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IL-4 receptor α in non-lipid rafts is the target molecule of strictinin in inhibiting STAT6 activation

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ABSTRACT

Strictinin has been shown to suppress interleukin (IL)-4-induced signal transducer and activator of transcription (STAT)-6 phosphorylation, which is a critical event for IgE class switching. However, it is unclear how strictinin inhibits STAT6 activation. Strictinin inhibited STAT6 phosphorylation by suppressing IL-4 receptor α (IL-4Rα) activation. Strictinin was bound to the cell surface and only localized in non-lipid raft fraction of the cells where IL-4Rα was also located. In addition, strictinin directly bound to IL-4Rα and inhibited binding of IL-4 to IL-4Rα. These results suggest that IL-4Rα locating in non-lipid raft region is a target molecule for strictinin in inhibiting STAT6 activation.

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1. Introduction

The prevalence of immunoglobulin E (IgE)-mediated allergic diseases such as atopic dermatitis, allergic rhinitis, food allergy, and asthma has been increasing for the last four decades. In many individuals, total serum IgE level generally correlates with the severity of the disease [1]. Thus, it is expected that inhibition of IgE production may be a useful strategy to abrogate the progression of IgE-mediated diseases.

Interleukin (IL)-4 and IL-13 are important cytokines in the early stages of allergic diseases because of their important role in initiation of B-cell isotype switching to IgE production [2–4]. The signaling of these cytokines is mediated by IL-4 receptor (IL-4R), a cell surface receptor. IL-4R divides into two types; the type I is complex of IL-4R α chain (IL-4Rα) and common gamma chain (γc) and is unique to IL-4; type II is composed of IL-4Rα and IL-13 receptor α 1 chain (IL-13Rα1). After IL-4 binds to the IL-4Rα, it induces the association of IL-4Rα with γc and initiates signaling by causing the activation of tyrosine kinases, janus kinase (JAK). Activated JAK phosphorylate tyrosines within the cytoplasmic domain of the IL-4Rα. IL-13 also uses the IL-4Rα to initiate signaling after binding IL-13Rα1. One signaling molecule important for IL-4 and IL-13 is a member of the signal transducers and activators of transcription (STAT) 6. STAT6 is recruited to the activated IL-4Rα in response to either IL-4 or IL-13 and is thereafter phosphorylated by the JAK. Phosphorylated STAT6 promotes activation of the ε germline promoter, leading to the expression of ε germline transcript (εGT) essential for IgE production [5,6]. STAT6-deficient mice are deficient in class switching to IgE and in the development of atopic immune responses [7–9]. These findings indicate that the inhibition of STAT6 activation can suppress the initiation of IgE synthesis by inhibiting IgE class-switch recombination. Thus, it is expected that an inhibitor of STAT6 activation may be useful to abrogate the progression of IgE-mediated diseases.

In a previous study, we reported that strictinin, an ellagitanins in a green tea, effectively suppressed IL-4-mediated εGT expression in human peripheral blood mononuclear cells from healthy or atopic donors and inhibited antigen-specific IgE production *in vivo* [10]. However, the precise suppressive mechanism of the strictinin remains unclear. We recently reported that (−)-epigallocatechin-3-*O*-gallate (EGCG), a major green tea polyphenol, binds to 67 kDa

Abbreviations: εGT, ε germline transcript; IFN-γ, interferon-γ; IL-4, interleukin-4; IL-4Rα, interleukin-4 receptor α; IL-13, interleukin-13; γc, common γ-chain; JAK, janus kinases; SPR, surface plasmon resonance; STAT6, signal transducers and activators of transcription 6.

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laminin receptor (67LR) on the cell surface and exerts several biological activities such as anti-cancer, anti-allergic, and anti-inflammatory effects [11–14]. Thus, it is thought that polyphenols exhibit their biological activities through interactions with cellular molecules. However, there is no evidence indicating the first target of strictinin for exerting its inhibitory effect on IgE production.

In this report, we show that strictinin interacts with the non-lipid rafts of plasma membrane and binds to non-lipid raft-associated IL-4Rα. To our knowledge, this is the first report to reveal a target molecule of an ellagitannin.

