Cellular Signalling 57 (2019) 58–64

Contents lists available at ScienceDirect

Cellular Signalling

journal homepage: www.elsevier.com/locate/cellsig

A steroid alkaloid derivative 02F04 upregulates thymic stromal lymphopoietin expression slowly and continuously through a novel Gq/11-ROCK-ERK1/2 signaling pathway in mouse keratinocytes

Yan Weng[a,b], Jingwen Wang[a], Zhifu Yang[a], Miaomiao Xi[a], Jialin Duan[a], Chao Guo[a], Ying Yin[a], Ryosuke Segawa[b], Takahiro Moriya[b], Takayuki Yonezawa[c], Byung Yoon Cha[c], Je-Tae Woo[d], Aidong Wen[a,*], Noriyasu Hirasawa[b,*]

[a] Department of Pharmacy, Xijing Hospital, Air Force Medical University, Xi'an 710032, Shaanxi, PR China
[b] Laboratory of Pharmacotherapy of Life-Style Related Diseases, Graduate School of Pharmaceutical Sciences, Tohoku University, Sendai 980-8578, Miyagi, Japan
[c] Research Institute for Biological Functions, Chubu University, Kasugai 487-8501, Aichi, Japan
[d] Department of Biological Chemistry, College of Bioscience and Biotechnology, Chubu University, Kasugai 487-8501, Aichi, Japan

ARTICLE INFO

Keywords:
02F04
Thymic stromal lymphopoietin
Gq/11
Rho-associated protein kinase
Extracellular signal-regulated kinase 1/2

ABSTRACT

Thymic stromal lymphopoietin (TSLP), a master switch of allergic inflammation, plays an important role in the pathogenesis of allergic diseases. Although many compounds upregulate TSLP expression in vivo or in vitro, most of them are pollutants or toxicants. In the previous study, for the first time, we found that a steroid alkaloid derivative 02F04, which has a unique skeletal structure compared with other TSLP-inducing chemicals, significantly induced TSLP production in mouse keratinocytes. However, it is not investigated thoroughly that how 02F04 produces TSLP and why. In this study, we did a detailed investigation on the inducible effect and underlying molecular mechanism of 02F04 on TSLP production. We found that the peak time of TSLP mRNA level induced by 02F04 at 48 h led to a slow and continuous TSLP production in PAM212 cells. Besides, 02F04-induced TSLP production was significantly suppressed by inhibitors of Rho-associated protein kinase (ROCK), guanine nucleotide-binding protein subunit alpha q/11 (Gq/11) and extracellular signal-regulated kinase 1/2 (ERK1/2) at not only protein but also mRNA levels, and by siRNA-mediated knockdown of Gq or G11. This suggested that ROCK, Gq/11 and ERK1/2 signaling pathways were involved in 02F04-induced TSLP production. Increase in the level of p-ERK1/2 induced by 02F04 was suppressed by both inhibitors of ROCK and Gq/11, indicating that ROCK and Gq/11 molecules were located at the upstream of ERK1/2 to regulate 02F04-induced TSLP production. Gq/11 was located at the upstream of ROCK because the specific Gq/11 inhibitor of YM-254890 significantly reduced 02F04-induced actin stress fiber formation. Taken together, 02F04 upregulates a slow and continuous TSLP production through a novel Gq/11-ROCK-ERK1/2 signaling pathway. The thorough understanding the effect and mechanism of 02F04 on TSLP production is expected to supply it as a novel TSLP-regulating compound and a potential new tool for investigating the role of TSLP in allergic disorders.

1. Introduction

Thymic stromal lymphopoietin (TSLP), a novel interleukin-7 (IL-7)-like cytokine that is mainly derived from epithelial cells, fibroblasts, and mast cells and exerts its biological functions through TSLP receptors on dendritic cells (DCs) [1], represents a key link between epithelial cells and DCs at the interface of allergic inflammation by participating in the programming of DC-mediated Th2 polarization [2].

Abbreviations: TSLP, thymic stromal lymphopoietin; IL-7, interleukin-7; DCs, dendritic cells; TNF-α, tumor necrosis factor-α; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; LXR, liver X receptor; MEMα, alpha minimal essential medium; FBS, fetal bovine serum; ELISA, enzyme-linked immunosorbent assay; MTT, thiazolyl blue tetrazolium bromide; G11, guanine nucleotide-binding protein subunit alpha-11; Gq, guanine nucleotide-binding protein subunit alpha q; Gna11 siRNA, G11-specific small interfering RNA; Gnaq siRNA, Gq-specific small interfering RNA; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; F-actin, filamentous actin; ROCK, Rho-associated protein kinase; MAPK, mitogen-activated protein kinase; ERK1/2, extracellular signal-regulated kinase 1/2; NF-κB, nuclear factor-κB; p-ERK1/2, phosphorylated ERK1/2

* Corresponding authors.
E-mail addresses: adwen-2004@hotmail.com (A. Wen), hirasawa@m.tohoku.ac.jp (N. Hirasawa).

https://doi.org/10.1016/j.cellsig.2019.01.005
Received 10 December 2018; Received in revised form 16 January 2019; Accepted 17 January 2019
Available online 19 January 2019
0898-6568/

TSLP expression has been reported to be increased at inflamed sites in patients with asthma [3], allergic rhinitis [4] or atopic dermatitis [5], and moreover, increased TSLP production in keratinocytes further accelerates allergic skin diseases such as atopic dermatitis [6] and psoriasis [7]. Hence, TSLP, as a master switch of allergic inflammation, plays an important role in the pathogenesis of allergic diseases especially inflammatory skin disorders.

TSLP production is known to be induced by exogenous and endogenous factors [8]. Inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and IL-1β, and some proteases have been identified as endogenous triggers for TSLP production [9,10]. Exogenous factors, including allergen exposure [11], viral infection [12], ligation of Toll-like receptor [13], treatment with physiologically active ligands of vitamin D receptors [14], and exposure to some mixed compounds such as diesel exhaust particles [15] and cigarette smoke [16], have also been found to induce the production of TSLP in vivo or in vitro. In addition, certain pure chemical compounds such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA) [17], dibutyl phthalate [18], and pentanoic acid [19] were demonstrated to significantly increase TSLP expression and exacerbate allergic skin inflammation. Moreover, some environmental compounds including xylene, toluene and trimethylbenzene [20], were also shown to trigger the production of TSLP and exacerbate contact sensitizers in allergic dermatitis. Although these compounds described above can upregulate TSLP expression in vivo or in vitro, most of them are pollutants or toxicants. Whether the common compounds have the potential to induce TSLP production remains unclear.

