Raffinose seems to play an important role in the LXR mediated lipid metabolism and differentiation in skin.

In liver, NR1D1, also called Rev-erbα, is involved in a variety of hepatic physiology through the regulation of the gene expression.. NR1D1 regulates plasma triglyceride homeostasis by repressing expression of ApoCⅢ and glucose homeostasis by inhibiting expression of glucose 6-phosphatase. NR1D1 also controls bile acid metabolism by participating in the circadian regulation of sterol regulatory element-binding protein (SREBP) activity. (Le Martelot et al., 2009; Ramakrishnan et al., 2006) In previous studies on the correlation between NR1D1 and LXRs in lipid metabolism, it has been reported that LXRs regulate transcriptional expression of NR1D1 in macrophage. (Fontaine et al., 2008) However, there is no known effect of NR1D1 on LXR signaling in relation to lipid metabolism. Here, we showed that overexpression of NR1D1 and NR1D1 agonist, GSK4112, induced expression of LXRα and SREBP1-c. NR1D1 also activates transcriptional activity of both LXRα and LXRβ. Non-alcoholic fatty liver disease (NAFLD) is one of the types of fatty liver that is formed by the accumulation of fat in the liver due to factors other than alcohol. As a strategy for treating NAFLD using LXR signaling,

the inhibition of LXRs activity by inverse agonist has been proposed as a therapeutic method by repressing liver fat accumulation. (Griffett et al., 2015; Huang et al., 2013) We thought that compounds that modulate NR1D1 activity have the potential to control LXR signaling and contribute to treating NAFLD. Thus, we performed screening to find a compound that regulates the transcriptional activity of NR1D1, and found Compound 1, which inhibits the activity of NR1D1. This Compound 1 inhibited the transcriptional activity of LXRs as expected and inhibited the expression of the LXR downstream genes. Taken together, these results indicate that NR1D1 modulation affects LXR signaling, suggesting that NR1D1 is involved in LXR mediated lipid metabolism.

In conclusion, we present AP-1 and NR1D1 as LXR signaling modulators and discussed the effects of their interaction with LXRs in skin and liver (Figure 15). However, further studies are required to determine whether changes in LXR signaling by these modulators are applicable to other tissues and diseases.

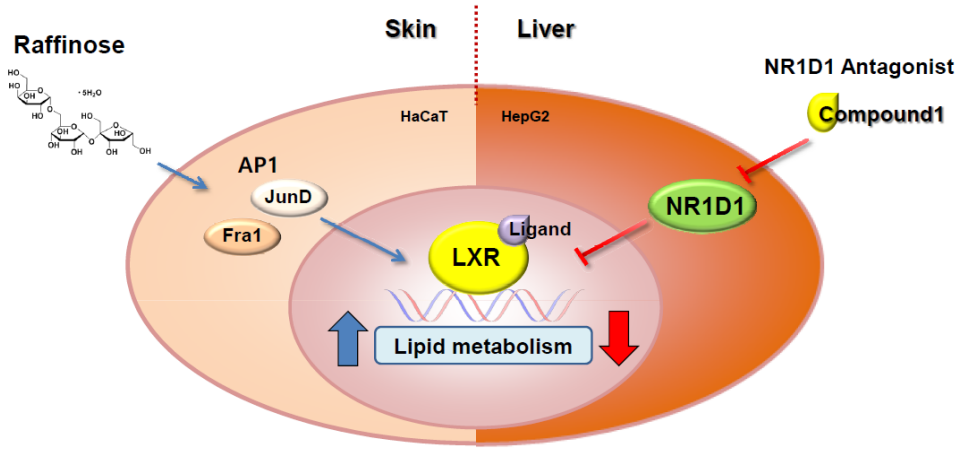


Figure 15. Schematic illustration of regulation of LXR mediated lipid metabolism by AP-1 and NR1D1 in skin and liver.