2. Materials and methods

2.1. Reagents

Strictinin (1-*O*-galloyl-4,6-(–)-hexahydroxydiphenoyl-β-D-glucose) was purchased from Nagara Science Co., Ltd. (Shizuoka, Japan). EGCG was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Glucose and ellagic acid were obtained from Wako Pure Chemical Industries (Osaka, Japan). Pyrogallol and gallic acid were obtained from Nacalai Tesque (Fukuoka, Japan). Human recombinant IL-4 (hrIL-4), human recombinant IFN-γ (hrIFN-γ), mouse recombinant IL-13 (mrIL-13), and human recombinant IL-4 receptor α (Gly24-His232; IL-4 binding domain) were obtained from R&D Systems Inc. (Minneapolis, MN, USA). Protein A Sepharose beads were obtained from Amersham Pharmacia Biotech (Buckinghamshire, UK). Rabbit anti-human STAT6 antibody (S-20), rabbit anti-human IL-4Rα antibody (C-20), rabbit anti-human JAK3 antibody (C-21), and horseradish peroxidase (HRP)-conjugated anti-phosphorylated tyrosine antibody (PY20) were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Mouse anti-human flotillin-1 antibody was bought from BD Transduction Laboratories™ (Tokyo, Japan). HRP-conjugated anti-rabbit IgG antibody and HRP-conjugated anti-Mouse IgG antibody were obtained from ICN Pharmaceuticals, Inc. (Aurora, OH, USA) and Zymed Laboratories, Inc. (San Francisco, CA, USA), respectively.

2.2. Cell culture

The human EBV-negative Burkitt's lymphoma cell line DND39 cells were maintained in complete RPMI 1640 medium (Gibco BRL, Gaithersburg, MD, USA) supplemented with 100 U/mL penicillin, 100 μg/mL of streptomycin, and 10% fetal bovine serum (FBS). Human cervical cancer cell line HeLa cells and mouse embryonic fibroblast cell line NIH3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Cosmobio Co., Ltd, Tokyo, Japan) supplemented with 5% FBS (Intergen Co., NY, USA), 100 U/mL penicillin G, 100 μg/mL streptomycin, and 10 mM Hepes buffer. All cell lines were cultured in a humidified atmosphere with 5% CO_2 at 37 °C.

2.3. Cell stimulation with cytokines

Cells were exposed to the hrIL-4 (100 U/mL) in the presence of 25 μM strictinin, its component parts (glucose, ellagic acid, pyrogallol, and gallic acid) or 25 μM EGCG for 30 min under serum-free conditions. To measure the effect of strictinin on IL-4Rα and JAK3 activation, cells were stimulated with hrIL-4 (100 U/mL) in the presence of strictinin for 10 min. Cells were lysed in lysis buffer and the cell extracts were immunoprecipitated with anti-STAT6 (S-20) followed by incubation with protein A-Sepharose beads (Amersham Bioscience). The washed immunoprecipitates were separated using an 8% acrylamide gel, and transferred onto nitrocellulose membranes (Schleicher & Schuell BioScience, Inc., NH, USA). Membranes were then probed with anti-phosphotyrosine (PY20). The bound antibody was detected using ECL (Amersham Pharmacia Biotech).

To evaluate the effects of strictinin on type II IL-4R, HeLa cells were exposed to the hrIL-4 (100 U/mL) and NIH 3T3 cells were exposed to mrIL-13 (10 ng/mL) in the presence of strictinin for 30 min under serum-free conditions. To assess the effect of strictinin on IFN-γ stimuli, DND39 cells were exposed to the hrIFN-γ (800 ng/mL) in the presence of strictinin for 30 min under serum-free conditions.