Recently, for the first time, we found that a steroid alkaloid derivative named 02F04 (shown in Fig. 1), also as an analogue of the Liver X receptor (LXR) endogenous ligand, induced a slow TSLP production in murine keratinocytes by activating traditional signaling pathways rather than nuclear receptors [21]. However, we didn't investigate thoroughly how slowly 02F04 produce TSLP and why. Though 02F04 has a unique skeletal structure compared with other TSLP-inducing chemicals, we didn't clarify clearly whether there is a novel molecular mechanism involved in 02F04-induced TSLP production and what the correlation between the novel and traditional signaling pathways is. Hence, in this study, we focused on these problems and did a detailed study on the inducible effect and the underlying novel molecular mechanism of 02F04 on TSLP production. The thorough understanding the effect and mechanism of 02F04 on TSLP production might supply it as a potential new tool for investigating the role of TSLP in inflammatory skin disorders.

2. Materials and methods

2.1. Materials

02F04, U0126 and Y-27632 were purchased from InterBioScreen LTD. (Cat. No. STOCK1N-53172; Moscow, Russia), Promega (Madison, WI, USA), and Nacalai Tesque Inc. (Kyoto, Japan), respectively. YM-254890 was a kind gift from Astellas Pharma Inc. (Tokyo, Japan) and Taiho Pharmaceutical CO., LTD. (Tokyo, Japan).

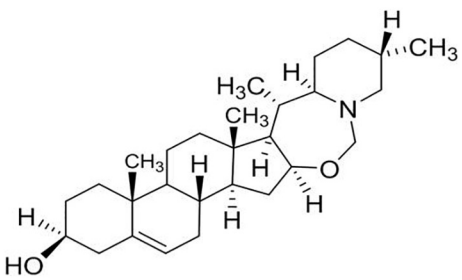

Fig. 1. Chemical structure of 02F04.

2.2. Cell culture

The murine keratinocyte cell line PAM212 [22] derived from BALB/c mouse skin was kindly provided by Dr. Yuspa (National Institutes of Health, Bethesda, MD, USA). Cells were cultured in alpha minimal essential medium (MEMα) supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin G potassium (15 μg/mL), and streptomycin (50 μg/mL) (Meiji Seika, Tokyo, Japan) in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C.

2.3. Cell seeding and treatment

Cells at different concentrations were seeded in various culture plates (Thermo Fisher Scientific) according to experimental requirements: 1×10^5 cells/mL in 48-well or 24-well culture plates for RNA interference; 1×10^5 cells/mL in 24-well culture plates for TSLP production; 1×10^5 or 0.25×10^5 cells/mL in 12-well culture plates for RNA extraction; 2×10^4 cells/mL in 12-well culture plates with gelatin-coated cover glass in it for filamentous actin (F-actin) staining; 1×10^5 cells/mL in 6-well culture plates for the preparation of cell lysates, respectively. The volumes of the cell suspensions seeded were 0.25, 0.5, 1 and 2 mL for a well of 48-, 24-, 12- and 6-well plate, respectively. The cells in different plates were then incubated at 37 °C, 5% CO_2 for 24 h except 32 h for RNA interference, and then stimulated with 02F04 in the presence or absence of different inhibitors for appropriate periods. All cells were used within 6 to 15 passaging cycles, and cell cultures were passaged every 3 to 4 days.

2.4. RNA interference

Reverse transfection was performed using Lipofectamine™ RNAiMAX reagent. Transfection complex was prepared and incubated for 15 min at room temperature according to the manufacturer's instructions when using. Then, the cell suspension was seeded in culture plate with complete growth medium without antibiotics, followed by addition of transfection complex. After incubated for 32 h at 37 °C, cells were washed twice with ice-cold PBS and then, cells in 24-well culture plate were collected with RNAiso plus and stored at −80 °C for total RNA extraction, while those in 48-well culture were firstly stimulated for 24 h by normal 10% FBS-MEMα with 02F04 in it and then, the supernatants were collected and stored at −20 °C for TSLP ELISA.

2.5. Cell viability

The viability of PAM212 cells was assessed using the thiazolyl blue tetrazolium bromide (MTT) cell proliferation assay. After removing the supernatant from each well, culture medium containing 10% MTT solution (5 mg/mL MTT in phosphate buffered saline) was added and the cells were incubated for another 4 h. Then, the resultant formazan crystals were dissolved in dimethyl sulfoxide and absorbance was measured at 570 nm using the iMark Microplate Absorbance Reader (Bio-Rad, Hercules, CA, USA). The chemicals were considered non-cytotoxic when cell viability was > 80%.

2.6. Enzyme-linked immunosorbent assay (ELISA) of TSLP

PAM212 cells were treated with 02F04 for 24 h in the presence or absence of YM-254890. At the end of the treatment time, supernatants of the culture media were collected and TSLP concentrations were determined with a DuoSet ELISA Development System (R & D Systems) according to the manufacturer's instructions.

2.7. RNA extraction and real-time PCR

At the end of the treatment period, total RNA was extracted from cells using RNAiso plus (Takara Bio, Shiga, Japan), and cDNA was

synthesized from total RNA by reverse transcription with PrimeScript™ RT master mix (Takara Bio). Real-time PCR was carried out using SYBR premix Ex Taq II (Takara Bio) in a TP800 Thermal Cycler Dice Real Time System (Takara Bio). Primers sequences used were as follows: glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (forward: 5′-TGTGTCCGTCGTGGATCTGA-3′; reverse: 5′-TTGCTGTTGAAGTCGC AGGAG-3′), TSLP (forward: 5′-AGCTTGTCTCCTGAAAATCGAG-3′, reverse: 5′-AGGTTTGATTCAGGCAGATGTT-3′), mGnaq (forward: 5′-ACT CTGGAGTCCATCATGG-3′; reverse: 5′-TGTATGGGATCTTGAGCG-3′), and mGna11 (forward: 5′-TCCTGCACTCACACTTGGTC-3′; reverse: 5′-GACAGGGACAGGAAGTGAGC-3′). After the reaction was completed, melting points were checked to define the identity of the final product. Cycle threshold values were calculated using the second derivative maximum method, and relative quantification was carried out by normalizing the expression of target genes to the reference GAPDH gene.

