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ORIGINAL ARTICLE

Exploring compounds to be used as cosmetic agents that activate peroxisome proliferator-activated receptor alpha

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Abstract

Objective: The human epidermis is formed by the proliferation and differentiation of keratinocytes adjacent to the basement membrane. The outermost layer, the stratum corneum, is equipped with a barrier function that prevents water evaporation, and intercellular lipids play an important role in this barrier function. When the barrier is functioning normally, evaporation is prevented; however, when barrier function is impaired, moisture evaporates, resulting in dry and rough skin. Therefore, maintenance of normal barrier function is critical for maintaining normal skin function. Peroxisome proliferator-activated receptor α (PPARα) is mainly not only involved in lipid metabolism in the liver but is also expressed in the epidermis and is involved in inducing keratinocyte differentiation, promoting lipid production, maintaining barrier function and suppressing skin inflammation. Hence, compounds that activate PPARα are expected to control skin function. Therefore, we identified PPARα activators from among extracts of natural resources that have been approved for use in humans and analysed the effects of these extracts on skin function.

Methods: First, extracts of 474 natural resources were screened using a PPARα activator screening cell line independently constructed in our laboratory. Next, reporter assays were performed using the Gal4-chimera system to evaluate whether these extracts act as ligands for PPARα. We then analysed their effect on primary normal human epidermal keratinocyte cells by using real-time RT-PCR. Finally, we evaluated PPARα activation effect by the combination of these extracts.

Results: We identified 36 extracts having the effect of activating PPARα. In particular, #419, a *Typha angustifolia* spike extract, showed concentration-dependent transcriptional activation through PPARα-LBD and was considered to be likely to contain a compound that is a ligand of PPARα. #419 increased the expression of PPARα target genes and genes related to skin function in primary cultured human epidermal keratinocytes. Finally, the use of #419 in combination with nine extracts increased PPAR activity more than twice as much as #419 alone treatment.

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Conclusions: These results showed that the reporter cell line could be useful for discovering extracts of natural resources and that the identified *Typha angustifolia* spike extract could be used in cosmetics that activate PPARα, which expected to improve skin function.

KEYWORDS

cosmetic materials, genetic analysis, keratinocyte, PPARα, skin barrier, skin physiology, *Typha angustifolia*

Résumé

Objectif: L'épiderme humain se forme grâce à la prolifération et à la différenciation des kératinocytes adjacents à la membrane basale. La couche externe, dite « couche cornée », possède une fonction barrière qui empêche l'évaporation de l'eau, dans laquelle les lipides intercellulaires jouent un rôle important. Lorsque la barrière fonctionne normalement, l'évaporation est évitée ; mais lorsqu'elle est altérée, l'évaporation a lieu et la peau, privée d'hydratation, devient sèche et rêche. Par conséquent, il est capital de maintenir cette fonction barrière normale pour que la peau conserve son fonctionnement normal. Le récepteur alpha activé par prolifératurs de peroxysomes (PPARα) intervient surtout non seulement dans le métabolisme lipidique du foie, mais également dans l'épiderme ; il joue en effet un rôle dans l'induction de la différenciation des kératinocytes, la promotion de la production lipidique, le maintien de la fonction barrière et la suppression de l'inflammation de l'épiderme. Par conséquent, les activateurs du PPAR-α devraient être déterminants pour une bonne fonction cutanée. Nous avons donc identifié des activateurs du PPAR-α parmi des extraits de ressources naturelles dont l'utilisation chez l'homme est approuvée, et nous avons analysé les effets de ces extraits sur la fonction cutanée.
Méthodes: Tout d'abord, des extraits de 474 ressources naturelles ont été sélectionnés à l'aide d'une lignée cellulaire de détection des activateurs du PPAR-α, construite indépendamment dans notre laboratoire. Ensuite, des tests de gènes rapporteurs ont été effectués à l'aide du système Gal4-chimera pour voir si ces extraits jouaient le rôle de ligands pour le PPAR-α. Nous avons ensuite analysé leur effet sur les cellules kératinocytaires épidermiques humaines normales primaires par RT-PCR en temps réel. Enfin, nous avons évalué l'effet d'activation du PPAR-α par l'association de ces extraits.
Résultats: Nous avons identifié 36 extraits ayant pour effet d'activer le PPAR-α. En particulier, le n° 419, un extrait d'épi de *Typha angustifolia*, a montré une activation transcriptionnelle dépendante de la concentration par le PPAR-α-LBD et a été considéré comme susceptible de contenir un composé qui est un ligand du PPAR-α. Le n° 419 a augmenté l'expression des gènes cibles du PPAR-α et des gènes liés au fonctionnement de la peau dans les kératinocytes épidermiques humains primaires mis en culture. Enfin, l'utilisation du n° 419 en association avec neuf extraits a augmenté de plus du double l'activité du PPAR par rapport au traitement par le n° 419 seul.
Conclusions: Ces résultats ont montré que la lignée cellulaire rapporteuse pourrait être utile pour découvrir des extraits de ressources naturelles et que l'extrait d'épi de *Typha angustifolia* identifié pourrait être utilisé dans des cosmétiques qui activent le PPAR-α, ce qui devrait améliorer la fonction cutanée.