2.4. Immunoprecipitation and immunoblot analysis

Cells were lysed in lysis buffer containing 10 mM Tris–HCl (pH 7.6), 50 mM NaCl, 200 mM sodium orthovanadate, 0.5% Triton-X 100, 1 mM phenylmethylsulfonyl fluoride, and 5 μg/mL aprotinin. Whole cell lysates were incubated with protein A Sepharose beads bound with rabbit polyclonal antibodies for IL-4Rα (C-20), JAK3 (C-21), or STAT6 (S-20) for 4 h at 4 °C. The beads were resuspended in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) buffer. After centrifugation, supernatants were subjected to immunoblot analysis. IFN-γ-induced STAT1 activation was evaluated with anti-STAT1 p84/p91 (E-23) and anti-pSTAT1 (Tyr701). The immunoprecipitants or cell lysates were loaded onto 8% SDS–PAGE gel and electrophoresis was performed under reducing conditions. The proteins were then electrotransferred onto nitrocellulose membrane, which were probed with HRP-conjugated anti-phosphotyrosine (PY20) or phosphotyrosine (4G10). The bound antibody was detected using ECL (Amersham Pharmacia Biotech).

2.5. Analysis of strictinin binding to cells using a surface plasmon resonance (SPR) biosensor

Analysis of the interaction between strictinin and DND39 cells was performed using the SPR biosensor SPR670 (Nippon Laser and Electronics Lab., Nagoya, Japan). DND39 cells were immobilized on the sensor chip, which was equilibrated in SPR running buffer, PBS (pH 7.4, 30 μL/min). Strictinin was diluted at 0, 25, or 50 μM in SPR running buffer in 60 μL injection volumes and at a flow rate of 30 μL/min. Binding was measured at 25 °C for 2 min followed by dissociation. The value of the angle presented in Fig. 3 corresponded with the binding strength.

2.6. Preparation of lipid raft fractions and measurement of strictinin in cell membrane

After treatment of DND39 cells with or without 25 μM strictinin for 24 h, lipid raft fractions were prepared by cell lysis using 1% Triton X-100 followed by sucrose gradient fractionation as described previously [15]. One-milliliter fractions were collected from the top of the gradient [designated fractions number 1 (top) through 13 (bottom)]. Low-buoyant density fractions 1–6 were designated lipid raft fractions, and high-buoyant density fractions 7–13 were designated non-lipid raft fractions. The protein concentration of each fraction was determined using the Pierce BCA protein assay kit (Rockford, IL, USA). Pooled lipid raft and non-lipid raft fractions were then internally normalized for protein content.

The level of strictinin in lipid raft and non-lipid raft fractions from strictinin-treated DND39 cells for 24 h was determined by HPLC with a coulometric array detector (ESA Inc., Chelmsford, CA, USA). In brief, 200 μg of lipid raft (fraction number 2 in Fig. 3B) and non-lipid raft (fraction number 11 in Fig. 3B) fractions were twice extracted by ethyl acetate. The combined ethyl acetate solutions were added to a 20% ascorbic acid solution, and dried under nitrogen gas and then redissolved in a 50% acetonitrile (ACN) aqueous solution. The resultant solution was injected onto