2.8. Western blotting

PAM212 cells were treated with 02F04 for 24 h in the presence or absence of Y-27632 or YM-254890. At the end of the treatment time, cells were lysed by ice-cold lysis buffer (20 mM HEPES buffer including 1% (*v*/v) Triton-X 100, 10% (v/v) glycerol, 1 mM EDTA, 50 mM NaF, 2.5 mM *p*-nitrophenyl phosphate, 10 mg/ml phenylmethylsulfonyl fluoride,1 mM Na_3VO_4, and 10 mg/mL leupeptin). Cell lysates were denatured, subjected to 10% (*w*/*v*) sodium dodecyl sulfate-polyacrylamide gel for electrophoresis (SDS-PAGE), and transferred to a nitrocellulose (NC) membrane. After blocking with 4% (w/v) Block Ace (DS Pharma Biomedical, Osaka, Japan), the NC membranes were incubated with phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) rabbit monoclonal antibodies (mAb) (1:1000, #9101s) or p44/42 MAPK (Erk1/2) rabbit mAb (1:1000, #9102s) as primary antibodies at 4 °C over night along with HRP-conjugated anti-rabbit IgG (1:2000, NA934V, GE Healthcare Life Sciences, Buckinghamshire, England) as secondary antibodies at 4 °C for 3 h, respectively. Then, blots were detected with ECL™ Western blotting detection reagents.

2.9. F-actin staining

PAM212 cells were treated with 02F04 for 6 h in the presence or absence of YM-254890. Then, the cells were fixed with 4% paraformaldehyde for 15 min followed by permeabilization with 0.1% TritonX-100 for 10 min at room temperature. Thereafter, F-actin was labeled with rhodamine-phalloidin (Cytoskeleton. Inc., Denver, CO, USA) at room temperature for 1 h, and nuclei with 4′, 6-diamidino-2-phenylindole (DAPI; Dojindo, Kumamoto, Japan) for 15 min. Fluorescence images were captured by a laser scanning confocal microscope (LSM700; Zeiss, Oberkochen, Germany) with Zen software (Zeiss).

2.10. Statistical analysis

The data are expressed as means ± standard errors of the mean (SEMs). Results were analyzed using a two-tailed paired Student's *t*-test to compare data between two groups and Dunnett's test for multiple comparisons. Differences with *p*-values $< .05$ were considered statistically significant.

3. Results

3.1. 02F04 induces a slow and continuous TSLP mRNA expression in PAM212 cells, but not in human epithelial cells

In the previous study, we found that 02F04 significantly induced TSLP production and elevated TSLP mRNA level at 24 h after treatment. Here, we further investigated TSLP gene expression during a 72 h-stimulation by 02F04. As shown in Fig. 2, mRNA level of TSLP was

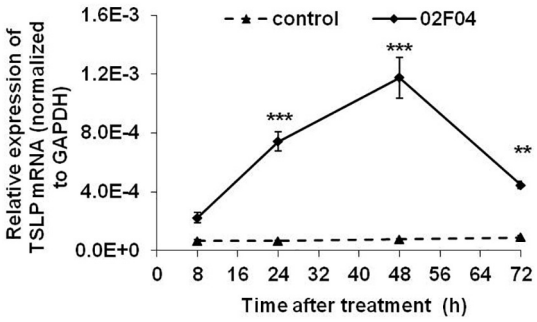

Fig. 2. Upregulation of TSLP mRNA expression by 02F04 in PAM212 cells. PAM212 cells were stimulated with 02F04 for the indicated time at 10 μM, and mRNA level of TSLP was determined by qRT-PCR. Data are shown as the mean ± SEM with three samples. Significance: $^{**}p < .01$, $^{***}p < .001$ vs. the corresponding control.

increased with the duration of 02F04 treatment at 10 μM until 48 h, then decreased gradually, but there was always a significant increase in the 02F04-stimulated group compared with the non-stimulated group ($p < .001$ for 24 and 48 h, and $p < .01$ for 72 h). Hence, 02F04 upregulated a very slow and continuous TSLP expression with the peak time of mRNA level at 48 h in PAM212 cells. However, when human keratinocytes HaCaT and A431, or human bronchial epithelial cells BEAS-2B were stimulated for 24 h with 02F04 at 0.3, 1, 3, 10, and 30 μM, TSLP production was not increased in any human epithelial cells even at a high concentration of 30 μM (data not shown).

3.2. Gq/11 signaling pathway regulated 02F04-induced TSLP production

To investigate whether 02F04-induced TSLP production involved Gq/11 signaling pathway, PAM212 cells were stimulated by 02F04 with or without YM-254890 (a specific Gq/11 inhibitor) for 24 h, and then protein and mRNA levels of TSLP were determined. Fig. 3A showed that YM-254890 at 10–100 nM significantly inhibited 02F04-induced TSLP production without any apparent cytotoxic effect. Similarly, 02F04-induced increase in TSLP mRNA was also significantly suppressed by YM-254890 (Fig. 3B). Furthermore, Fig. 3C demonstrated that mRNA expression of *Gnaq* and *Gna11* can be suppressed specifically by Gq and G11 siRNA, respectively. Accompanied by the decrease in gene expression of *Gnaq* and *Gna11*, 02F04-induced TSLP production was also significantly reduced (Fig. 3D). Therefore, 02F04-induced TSLP production was modulated by Gq/11 signaling pathway.

3.3. Extracellular signal-regulated kinase 1/2 (ERK1/2) and Rho-associated protein kinase (ROCK) signaling pathways were involved in 02F04-induced TSLP production

In the previous study, we only demonstrated that at the protein level, both Y-27632 (a ROCK inhibitor) and U0126 (an ERK1/2 inhibitor) significantly inhibited 02F04-induced TSLP production in a concentration-dependent manner. Here, the effect of the inhibitors on 02F04-induced TSLP mRNA level was determined. Fig. 4 A and B showed that both U0126 and Y-27632 significantly reduced TSLP expression induced by 02F04 at the transcriptional level ($p < .05$ for U0126, and $p < .001$ for Y-27632). Hence, we further confirmed from the gene level that ROCK and ERK1/2 signaling pathways participate in 02F04-induced TSLP production.