INTRODUCTION

The human epidermis is composed of four layers: the stratum basale, stratum spinosum, stratum granulosum and stratum corneum, in order from the deepest to the most superficial. These layers are formed by the migration of keratinocytes adjacent to the basement membrane into the outer layer during differentiation and maturation [1]. The outermost layer, the stratum corneum, functions as a barrier to prevent water evaporation [2], and intercellular lipids composed of ceramides and cholesterol play an important role in this barrier function [3–6]. When the barrier is functioning normally, evaporation is prevented; however, when barrier function is impaired, moisture is evaporated and the skin becomes dry and rough. Therefore, normal barrier function is crucial for maintaining normal skin function [7,8]. Thus, in order to maintain the health of the skin, the use of cosmetic materials that can maintain the homeostasis of the skin barrier function is attracting attention.

Peroxisome proliferator-activated receptor (PPAR) is a nuclear receptor that controls transcription in a ligand-dependent manner, and there are three subtypes—PPARα, δ and γ—in mammals [9]. PPAR heterodimerizes with the retinoid X receptor α (RXRα), which takes 9-cis-retinoic acid as a ligand; PPAR/RXRα heterodimer binds to PPAR responsive element (PPRE) with a motif consisting of 5′-AGGTCA-3′ aligned one base apart in the same direction [10–14]. PPARα is mainly involved in lipid metabolism in the liver, but it is also known to be involved in inducing human keratinocyte differentiation, promoting lipid production, maintaining barrier function and suppressing skin inflammation [15–18]. In the epidermis, PPARα activation induces keratinocyte differentiation; elevates the expression of involucrin, filaggrin, loricrin and other substances composing the stratum corneum; stimulates lipid production; coordinates the profile of fatty acids and lipids, such as ceramide and cholesterol sulphate; and may maintain skin function homeostasis [16,19–21]. Indeed, it has been reported that activation of PPARα in a mouse model of skin barrier disruption exhibits a restorative effect on skin barrier function by increasing lipid production, lamellar body secretion and intercellular lipid levels [19,20,22]. In addition, PPARα has been reported to exert anti-inflammatory effects in skin conditions, such as irritant and allergic contact dermatitis and UV-induced erythema, by inhibiting the expression of inflammatory cytokines, such as IL-6, suppressing catalase-mediated ROS production and other effects [23–26]. Based on such findings, it is thought that compounds that activate PPARα can be used as materials for functional cosmetics. However, due to regulatory limitations, it is difficult to develop skin care cosmetics containing PPAR activators used as pharmaceuticals such as fibrates [27].

We previously developed a cell-based PPARα activator screening system that can control human PPARα expression using a tetracycline induction system for conveniently and rapidly measuring the transcriptional activation ability of PPARα [28,29]. Therefore, in this study, extracts of 474 natural resources were screened for the ability to activate PPARα, and their effects on skin function were evaluated.

MATERIALS AND METHODS

Reagents

GW7647, GW6471 and T0901317 were purchased from Sigma-Aldrich, 9-cis-retinoic acid was purchased from Fujifilm Wako Pure Chemical Corp., and rosiglitazone was purchased from Alexis Biochemicals. GW501516 was synthesized as described previously [30].

Extract preparation of natural resources

Extracts of natural resources (474 species in total), mainly from plants, were used. Dried resources were extracted with water, ethanol, 1,3-butylene glycol or a mixture of these according to the cosmetic raw material standards for each resource, and were used as samples.

Cell culture

HepG2 human hepatoblastoma cells were cultured in Dulbecco's modified Eagle medium (DMEM; Nacalai Tesque) containing 10% foetal bovine serum (FBS; Biowest), 100 IU/ml penicillin and 100 μg/ml streptomycin (Nacalai Tesque). HepG2-tet-off-hPPARα-Luc cells [29] were cultured in DMEM containing 10% FBS, 2 μg/ml tetracycline (Wako Pure Chemical), 0.5 μg/ml puromycin (Sigma-Aldrich), 300 μg/ml G418 (Nacalai Tesque), 2 μg/ml blasticidin S (Kaken Pharmaceutical), 100 IU/ml penicillin and 100 μg/ml streptomycin. For ligand treatment, cells were cultured in DMEM supplemented with 10% charcoal/dextran-treated FBS (Thermo Scientific). Primary NHEK cells (PromoCell) were cultured in Keratinocyte Growth Medium 2 (PromoCell).

Luciferase assays using a human PPARα reporter cell line

HepG2-tet-off-hPPARα-Luc cells (4×10^4 cells/well) were seeded in 96-well plates and incubated in DMEM supplemented with 10% charcoal dextran-treated FBS

with or without 2 μg/ml tetracycline. The cells were treated with various concentrations of test samples. Firefly luciferase activity was quantified using a luciferase assay system (Promega) and a luminometer (Berthold Technologies). To evaluate the specificity of test samples for PPARα, luciferase activity levels were determined in cells cultured in (Tet−) medium and divided by those observed in cells cultured in (Tet+) medium as described previously [29].