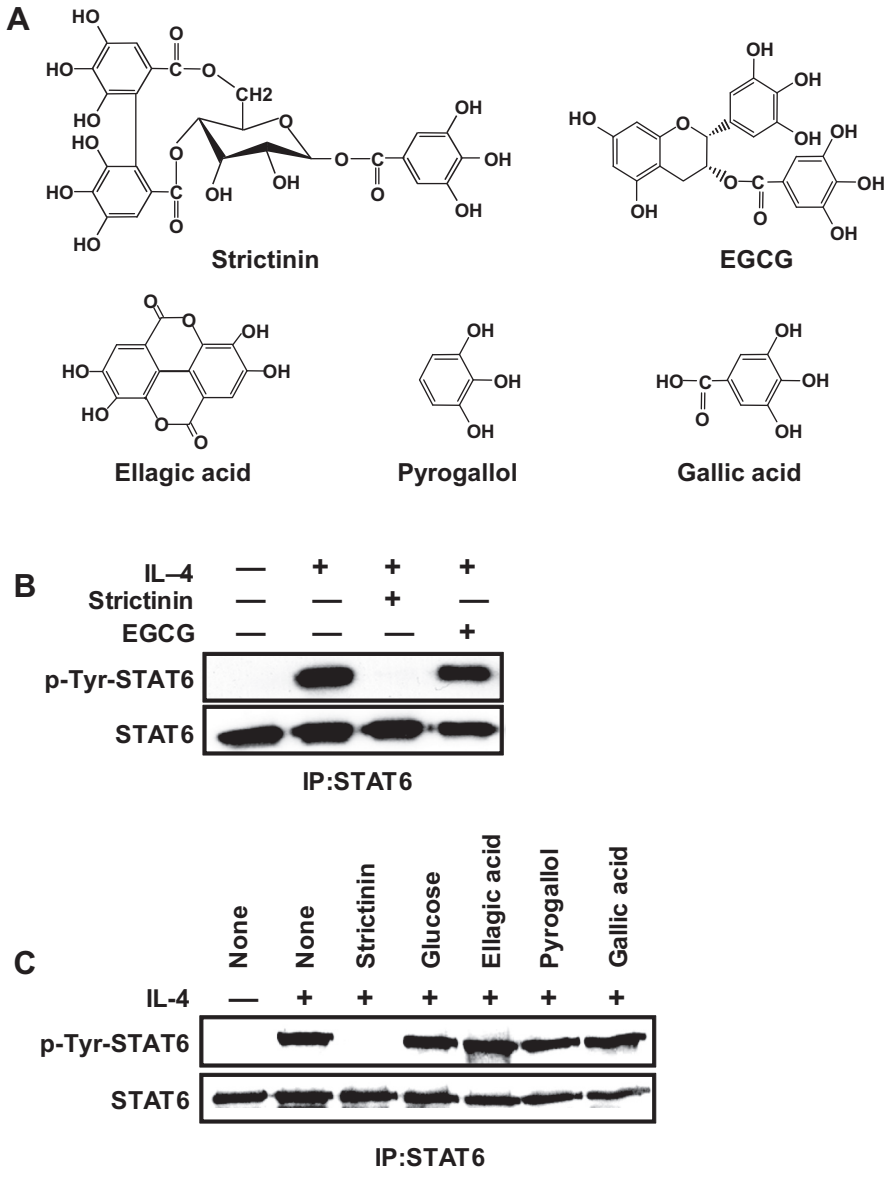

Fig. 1. Structure–activity relationships of strictinin for IL-4-induced tyrosine phosphorylation of STAT6. (A) The chemical structure of strictinin, its component (glucose, ellagic acid, pyrogallol, and gallic acid), and EGCG. (B) DND39 cells were treated with strictinin or EGCG in the presence of hrIL-4 for 30 min. (C) Cells were treated with strictinin, glucose, ellagic acid, pyrogallol, or gallic acid in the presence of hrIL-4 for 30 min. STAT6 was immunoprecipitated, separated by 8% SDS–PAGE, and immunoblotted with anti-phosphotyrosine antibody. Shown in the lower panel are protein levels from the same filter blotted again with the anti-STAT6 antibody.

the HPLC with TSK gel ODS 80Ts reversed-phase column (4.6 × 250 mm, Tosoh, Tokyo, Japan). The column was eluted at 30 °C with 0.1 M NaH_2PO_4 buffer (pH 2.5) containing 0.1 mM EDTA·2Na-ACN (87:13) at a flow rate of 0.6 μL/min. The eluent was monitored electrochemically at an applied potential of +660 mV versus Ag/AgCl.

2.7. Localization of IL-4Rα and JAK3

The localization of IL-4Rα and JAK3 was assessed in DND39 cells treated with or without IL-4 (25 U/mL) for 24 h. After lysis of cells, the lysates were used for sucrose gradient centrifugation as described previously. Lipid raft or non-lipid raft fractions were loaded onto 8% SDS–PAGE gel, and electrophoresis was performed under reducing conditions. After that, immunoblot analysis was performed using rabbit polyclonal antibodies against IL-4Rα and JAK3 followed by HRP-conjugated rabbit IgG. To confirm the location of lipid rafts in recovered and pooled sucrose gradient fractions, both low- and high-buoyant density fractions were subjected to 8% SDS–PAGE and immunoblotted using an antibody against flotillin-1 as raft marker.