3.4. Gq/11 was the upstream of ROCK signaling pathway in 02F04-induced TSLP production

Next, PAM212 cells were treated with 02F04 for 6 h in the presence or absence of YM-254890, and F-actin staining was used to evaluate whether there was a correlation between 02F04-induced Gq/11 and

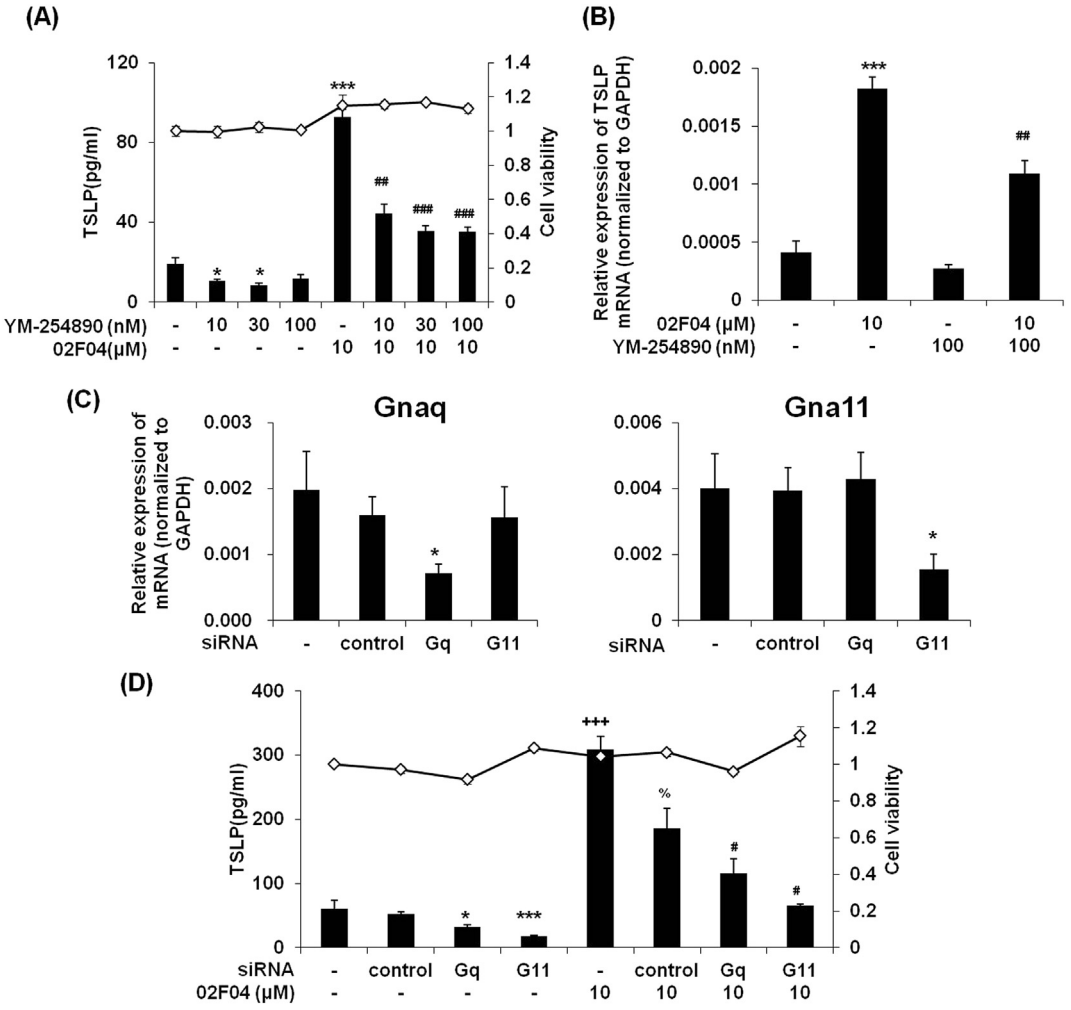

Fig. 3. 02F04-induced TSLP production involves the Gq/11 signaling pathway. PAM212 cells were stimulated with 02F04 (10 μM) for 24 h in the presence or absence of YM-254890 at the indicated concentrations. TSLP levels in the culture supernatant and cell viability were determined by ELISA and the MTT assay, respectively (A). TSLP mRNA level was determined by qRT-PCR (B). Additionally, PAM212 cells were transfected with siRNA of control, Gq or G11 at 50 nM for 32 h, then mRNA levels of Gnaq and Gna11 were determined directly by qRT-PCR (C), or cells were further stimulated with 02F04 (10 μM) for another 24 h (D) and then, TSLP levels in the culture supernatant and cell viability were determined by ELISA and the MTT assay, respectively (D). Data are shown as the mean ± SEM with four samples for A and D, three for B and C. Significance: A, B: $^{*}p < .05$, $^{***}p < .001$ vs. the non-stimulated group; $^{\#\#}p < .01$, $^{\#\#\#}p < .001$ vs. 02F04 alone; C, D: $^{*}p < .05$, $^{***}p < .001$ vs. the control siRNA group; $^{\#}p < .05$ vs. 02F04 plus control siRNA-stimulated group; $^{+++}p < .001$ vs. the non-stimulated group; $^{\%}p < .05$ vs. 02F04 alone. (A,D: ■: TSLP production; -◇-: Cell viability).