Gal4-chimera reporter gene assay

Luciferase assays were performed as described previously [29]. Briefly, HepG2 cells were transfected using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's instructions. HepG2 cells (3×10^4 cells/well) were seeded in 96-well plates and incubated in DMEM supplemented with 10% charcoal dextran-treated FBS. The cells were transfected with 100 ng 4xUAS-tk-Luc (a reporter plasmid regulated by GAL4 fusion protein) and either 10 ng pBIND-hPPARα-LBD, pBIND-hPPARβ/δ-LBD, pBIND-hPPARγ1-LBD, pBIND-hRXRα-LBD, pBIND-hLXRα or pBIND-hLXRβ expression vector (expression plasmids for GAL4-hPPAR, hRXR or hLXR chimera protein) [29,31]. Then, the cells were treated with various concentrations of the test samples. To block PPARα, cells were pretreated with GW6471, a PPARα antagonist, for 1 h before adding the test samples. After 24 h, both firefly and *Renilla* luciferase activities were quantified using the Dual-Luciferase® reporter assay system (Promega) on a luminometer.

TABLE 1 Primers used for real-time PCR

Gene	Sequence
Beta 2 microglobulin	
For. primer:	TATCCAGCGTACTCCAAAGA
Rev. primer:	GACAAGTCTGAATGCTCCAC
PPIA	
For. primer:	GCGTCTCCTTTGAGCTGTTT
Rev. primer:	TCACCACCCTGACACATAAACC
PPARα	
For. primer:	CTATCATTTGCTGTGGAGATCG
Rev. primer:	AAGATATCGTCCGGGTGGTT
PDK4	
For. primer:	TTCCAGACCAACCAATTCACA
Rev. primer:	CCTGGTGTTCAACTGTTGCC
PLIN2	
For. primer:	TGAGATGGCAGAGAACGGTGTG
Rev. primer:	GGCATTGGCAACAATCTGAGT
AQP3	
For. primer:	GATCAAGCTGCCCATCTACAC
Rev. primer:	CCAGAGGGGTAGGTAGCAAAG
HAS3	
For. primer:	ACTACATCCAGGTGTGCGACT
Rev. primer:	CCAAGGGCCCACTAATACACT
SULT2B1b	
For. primer:	TCATCACCTACCCCAAGTCAG
Rev. primer:	AAGATCTGGATGGGAAGATGG

Note: Sequences of forward (For.) and reverse (Rev.) primers for each target are shown. Sequences are 5′ to 3′.

RNA extraction and quantitative real-time RT-PCR

The total RNA samples were isolated from the cells using the QuickGene RNA cultured cell HC kit S (KURABO) according to the manufacturer's instructions. First-strand cDNA was synthesized from total RNA of each cell sample using the SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen). The cDNAs were used as templates for individual PCR reactions using specific primer sets (Table 1). PCR reactions were carried out using QuantiTect™ SYBR® Green PCR Kit (Qiagen). Quantitative PCR analysis was performed using the CFX384 Touch Real-Time PCR Detection System (Bio-Rad Laboratories). Beta 2 microglobulin was used to normalize each expression data set.

Statistical analysis

Statistical analyses were performed using an unpaired Student's *t*-test with GrpahPad Prism 9.

RESULTS

Screening extracts of natural resources that activate PPARα

To identify extracts of natural resources controlling skin function, we used a previously constructed human PPARα activator screening cell line [29]. This cell line was established using a tetracycline (Tet) induction system (Tet-off system), which can control PPARα expression in the presence or absence of tetracycline in the culture medium. That is, PPARα expression is suppressed by adding tetracycline to the medium (Tet+) and induced by removing tetracycline from the medium (Tet−) [28]. Furthermore, the ability of the test extracts to activate PPARα can be conveniently evaluated by

luciferase assay, because a luciferase gene with PPRE as an enhancer has been integrated into the genome. Thus, by using this screening cell line, the specificity for PPARα can be assessed by comparing the luciferase activity when PPARα expression is induced (Tet−) or suppressed (Tet+) [29].

For screening, extracts of 474 natural resources available from those described in 'Standards for Cosmetics' (Notification No. 331 of the Ministry of Health and Welfare on September 29, 2000) were used, and screening was performed using the above-mentioned screening cell lines. In the first step, to identify potential extracts that stimulate PPARα transactivation activities, we evaluated 474 extracts using the screening cells in Tet− medium for PPARα expression. The screening cells were cultured in the absence of tetracycline, each extract was added at a final concentration of 1% in a state in which PPARα was expressed (Tet−), and luciferase activity was evaluated 24 h after addition. Consequently, 150 extracts with more than 20% activity relative to that of the positive control, 0.1 μM GW7647 (PPARα agonist), were obtained (Figure 1a). Next, to evaluate the specificity of the 150 extracts for PPARα, a second-step screening was performed with (Tet−) and without (Tet+) PPARα expression and evaluated based on the ratio of luciferase activity at the time of induction (Tet−) and suppression (Tet+). As a result, 36 extracts with more than 15% activity relative to that of GW7647 (0.1 μM) were obtained (Figure 1b). These 36 extracts showed increased activity with PPARα expression compared to when PPARα was not expressed, and they are expected to have the desired effect of activating PPARα.