2.8. Binding of strictinin to recombinant IL-4Rα

Binding affinities of strictinin and EGCG toward IL-4Rα were measured using the ProteOn XPR36 Protein Interaction Array system with the GLH Sensor Chip (Bio-Rad, Hercules, CA, USA). A solution of 0.05% Tween 20 and 5% DMSO in PBS (pH 7.4) was used a running buffer at a flow rate of 50 μL/min. Recombinant IL-4Rα (50 μg/mL in 10 mM sodium acetate pH 5) was immobilized on the sensor chip, which was then equilibrated in PBS. Strictinin or EGCG was injected as the ligand at five concentrations within a range of 6.25–100 μM for IL-4Rα binding. We analyzed binding kinetics with Bio-Rad ProteON Manager™ software (Bio-Rad) and calculated dissociation constants K_D which were determined from the rate constants, K_d (1/s) and K_a (1/Ms), K_d/K_a.

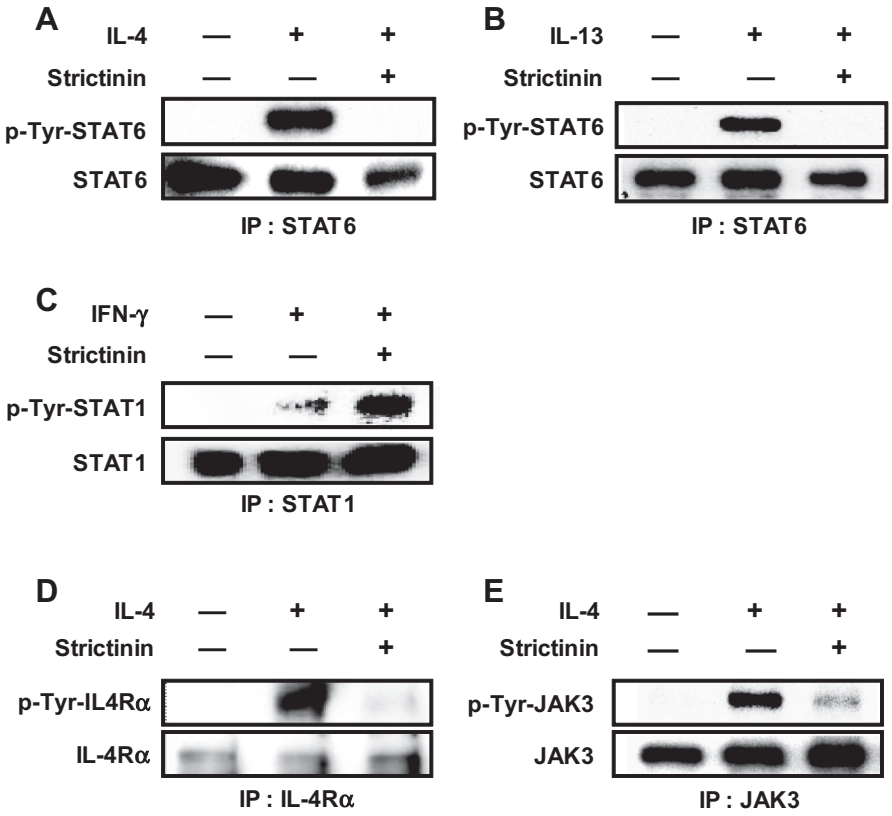

Fig. 2. Strictinin inhibits IL-4 and IL-13 signaling through suppression of IL-4Rα activation. (A) HeLa cells were treated with hrIL-4 for 30 min in the presence of strictinin. (B) NIH-3T3 cells were treated with mrIL-13 and strictinin for 30 min. Cell lysates were then immunoprecipitated with anti-STAT6 antibody and analyzed by Western blotting using an anti-phosphotyrosine antibody (PY20). (C) DND39 cells were treated with hrIFN-γ and strictinin for 30 min. Cell lysates were then immunoprecipitated with anti-STAT1 antibody and analyzed by Western blotting using an anti-pTyr-STAT1 antibody. (D and E) DND39 cells were treated with strictinin in the presence of hrIL-4 for 10 min and lysed in lysis buffer. The lysates were then immunoprecipitated with an antibody against IL-4Rα or JAK3. Immunoprecipitates were analyzed by 8% SDS–PAGE and Western blotting using PY20 to detect phosphorylated IL-4Rα and JAK3. Blot was then reprobed with anti-IL-4Rα and anti-JAK3 antibodies.