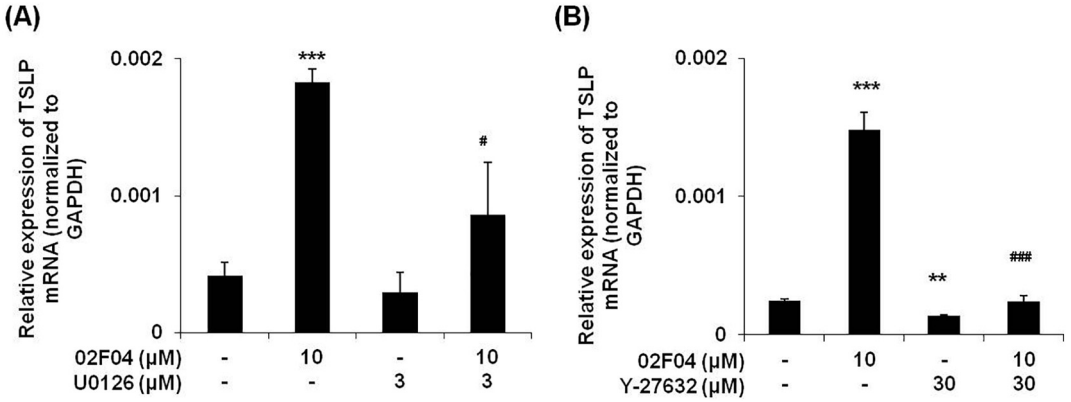

Fig. 4. Effects of U0126 and Y-27632 on 02F04-induced TSLP expression. PAM212 cells were stimulated with 02F04 (10 μM) for 24 h in the presence or absence of 3 μM U0126 (A) or 30 μM Y-27632 (B). TSLP mRNA level was determined by qRT-PCR. Data are shown as the mean ± SEM with three samples. Significance: $^{**}p < .01$, $^{***}p < .001$ vs. the non-stimulated group; $^{\#}p < .05$, $^{\#\#\#}p < .001$ vs. 02F04 alone.

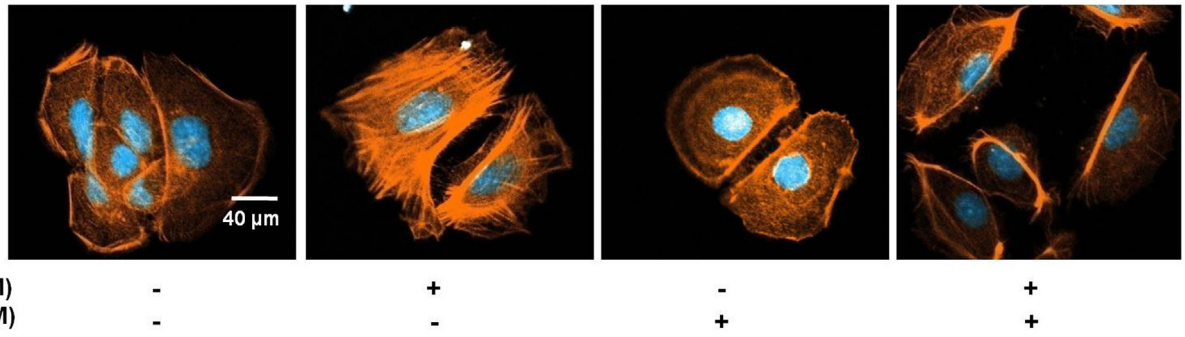

Fig. 5. Effect of Gq/11 inhibitor on 02F04-induced polymerization of actin fibers. PAM212 cells were stimulated for 6 h with 02F04 (10 μM) in the presence or absence of YM-254890 (10 μM). F-actin expression was measured with a laser scanning confocal microscope.

ROCK signaling pathways. It was shown in Fig. 5 that compared with the non-stimulated group, 02F04 induced the clear formation of dense F-actin filaments surrounding the nucleus after stimulation for 6 h, but exposure to YM-254890 for the same time significantly reduced 02F04-induced polymerization of actin fibers. This result demonstrated that Gq/11 was located at the upstream of ROCK signal transduction to regulate 02F04-induced TSLP production.

3.5. ROCK and Gq/11 were the upstreams of ERK1/2 signaling pathway in 02F04-induced TSLP production

To understand the correlation between different signaling pathways and ERK1/2 signaling cascade in 02F04-induced TSLP production, PAM212 cells were stimulated with 02F04 for 24 h in combination with the inhibitors of ROCK or Gq/11, and the protein level of phosphorylated ERK1/2 (p-ERK1/2) was determined. Fig. 6A and B showed that Y-27632 at 10 or 30 μM, and YM-254890 at 30 or 100 nM, not only inhibited basal p-ERK1/2 expression of PAM212 cells compared with the control group, but also suppressed 02F04-induced phosphorylation of ERK1/2. The inhibitory effects on p-ERK1/2 expression were consistent with the reduction of TSLP expression produced by the two inhibitors in Figs. 4B and 3A, B. This indicated that intracellular signaling molecules of ROCK and Gq/11 were the upstreams of ERK1/2 signaling pathway in 02F04-induced TSLP production. Combining with the result in 3.4 that Gq/11 was the upstream of ROCK signaling pathway in 02F04-induced TSLP production, we can draw a conclusion that 02F04 upregulates TSLP expression through a Gq/11-ROCK-ERK1/2 signaling pathway.

4. Discussion

In the previous study, we reported, for the first time, that a steroid alkaloid derivative 02F04 induced TSLP production in mouse epithelial cells of keratinocytes including PAM212 and KCMH-1 cells. In this present study, we further found that 02F04 can not increase TSLP production in human epithelial cells, but upregulates a slow and continuous TSLP production with the peak time of TSLP mRNA expression at 48 h through a novel Gq/11-ROCK-ERK1/2 signaling pathway in PAM212 cells. It is well known that there are both parallels and discrepancies between mouse and human epidermal cells. Therefore, the different responses to 02F04 in mouse and human epidermal cells are contributable to the species specificity.