We then performed reporter assays with the Gal4-chimera system to assess whether these 36 extracts act as ligands for PPARα. The Gal4-chimera system utilizes a fusion protein that contains a ligand-binding domain (LBD) of PPARα and a DNA-binding domain of Gal4, a yeast transcription factor. This plasmid and a reporter plasmid with luciferase downstream of a Gal4 response element are co-transfected into HepG2 cells. Next, samples that are to be tested for responsiveness to the PPARα LBD are preprocessed and then subjected to luciferase assays [29]. Using this system, the luciferase activities were measured when adding one of the 36 extracts at a final concentration of 1%. As a result, more than five times, the activity of vehicle treatment was observed for two extracts: #272 and #419 (Figure 1c). Thus, the screened extracts #272 and #419 might act on the PPARα LBD to activate PPARα.

Analysis of the effects of screened extracts on nuclear receptors

Since it was shown that #272 and #419 may contain compounds that activate PPARα, we conducted more detailed analyses. To evaluate the specificity of #272 and #419 for PPARα, the activity against each PPAR subtype was evaluated. As a result, PPARα, as well as PPARγ and PPARδ, were activated by #272, whereas #419 activated only PPARα (Table 2). In addition, the effects on other nuclear receptors were evaluated [32,33]. It was determined that #272 also had activating effects on RXRα, liver X receptor α (LXRα) and LXRβ, whereas #419 did not activate them, indicating PPARα-specific activation (Table 2).

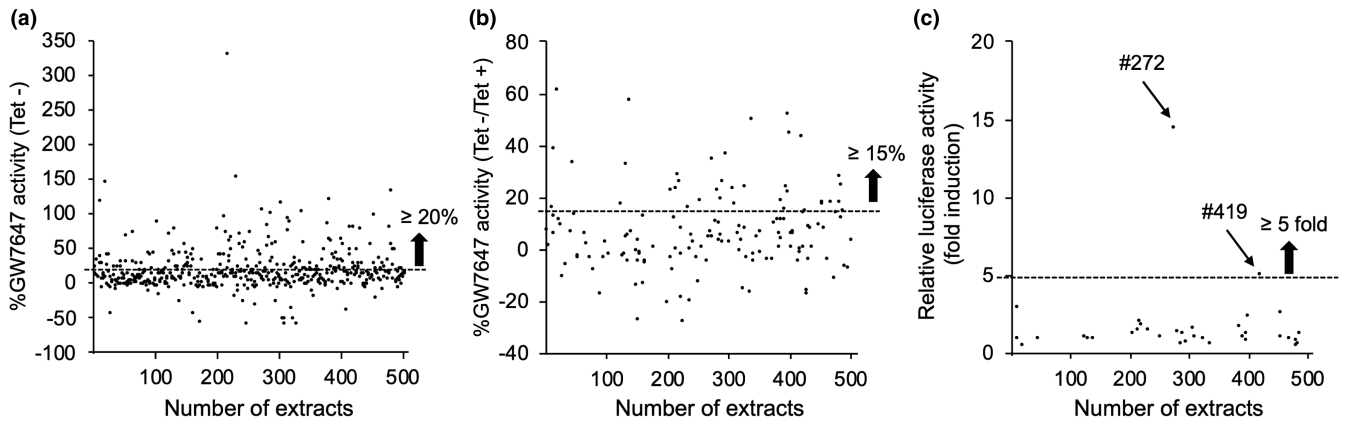


FIGURE 1 Screening of extracts of natural resources for PPARα activators. (a) HepG2-tet-off-hPPARα-Luc cells cultured in the absence of tetracycline (Tet−) were incubated with 0.1 μM GW7647 (100% control) or one of 474 extracts (1% final concentration each) for 24 h. Luciferase activity in each well was measured. (b) HepG2-tet-off-hPPARα-Luc cells cultured in the Tet+ or Tet− medium were treated with 0.1 μM GW7647 (100% control) or one of 150 extracts (1% final concentration each) for 24 h. The cells were used for reporter gene assays. (c) HepG2 cells were co-transfected with 4xUAS-tk-luc and pBIND-hPPARα. Transfected cells were treated with one of 36 extracts (1% final concentration each) for 24 h. The cells were used for reporter gene assays. Luciferase activity from reporter plasmids was normalized to *Renilla* luciferase activity. Values are expressed as fold induction of the vehicle set as 1

TABLE 2 Transcriptional activation of GAL4-nuclear receptors attributable to extracts #272 and #419