2.9. Assay for cell surface binding of IL-4

IL-4 binding to IL-4Rα was assessed by fluorescent immunostaining. After the treatment with strictinin or EGCG (25 μM) for 30 min, DND39 cells were blocked with 3% BSA-PBS, incubated with biotinylated anti-human IL-4 (PharMingen, San Diego, CA, USA) and then further incubated with quantum dot 605 streptavidin conjugates (Invitrogen, Garlsbad, CA, USA). Detection of IL-4 on the cell surface was performed using fluorescent microscopy (BZ-HIA/HIT2/HIM2; Keyence, Osaka, Japan). IL-4 binding signals were analyzed using by Image-J software.

2.10. Statistical analysis

Data were analyzed by analysis of variance using Graphpad Prism 5.0 software (Graphpad Software Inc., San Diego, CA, USA). Mean values were statistically significantly from the control group when $P < 0.05$.

3. Results

3.1. Structure–function relationship of strictinin on IL-4-induced STAT6 activation

IL-4 binds to IL-4Rα and induces activation of STAT6, which plays an essential role in IgE class switching [16,8]. We examined the effect of strictinin (Fig. 1A) on IL-4-induced phosphorylation of STAT6 in DND39 human Burkitt lymphoma B cells (Fig. 1B). Strictinin inhibited IL-4-induced STAT6 phosphorylation while EGCG, a major green tea polyphenol with a galloyl group, did not. To identify which components of strictinin have an inhibitory effect on STAT6 phosphorylation, we investigated the effect of its components (glucose, ellagic acid, pyrogallol or gallic acid) on IL-4-induced STAT6 phosphorylation (Fig. 1C). Only strictinin had a suppressive effect on STAT6 phosphorylation whereas its components had no inhibitory effect. These results indicate that galloyl glucose may be an important structure for the inhibitory effect of strictinin.

3.2. Strictinin inhibits IL-4 signaling by suppressing activation of IL-4Rα

IL-4R is divided into two types according to its different subunits. Type I IL-4R uses IL-4Rα and γc as a component of their receptors and type II IL-4R consists of IL-4Rα and IL-13Rα1 [17]. Type I IL-4R is mainly expressed in hematopoietic cells, whereas type II IL-4R is ubiquitously expressed [18]. To confirm the involvement of γc on the inhibitory effect of strictinin on IL-4-induced STAT6 activation, we examined the effect of strictinin on type II IL-4R signaling. Strictinin inhibited IL-4-induced STAT6 phosphorylation in HeLa cells, which expressed type II IL-4R [19] (Fig. 2A). Because Type II IL-4R could bind with IL-13 [20], we also evaluated the effect of strictinin on IL-13-induced STAT6 phosphorylation in NIH3T3 cells and found that IL-13-induced STAT6 phosphorylation was inhibited by strictinin (Fig. 2B), suggesting that strictinin exerts its inhibitory ability independent of γc. To explore whether the inhibitory effect of strictinin is specific for IL-4R, we investigated whether strictinin inhibits other cytokine signaling; strictinin did not suppress phosphorylation of STAT1 induced by IFN-γ