Mitogen-activated protein kinase (MAPK) and nuclear factor-κB (NF-κB) signaling transduction pathways are known to regulate TSLP production. Our previous study indicated that 02F04 induced a slow and long-term activation of p-ERK1/2, but NF-κB signaling pathway only weakly involved in 02F04-induced TSLP production [21]. Recently, we also found that the novel TSLP production pathways of ROCK and Gq/11 signaling cascades were involved in pentanoic acid-induced TSLP production in PAM212 cells [19]. Protein level in the supernatants of the culture media reflected not only the production of proteins in cells, but also their secretion and release from the cells. When 02F04-induced TSLP protein level in the supernatants was decreased by an inhibitor of the intracellular signaling molecule, it is still unclear that the reduced TSLP protein was attributable to the effect of the inhibitor on decreasing TSLP mRNA expression, or suppressing the secretion of TSLP from the cells. Hence, in order to investigate whether a signaling pathway is involved in 02F04-induced TSLP production, not only protein but also gene expression of TSLP should be determined. In this study, the upregulation of TSLP by 02F04 treatment in PAM212 cells was significantly suppressed by inhibitors of Gq/11, ROCK and ERK1/2 at not only protein but also mRNA levels. Besides, siRNA-mediated knockdown of Gq or G11 also suppressed 02F04-induced TSLP production. All of these results suggested that Gq/11, ROCK and ERK1/2 signaling pathways are involved in 02F04-induced TSLP production. In our previous study, we demonstrated that KCMH-1 cells, another mouse keratinocytes, can produce 200-fold more TSLP than PAM212 cells without stimulation and 02F04 also upregulates TSLP expression in KCMH-1 cells [23]. Next, we found that TSLP protein level in KCMH-1 cells wasn't inhibited by Y27632 (data not shown). This indicated that Y27632 did not inhibit the translation and secretion of TSLP. Therefore, the reduce in 02F04-induced TSLP protein by Y27632 in PAM212 cells was totally contributable to the decreased mRNA level of TSLP. In other words, 02F04 increased TSLP production at a direct transcription level rather than by a secretion mechanism even after treatment for a long time of 24 to 48 h, and Y27632 inhibited 02F04-induced transcription of TSLP directly. It was notable that the inhibitors of ERK1/2, ROCK and Gq/11 also reduced basal expression of TSLP, indicating that activation of these molecules was essential for maintaining basal TSLP levels.

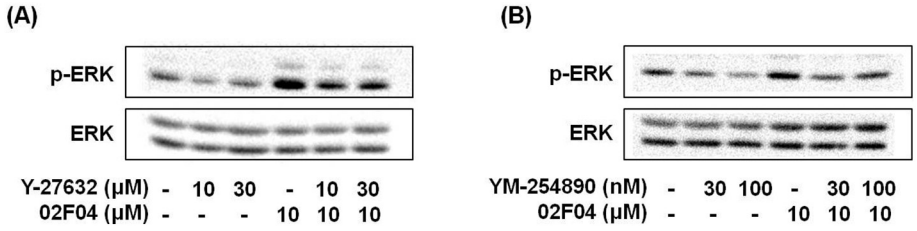

Fig. 6. Effect of ROCK and Gq/11 inhibitor on 02F04-induced phosphorylation of ERK1/2. PAM212 cells were stimulated with 02F04 (10 μM) for 24 h in the presence or absence of Y-27632 (A) or YM-254890 (B) at the indicated concentrations. Phosphorylation of ERK was measured by western blotting.

Various intracellular proteins can initiate the inflammatory response. MAPK is a central molecule mediating signaling pathways in innate immunity [24]. Activation of MAPK members, especially ERK1/2, plays an important role in the induction of inflammatory [25]. Hence, the signaling molecules which involved in inflammation are prone to relate to ERK1/2 signaling cascade. For example, four G protein subfamilies (Gq/11, Gi/o, $G4_{12/13}$, and Gs) influence the activity of the ERK1/2 cascade, with stimulatory effect for the former three and stimulatory or inhibitory effect for Gs [26]. Besides, there are also some relevant but controversial signaling molecules. Some study demonstrated that ROCK signal pathway activated ERK1/2 [27], while other showed that inhibiting ERK1/2 activation blocked the fibronectin-induced increase in F-actin protein expression [28]. We have previously demonstrated that 02F04 induced phosphorylation of ERK1/2 after stimulation at 2 h and reached a maximum at 24 h [21]. In this study, protein level of p-ERK1/2 produced by 02F04 was suppressed by both inhibitors of ROCK and Gq/11 accompanied by the decreased protein and mRNA levels of TSLP, indicating that ROCK and Gq/11 molecules were located at the upstream of ERK1/2 signal transduction to regulate 02F04-induced TSLP production. Therefore, the slow activation of p-ERK1/2 and the crosstalk between the above signaling pathways are probably involved in the slow production of TSLP induced by 02F04.

A previous study showed that Rho can be activated by the Gq/11 signaling pathway [29]. Additionally, the Rho/ROCK signaling pathway promotes actin polymerization in keratinocytes [30,31], and Gq/11 proteins have been implicated in actin polymerization [32]. Thus, we investigated the relevance between Gq/11 and ROCK signaling pathways in 02F04-induced TSLP production. We found that Gq/11 was located at the upstream of ROCK because the specific Gq/11 inhibitor of YM-254890 significantly reduced 02F04-induced actin stress fiber formation. Therefore, another possible reason for the slow production of TSLP induced by 02F04 may derive from the complicated crosstalk between Gq/11 and ROCK signaling pathways. Conclusively, the unique skeletal structure of 02F04 contributes to a novel mechanism of Gq/11-ROCK-ERK1/2 signaling pathway to induce a slow TSLP production, which is completely different from other stimulants such as TPA.

Keratinocytes represent > 95% cells in the skin and are therefore the predominant cell type. They have the potential to secrete a wide variety of cytokines such as TSLP through nuclear receptors. Besides, several membrane receptors, including protease-activated receptor 2 [33], epidermal growth factor receptor [10], TLR [34] and histamine receptor [35], have also been shown to involve in TSLP production in keratinocytes. Hence, keratinocytes might recognize different stimuli probably via different receptors. We have previously reported that pentanoic acid also induced a slow production of TSLP at mRNA and protein levels in PAM212 cells [19], which was similar to 02F04. More intriguingly, we also found that 02F04- and pentanoic acid-induced TSLP production was not regulated by the receptors related to their structures, in other words, NRs [21] or fatty acid receptors [19], respectively, but by the similar molecular mechanisms of activating Gq/11 and Rho/ROCK signaling pathways. Hence, we boldly speculate that PAM212 cells might recognize 02F04 and pentanoic acid probably via the similar but not unidentified membrane receptors. No matter whether the related receptors are consistent or not, a better understanding of the specific receptor involved in 02F04-induced TSLP production may allow us to develop novel therapeutic and preventive strategies for allergic diseases. Although Gq/11 signaling pathway involved in 02F04-induced TSLP production, it is unclear whether 02F04 activates Gq/11-coupled receptor, a subfamily of GPCRs. Thus, additional studies are urgently required to confirm this mechanism.