	Ligands		#272		#419	
	Fold induction (vs. vehicle)		Fold induction (vs. vehicle)		Fold induction (vs. vehicle)	
NR	Mean	SE	Mean	SE	Mean	SE
PPARα	64.97	7.15	22.57	4.21	2.99	0.27
PPARγ	54.44	3.46	17.36	2.92	1.37	0.06
PPARδ	172.36	4.69	8.71	0.16	0.85	0.09
RXRα	6.69	1.78	6.84	1.71	0.71	0.10
LXRα	514.70	32.75	9.28	0.92	0.71	0.10
LXRβ	39.35	5.25	3.38	0.98	0.45	0.11

Note: HepG2 cells were co-transfected with 4xUAS-tk-luc and pBIND-hPPARα, pBIND-hPPARγ, pBIND-hPPARδ, pBIND-hRXRα, pBIND-hLXRα or pBIND-hLXRβ. Transfected cells were treated with 1% of hit samples #272 and #419 or their ligands, 0.1 μM GW7647, 0.1 μM GW501516, 10 μM rosiglitazone, 10 μM 9-cis-RA or 1 μM T0901317 for 24 h. Then, the cells were used for reporter gene assays. Luciferase activity from reporter plasmids was normalized to *Renilla* luciferase activity. Values are expressed as fold induction compared to the vehicle set at 1. Individual values are shown ($n = 3–5$).

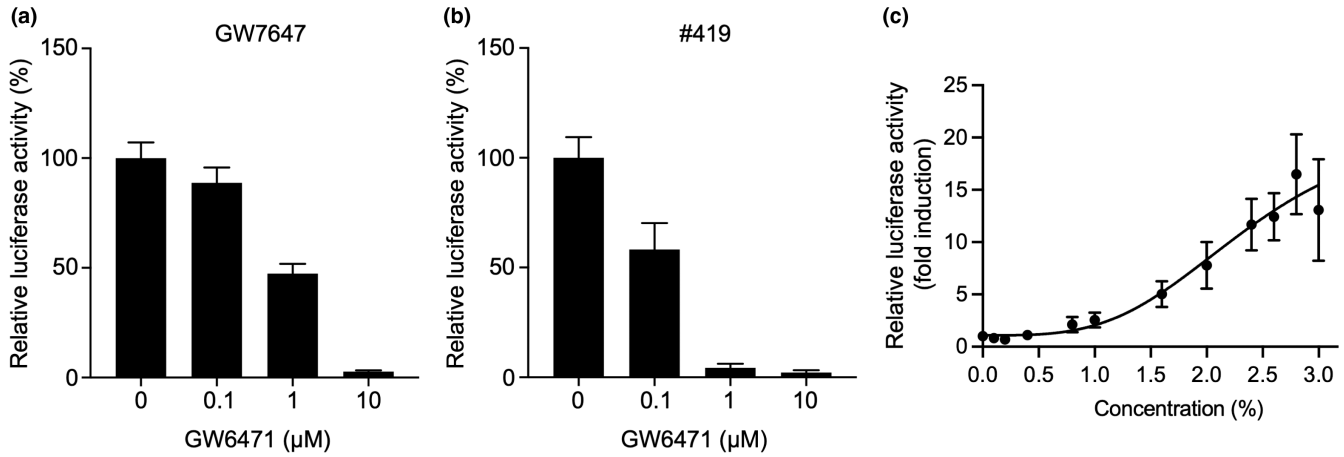


FIGURE 2 Transcriptional activation of luciferase activity by extract #419. HepG2 cells were co-transfected with 4xUAS-tk-luc and pBIND-hPPARα. (a, b) Transfected cells were pretreated with GW6471, a PPARα antagonist, for 1 h and then treated with 0.1 μM GW7647 (a) or 1% extract #419 (b) for 24 h. Luciferase activities from reporter plasmids were normalized to *Renilla* luciferase activity. Percent inhibition values were calculated based on the absence of GW6471. Values represent the means ± SE ($n = 3$). (c) Transfected cells were treated with various concentrations of extract #419 for 24 h and used for reporter gene assays. Luciferase activity from reporter plasmids was normalized to *Renilla* luciferase activity. Values are expressed as fold induction relative to the vehicle set at 1. Values represent means ± SE ($n = 5$)

Therefore, we proceeded with the analysis of #419, which was expected to be a PPARα-specific agonist. Next, we used the Gal4-chimera system to evaluate whether PPARα activation by #419 was suppressed by GW6471, an antagonist of PPARα. GW6471 suppressed both 1% #419 and PPARα ligand GW7647 (0.1 μM) in a concentration-dependent manner (Figure 2a,b). Then, the effect of the concentration of #419 on PPARα-LBD activation was evaluated. A concentration-dependent increase in PPARα-LBD activation was observed with #419 (Figure 2c). From the above, it was indicated that the extract #419 contains compounds that could be ligands of PPARα.