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국 문 초 록

Liver X receptors (LXRs)은 여러 조직 내에 발현하여 대표적으로 지질, 포도당, 콜레스테롤 등 인체의 신진대사 항상성 유지에 중요한 역할을 하는 핵 수용체이다. LXR은 지질 대사에 의해 유발되는 다양한 병리학적 질환의 치료적 타겟으로서 제시 되어 왔으며, LXR의 활성을 조절하는 조절자의 발견과 그 기전에 대한 연구는 계속적으로 진행되어 왔다. 이번 연구에서도 우리는 피부와 간에서 LXR 활성을 조절하는 조절자와 그 분자적 기전을 밝혀내는 것을 목표로 삼았다. 우리는 피부와 간에서 LXR의 활성을 조절하는 두 가지 사례를 제시하였다. 첫째로, 올리고당의 한 종류 인 Raffinose가 각질형성세포에서 LXR의 전사 활성을 유도하며, 지질 대사, 보습, 분화와 관련된 유전자의 발현을 증가시킴을 in vitro 실험뿐만 아니라 동물 모델을 통해서 확인하였다. 하지만, Raffinose의 이러한 효과가 LXR의 리간드로서 나타나는 결과가 아니라는 사실을 알게 되었다. 본 연구진은 Raffinose에 의한 LXR 활성 조절기전을 밝히기 위해 각질 세포의 생존과 분화에 중요한 역할을 하는 Activator proteins (AP-1)과 LXR 간의 상관 관계에 초점을 맞추었다. Raffonose와 LXR 합성 리간드 인 TO901317은 JunD와 Fra1의 발현을 유도하였고, Involucrin과 Loricrin의 조절 프로모터상에 있는 AP-1 motif에 c-Jun의 DNA 결합이 증가됨을 확인하였다. 흥미롭게도 LXR은 또한 Raffinose와 TO901317처리에 의하여 AP-1 motif에 결합이 증가하였다. AP-1이 LXR 신호전달에 미치는 영향을 확인한 결과 JunD와 Fra1이 LXR의 전사 활성을 증가시키며, LXR 매개 지질 대사 관련 유전자의 발현 또한 증가시킨다는 것을 관찰하였다. 결론적으로

첫번째 사례에서 본 연구진은 Raffinose에 의하여 유도된 AP-1 family (c-Jun, JunD와 Fra1)이 LXR의 신호전달을 활성화 시키고, 또한 각질세포 분화를 유도한다는 것을 확인하였다. 두 번째 사례는 물질대사가 활발한 조직에 높은 발현하는 NR1D1이 간에서 LXR 신호 전달에 미치는 영향을 규명하고자 하였다. NR1D1의 과 발현과 NR1D1의 agonist인 GSK4112의 처리가 LXRα와 SREBP1-c의 발현을 증가시켰다. 또한 NR1D1은 LXRα와 LXRβ의 전사 활성을 강화시킨다는 것을 관찰하였다. 본 연구진은 이 결과를 바탕으로 새로운 NR1D1 활성 조절하는 화합물을 찾고 LXR 신호전달에 미치는 영향을 확인하고자 하였다. 우리는 스크리닝을 통하여 NR1D1의 전사활성을 저해하는 Compound1을 찾았다. 간암 세포주인 HepG2에서 Compound1의 처리는 리간드에 의한 LXR 매개 전사활성을 저해하였고, LXRα와 그 하위 유전자의 발현을 저해함을 관찰하였다. 두 번째 사례에서는 NR1D1이 LXR의 공동 활성 조절자로서 작용하며, NR1D1의 전사활성을 저해하는 새로운 Compound1의 발견은 LXR 매개 지질 대사를 저해 할수 있는 가능성을 제시하였다. 이 두 가지 사례를 종합하여, 본 연구진은 각 피부와 간에서 AP-1과 NR1D1이 LXR의 공동 활성 조절자로서 지질 대사를 조절한다는 것을 제시하였다. 이는 다양한 질환 모델에서 LXR을 치료적 타겟으로 이용한 치료적 전략을 넓힐 수 있는 가능성을 제시할 수 있었다.

주요어: LXR, AP-1, NR1D1, 지질 대사, 각질 세포 분화