5. Conclusions

02F04, with a unique steroidal skeletal structure which is different from other TSLP-inducing chemicals reported previously, induces a slow and continuous TSLP production by a novel Gq/11-ROCK-ERK1/2 signaling pathway in PAM212 cells. The thorough understanding the effect and mechanism of 02F04 on TSLP production is expected to supply it as a potential new tool for investigating the role of TSLP in allergic disorders.

Conflict of interest statement

The authors declare that there is no conflict of interest.

Acknowledgments

The work is supported, in part, by Science and Technology Development Fund of Air Force Medical University, PR China (2018XD062) and the grants from the National Natural Science Foundation of China (No. 81470174, 81774190 and 81573549). The authors would like to thank the Ministry of Education, Culture, Sports, Science, and Technology, Japan (MEXT) for their support through the Monbukagakusho Scholarship (No. 142537).

References

[1] A. Cianferoni, J. Spergel, The importance of TSLP in allergic disease and its role as a potential therapeutic target, Expert. Rev. Clin. Immunol. 10 (2014) 1463–1474.
[2] Y.J. Liu, V. Soumelis, N. Watanabe, T. Ito, Y.H. Wang, W. Rde Malefyt, M. Omori, B. Zhou, S.F. Ziegler, TSLP: an epithelial cell cytokine that regulates T cell differentiation by conditioning dendritic cell maturation, Annu. Rev. Immunol. 25 (2007) 193–219.
[3] S. Ying, B. O'Connor, J. Ratoff, Q. Meng, K. Mallett, D. Cousins, D. Robinson, G. Zhang, J. Zhao, T.H. Lee, C. Corrigan, Thymic stromal lymphopoietin expression is increased in asthmatic airways and correlates with expression of Th2-attracting chemokines and disease severity, J. Immunol. 174 (2005) 8183–8190.
[4] M. Miyata, K. Hatsushika, T. Ando, N. Shimokawa, Y. Ohnuma, R. Katoh, H. Suto, H. Ogawa, K. Masuyama, A. Nakao, Mast cell regulation of epithelial TSLP expression plays an important role in the development of allergic rhinitis, Eur. J. Immunol. 38 (2008) 1487–1492.
[5] H.K. Jessup, A.W. Brewer, M. Omori, E.A. Rickel, A.L. Budelsky, B.R. Yoon, S.F. Ziegler, M.R. Comeau, Intradermal administration of thymic stromal lymphopoietin induces a T cell- and eosinophil-dependent systemic Th2 inflammatory response, J. Immunol. 181 (2008) 4311–4319.
[6] M. Li, N. Messaddeq, M. Teletin, J.L. Pasquali, D. Metzger, P. Chambon, Retinoid X receptor ablation in adult mouse keratinocytes generates an atopic dermatitis triggered by thymic stromal lymphopoietin, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 14795–14800.
[7] E. Volpe, L. Pattarini, C. Martinez-Cingolani, S. Meller, M.H. Donnadieu, S.I. Bogiatzi, M.I. Fernandez, M. Touzot, J.C. Bichet, F. Reyal, M.P. Paronetto, A. Chiricozzi, S. Chimenti, F. Nasorri, A. Cavani, A. Kislat, B. Homey, V. Soumelis, Thymic stromal lymphopoietin links keratinocytes and dendritic cell-derived IL-23 in patients with psoriasis, J. Allergy Clin. Immunol. 34 (2014) 373–381 e4.
[8] T. Takai, TSLP expression: cellular sources, triggers, and regulatory mechanisms, Allergol. Int. 61 (2012) 3–17.
[9] R. Segawa, N. Hirasawa, Exacerbation of allergic diseases by chemicals: role of TSLP, J. Pharmacol. Sci. 124 (2014) 301–306.
[10] R. Segawa, K. Shigeeda, T. Hatayama, J. Dong, N. Mizuno, T. Moriya, M. Hiratsuka, N. Hirasawa, EGFR transactivation is involved in TNF-α-induced expression of thymic stromal lymphopoietin in human keratinocyte cell line, J. Dermatol. Sci. 89 (2018) 290–298.
[11] B. Zhou, M.R. Comeau, T. De Smedt, H.D. Liggitt, M.E. Dahl, D.B. Lewis, D. Gyarmati, T. Aye, D.J. Campbell, S.F. Ziegler, Thymic stromal lymphopoietin as a key initiator of allergic airway inflammation in mice, Nat. Immunol. 6 (2005) 1047–1053.
[12] H.C. Lee, M.B. Headley, Y.M. Loo, A. Berlin, M. Gale Jr., J.S. Debley, N.W. Lukacs, S.F. Ziegler, Thymic stromal lymphopoietin is induced by respiratory syncytial virus-infected airway epithelialcells and promotes a type 2 response to infection, J. Allergy Clin. Immunol. 130 (2012) 1187–1196 e5.
[13] R. Segawa, N. Mizuno, T. Hatayama, D. Jiangxu, M. Hiratsuka, Y. Endo, N. Hirasawa, Lipopolysaccharide-activated leukocytes enhance thymic stromal lymphopoietin production in a mouse air-pouch-type inflammation model, Inflammation 39 (2016) 1527–1537.
[14] M. Li, P. Hener, Z. Zhang, S. Kato, D. Metzger, P. Chambon, Topical vitamin D3 and low-calcemic analogs induce thymic stromal lymphopoietin in mouse keratinocytes and trigger an atopic dermatitis, Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 11736–11741.
[15] B. Bleck, D.B. Tse, M.A. Curotto de Lafaille, F. Zhang, J. Reibman, Diesel exhaust particle-exposed human bronchial epithelial cells induce dendritic cell maturation and polarization via thymic stromal lymphopoietin, J. Clin. Immunol. 28 (2008) 147–156.
[16] Y. Nakamura, M. Miyata, T. Ohba, T. Ando, K. Hatsushika, F. Suenaga, N. Shimokawa, Y. Ohnuma, R. Katoh, H. Ogawa, A. Nakao, Cigarette smoke extract