Analysis of the effect of the extract #419 on skin function

Since #419 was shown to have a specific activating effect on PPARα, we next analysed its effects on skin function. Primary normal human epidermal keratinocyte (NHEK) cells were treated with either #419 or GW7647 (positive control). Then, mRNA was collected, and the expression of PPARα target genes and genes related to skin function was analysed by real-time RT-PCR. Neither #419 nor GW7647 affected the expression levels of the internal standard cyclophilin A (PPIA) or PPARα (Figure 3a,b). #419 increased the expression levels of pyruvate dehydrogenase kinase 4

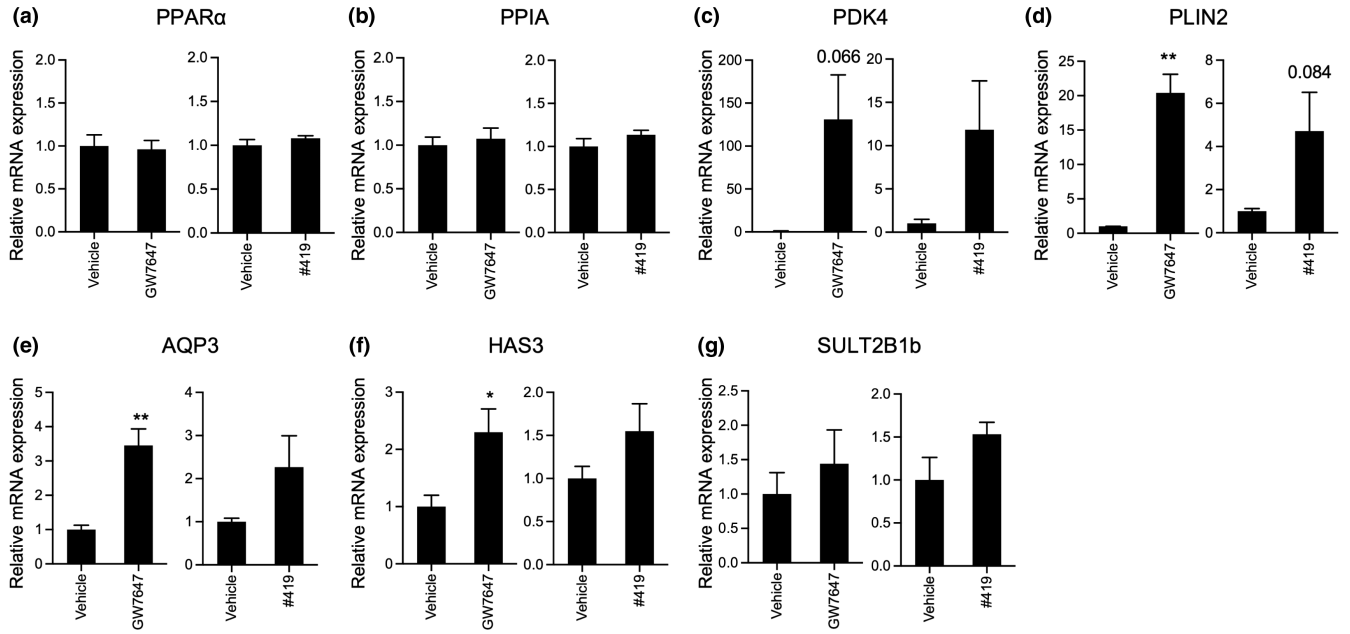
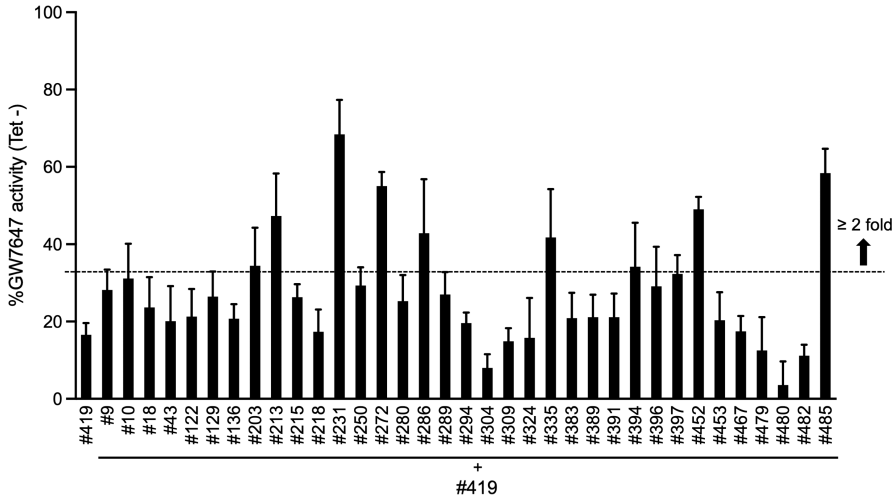


FIGURE 3 Extract #419 increases mRNA expression of hPPARα target genes and skin-related genes in NHEK cells. NHEK cells were treated with 1 μM GW7647, 0.4% extract #419 or vehicle for 72 h. Human PPARα (a), PPIA (b), PDK4 (c), PLIN2 (d), AQP3 (e), HAS3 (f) and SULT2B1b (g) mRNA levels were measured using real-time RT-PCR and normalized to beta 2 microglobulin mRNA levels relative to the vehicle set at 1. Values are expressed as means ± SE ($n = 3$–4). Significant differences between the values compared with the vehicle were determined using an unpaired Student's *t*-test (*$p < 0.05$, **$p < 0.01$)