induces thymic stromal lymphopoietin expression, leading to T(H)2-type immune responses and airway inflammation, J. Allergy Clin. Immunol. 122 (2008) 1208–1214.
[17] N. Hirasawa, Y. Ohsawa, K. Ishihara, T. Seyama, J. Hong, K. Ohuchi, Analysis of the mechanism for the development of allergic skin inflammation and the application for its treatment: establishment of a modified allergic dermatitis model in mouse ear lobes by application of 12-O-tetradecanoyl phorbol 13-acetate: putative involvement of thymic stromal lymphopoietin and roles of histamine, J. Pharmacol. Sci. 110 (2009) 245–250.
[18] R.P. Larson, S.C. Zimmerli, M.R. Comeau, A. Itano, M. Omori, M. Iseki, C. Hauser, S.F. Ziegler, Dibutyl phthalate-induced thymic stromal lymphopoietin is required for Th2 contact hypersensitivity responses, J. Immunol. 184 (2010) 2974–2984.
[19] N. Mizuno, K. Abe, Y. Morishita, S. Yamashita, R. Segawa, J. Dong, T. Moriya, M. Hiratsuka, N. Hirasawa, Pentanoic acid induces thymic stromal lymphopoietin production through Gq/11 and Rho-associated protein kinase signaling pathway in keratinocytes, Int. Immunopharmacol. 50 (2017) 216–223.
[20] N. Satou, K. Ishihara, M. Hiratsuka, H. Tanaka, Y. Endo, S. Saito, Y. Iwakura, W.J. Leonard, N. Hirasawa, Induction of thymic stromal lymphopoietin production by xylene and exacerbation of picryl chloride-induced allergic inflammation in mice, Int. Arch. Allergy Immunol. 157 (2012) 194–201.
[21] Y. Weng, N. Mizuno, J. Dong, R. Segawa, T. Yonezawa, B.Y. Cha, J.T. Woo, T. Moriya, M. Hiratsuka, N. Hirasawa, Induction of thymic stromal lymphopoietin by a steroid alkaloid derivative in mouse keratinocytes, Int. Immunopharmacol. 55 (2017) 28–37.
[22] S.H. Yuspa, P. Hawley-Nelson, B. Koehler, J.R. Stanley, A survey of transformation markers in differentiating epidermal cell lines in culture, Cancer Res. 40 (1980) 4694–4703.
[23] R. Segawa, S. Yamashita, N. Mizuno, M. Shiraki, T. Hatayama, N. Satou, M. Hiratsuka, M. Hide, N. Hirasawa, Identification of a cell line producing high levels of TSLP: Advantages for screening of anti-allergic drugs, J. Immunol. Methods 402 (2014) 9–14.
[24] W. Shi, X. Hou, H. Peng, L. Zhang, Y. Li, Z. Gu, Q. Jiang, M. Shi, Y. Ji, J. Jiang, MEK/ERK signaling pathway is required for enterovirus 71 replication in immature dendritic cells, Virol. J. 11 (2014) 227.
[25] S. Pastore, F. Mascia, F. Mariotti, C. Dattilo, V. Mariani, G. Girolomoni, ERK1/2 regulates epidermal chemokine expression and skin inflammation, J. Immunol. 174 (2005) 5047–5056.
[26] P.H. Sugden, A. Clerk, Regulation of the ERK subgroup of MAP kinase cascades through G protein-coupled receptors, Cell. Signal. 9 (1997) 337–351.
[27] V.M. Zohrabian, B. Forzani, Z. Chau, R. Murali, M. Jhanwar-Uniyal, Rho/ROCK and MAPK signaling pathways are involved in glioblastoma cell migration and proliferation, Anticancer Res. 29 (2009) 119–123.
[28] J.H. Park, J.M. Ryu, S.P. Yun, M.O. Kim, H.J. Han, Fibronectin stimulates migration through lipid raft dependent NHE-1 activation in mouse embryonic stem cells: involvement of RhoA, Ca(2+)/CaM, and ERK, Biochim. Biophys. Acta 1820 (2012) 1618–1627.
[29] J. Jin, Y. Mao, D. Thomas, S. Kim, J.L. Daniel, S.P. Kunapuli, RhoA downstream of G(q) and G(12/13) pathways regulates protease-activated receptor-mediated dense granule release in platelets, Biochem. Pharmacol. 77 (2009) 835–844.
[30] V.M. Braga, Cell-cell adhesion and signalling, Curr. Opin. Cell Biol. 14 (2002) 546–556.
[31] A. Vaezi, C. Bauer, V. Vasioukhin, E. Fuchs, Actin cable dynamics and Rho/Rock orchestrate a polarized cytoskeletal architecture in the early steps of assembling a stratified epithelium, Dev. Cell 3 (2002) 367–381.
[32] S. Taboubi, F. Garrouste, F. Parat, G. Pommier, E. Faure, S. Monferran, H. Kovacic, M. Lehmann, Gq-coupled purinergic receptors inhibit insulin-like growth factor-I/phosphoinositide 3-kinase pathway-dependent keratinocyte migration, Mol. Biol. Cell 21 (2010) 946–955.
[33] A. Briot, C. Deraison, M. Lacroix, C. Bonnart, A. Robin, C. Besson, P. Dubus, A. Hovnanian, Kallikrein 5 induces atopic dermatitis-like lesions through PAR2-mediated thymic stromal lymphopoietin expression in Netherton syndrome, J. Exp. Med. 206 (2009) 1135–1147.
[34] A.T. Vu, T. Baba, X. Chen, T.A. Le, H. Kinoshita, Y. Xie, S. Kamijo, K. Hiramatsu, S. Ikeda, H. Ogawa, K. Okumura, T. Takai, Staphylococcus aureus membrane and diacylated lipopeptide induce thymic stromal lymphopoietin in keratinocytes through the Toll-like receptor 2-Toll-like receptor 6 pathway, J. Allergy Clin. Immunol. 126 (2010) 985–993.
[35] K. Schaper, K. Rossbach, B. Köther, H. Stark, M. Kietzmann, T. Werfel, R. Gutzmer, Stimulation of the histamine 4 receptor upregulates thymic stromal lymphopoietin (TSLP) in human and murine keratinocytes, Pharmacol. Res. 113 (2016) 209–215.