FIGURE 4 Evaluation of transcriptional activity of extract #419 in combination with other extracts. HepG2-tet-off-hPPARα-Luc cells cultured in Tet− medium were incubated with 0.1 μM GW7647 (100% control) or extract #419 with or without 35 individual extracts (0.5% final concentration each) for 24 h. Luciferase activity in each well was measured. Values are expressed as means ± SE ($n = 3$–8)



(PDK4) [33,34] and perilipin 2 (PLIN2), also known as adipose differentiation-related protein [28], which are target genes of PPARα, as well as GW7647 (Figure 3c,d). Therefore, #419 activated PPARα in NHEK cells as expected.

Next, the effect of #419 on the expression of skin function genes was analysed. It was determined that #419 increased the expression levels of skin function-related genes (Figure 3e–g), including aquaporin 3 (AQP3), which is involved in the transport of water molecules and glycerin [35], sulfotransferase family 2B member 1 isoform b (SULT2B1b), which synthesizes the cholesterol sulphate that constitutes the intercellular lipids of the stratum corneum [36], and hyaluronan synthase 3 (HAS3) [37]. These results indicated that the extract #419 activated PPARα in keratinocytes, and the sample may affect the expression of genes involved in skin function.

Evaluation of PPARα activation effect by the combination of #419 and other extracts

These results indicate that #419 contains compounds that could act as ligands for PPARα and in keratinocytes.

TABLE 3 List of extracts activated the PPARα activity

Number	Name
203	*Achillea millefolium* extract
213	*Uncaria gambir* extract
231	*Scutellaria baicalensis* root extract
272	*Sanguisorba officinalis* root extract
286	*Rubus suavissimus* leaf extract
335	*Camellia sinensis* leaf extract
394	*Punica granatum* flower extract
419	*Typha angustifolia* spike extract
452	*Euterpe oleracea* fruit extract
485	*Rubus idaeus* juice

Although reporter assays with the Gal4-chimera system showed no activity, 35 extracts that were active in the secondary screening using the PPARα activator screening cell line that expressed the full length of PPARα might affect the transcriptional activity of PPARα through involvement in post-translational modification or its expression. In other words, it is suggested that some of the extracts selected in the secondary screening may activate PPARα at a different point of action than #419; thus, the combination of these extracts may further enhance the effects of PPARα activity. Therefore, we evaluated the activities of PPARα when #419 and the screened extract were combined.

The activating effects of the combination of #419 with 35 extracts identified in the secondary screening were evaluated by reporter assay using the PPARα activator screening cell line. The results showed that nine extracts increased activation by more than twofold when used in combination with #419 compared with #419 alone treatment (16.6% alone vs. in combination with extract #203: 34.4%, #213: 47.3%, #231: 68.4%, #272: 55.0%, #286: 42.8%, #335: 41.7%, #394: 34.2%, #452: 49.1% and #485: 58.4%; Figure 4). Therefore, by combining these extracts, further improvement of PPARα activation can be expected.

DISCUSSION

Here, we used our constructed PPARα reporter cell line [29] to evaluate the effect of extracts of natural resources on the activity of PPARα. Of 474 extracts, 36 were found to potentially contain compounds that enhance PPARα activity. In particular, #419 was considered likely to contain compounds that are ligands of PPARα, because #419 showed concentration-dependent transcriptional activation in PPARα-LBD, and the activation of which was suppressed by an antagonist of PPARα.

The compound in #419 that exerted a PPARα-activating effect was *Typha angustifolia* spike extract, which has been reported to have anti-inflammatory and antioxidant effects (Table 3) [38–40]. However, no association with PPAR has been reported so far. Our findings revealed that #419 increased the expression levels of PPARα target genes PDK4 and PLIN2 in NHEKs. PDK4 is a factor involved in energy metabolism [33,34] and is widely known as a target gene of PPARα. PLIN2 is a factor involved in the formation and storage of lipid droplets in the skin and has a protective function against skin irritation [41,42]. PLIN2 is also a target gene of PPAR [28], and in fact, PPAR ligands have been reported to induce PLIN2 expression in sebaceous gland cells (sebocytes) to drive the formation of lipid droplets [43].

Extract #419 also increased the expression levels of some genes involved in skin function, including SULT2B1b, a cholesterol sulphate synthase involved in maintaining barrier function homeostasis. This suggests that the synthesis of cholesterol sulphate, which constitutes the intercellular lipids in the stratum corneum, may be promoted, and the barrier function may be improved. This is consistent with reports to date of increased expression of SULT2B1b upon activation of PPARα, which has also been implicated in the homeostasis of skin barrier function [36]. In addition, extract #419 also increased the amount of water molecules and the expression of AQP3, which is involved in glycerin transport, and HAS3, a hyaluronan synthase. AQP3, which is present in the epidermis, is expressed from the basal to the granular layers with cellular nuclei and is thought to be involved in the trafficking of water molecules and glycerin from the dermis to the epidermis [35]. Abnormal skin dryness has been observed in AQP3 knockout mice, and the glycerol content of AQP3 and epidermis has been shown to play key roles in skin moisturization and barrier function [44–47]. Hyaluronic acid (HA) is a type of glycosaminoglycan, which is a polymer of macromolecules involved in water retention and maintenance of morphology in many tissues, including the cartilage and dermis. In the epidermis, HA is synthesized mainly by HAS3 and is known to be located anywhere from the basal lamina to the stratum corneum, where it is involved in water maintenance, cell growth and migration, and wound healing [48]. Among the effects induced by hyaluronan, signalling via an interaction with CD44 in the plasma membrane of epidermal keratinocytes is known to be involved in promoting cholesterol synthesis, inducing epidermal keratocyte differentiation, and promoting cell growth [37]. Both AQP3 and HAS3 have been reported to have elevated expression upon activation of PPARα [49–51]. Thus, it is suggested that both AQP3 and HAS3 are factors related to the moisturizing properties of the skin, which may be improved by #419.

It is important to identify the main components of #419 that activates PPARα. Rigano et al. reported that many natural products such as terpenes, polyketides, phenylpropanoids, polyphenols and alkaloids, activated PPARα [52]. In the case of *Typha angustifolia*, Chen et al. reported that the chemical constituents of the pollen of *Typha angustifolia* are flavonoids, such as quercetin, kaempferol, kaempferol-3-*O*-neohesperidoside (K3ON), isorhamnetin, isorhamnetin-3-*O*-neohesperidoside (I3ON), typhaneoside and naringenin [38]. Several studies have reported that alkaloids, such as isorhamnetin, naringenin, kaempferol and quercetin, activated PPARα activity and induced PPARα-regulated genes [53–55]. In addition, one of the main components of the pollen of *Typha angustifolia* is arachidonic acid, a PPARα ligand [56,57]. Indeed, arachidonic acid up-regulated the transcriptional activity of PPARα in the Gal4-chimera system (Figure S1) [58–60]. These reports and our result suggest that the above components of *Typha angustifolia* might be important in activating PPARα.

Although reporter assays with the Gal4-chimera system showed no activity, 35 extracts that were active in an assessment system that expressed the full length of PPARα might affect the transcriptional activity of PPARα through involvement in post-translational modification or its expression. In particular, the nine extracts that we determined could enhance the activity of PPARα when used in conjunction with #419 have been discussed in previous studies, as shown below (Table 3). #203, an extract of *Achillea millefolium*, has an anti-inflammatory effect through increasing PPARγ expression and inhibiting NFκB expression [61]. #213, an extract of *Uncaria gambir*, may increase the expression of PPARγ [62]. #231 is *Scutellaria baicalensis* root extract, and it has been reported that wogonin, one of its constituents, activates PPARα [63]. #272 is *Sanguisorba officinalis* root extract, and its tannins elevate PPARδ expression [64]. #286 is *Rubus suavissimus* leaf extract, which has been reported to suppress the decrease in PPARα and PPARγ expression in hamsters due to a high-fat diet challenge [65]. #335 is tea (*Camellia sinensis*) leaf extract, which can activate PPARα [66]. #394 is *Punica granatum* flower extract, which has been reported to elevate the expression of PPARγ [67]. #452, *Euterpe oleracea* fruit extract, also elevates PPARα expression [68]. #485 is raspberry (*Rubus idaeus*) juice, which can activate the PPARα signalling pathway [69]. Thus, since these nine extracts may affect PPAR expression and signalling pathways, there is a possibility that the PPARα LBD-based Gal4-chimera system did not show activity and that they may have been observed to be active in assays using reporter-genes with PPRE enhancers. Further analysis of the detailed mechanisms shown here is necessary.

CONCLUSIONS

We searched extracts of natural resources for those with potential to activate PPARα and found that #419, *Typha angustifolia* spike extract, activated PPARα in keratinocytes. Among the genes with elevated expression in response to #419, AQP3 transports glycerin from the dermis to the epidermis; glycerin increases the amount of lipid synthesis in the epidermis; SULT2B1b synthesizes cholesterol sulphate, which is involved in the maintenance of barrier function homeostasis; the interaction between hyaluronan and CD44 is involved in promoting cholesterol synthesis, inducing differentiation of epidermal keratinocytes and promoting proliferation; and PLIN2 is a factor related to the formation and storage of lipid droplets in the skin and protects against skin irritation. Therefore, through these functions, #419, *Typha angustifolia* spike extract, could be used in cosmetics that activate PPARα, which expected to improve skin function.

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CONFLICT OF INTEREST

This study was a collaboration study between Graduate School of Pharmaceutical Sciences, Osaka University and Maruho Co., Ltd. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication. This study was funded by Maruho Co., Ltd.

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