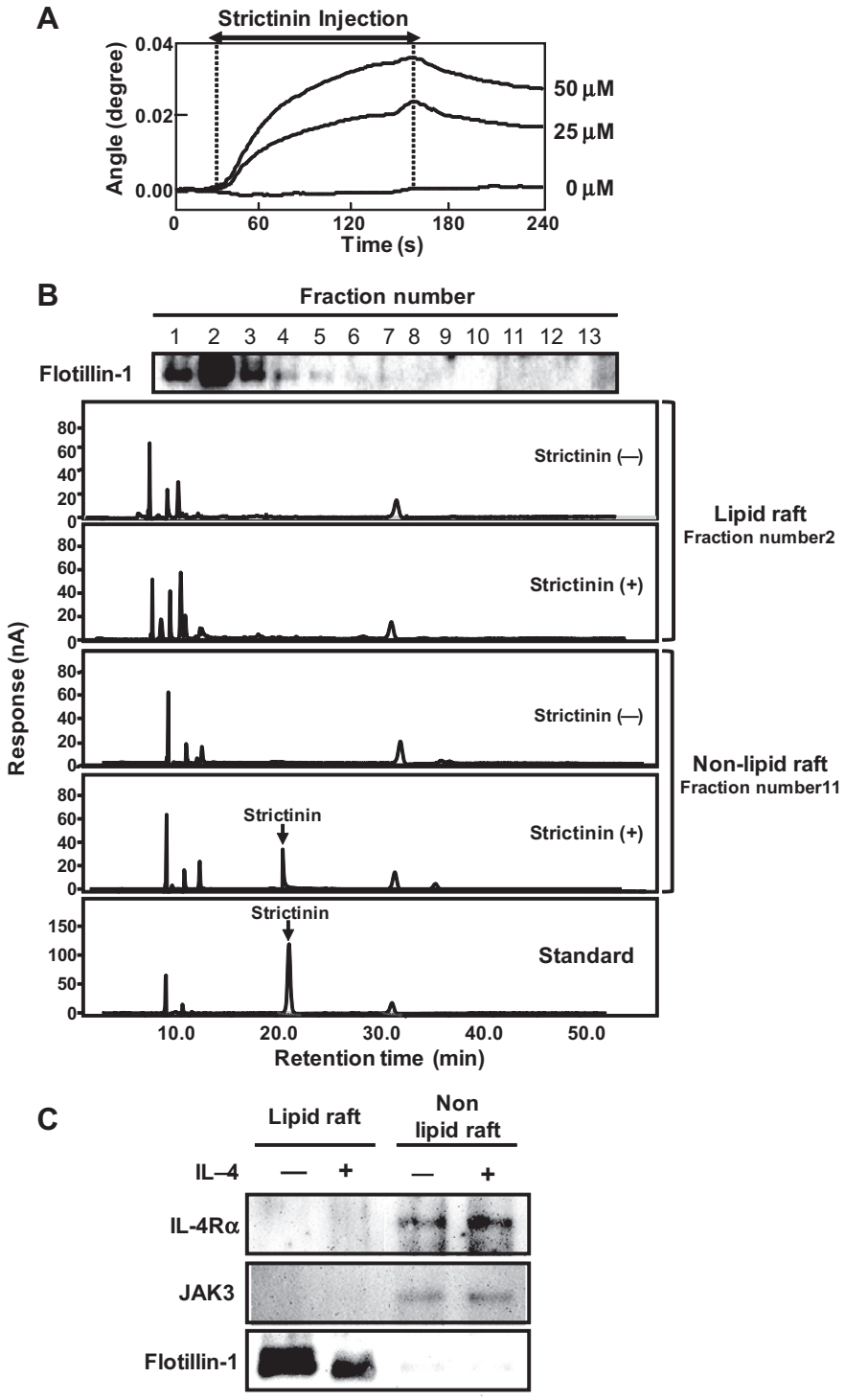

Fig. 3. Strictinin interacts with non-lipid raft regions. (A) DND39 cells were fixed on the sensor chip. The cell surface interaction of strictinin with immobilized DND39 cells was measured using a SPR biosensor. Strictinin was injected for the indicated interval in the figure. (B) DND39 cells were treated with or without strictinin. The cells were lysed with cold 1% Triton X-100 followed by a sucrose gradient centrifugation. The low-buoyant density fractions were designated as lipid raft fractions (number 1–6) and the high-buoyant density fractions were designated as non-lipid raft fractions (number 7–13). Each fraction was subjected to Western blotting. Flotillin-1 was used as a lipid raft marker. Lipid raft (fraction number 2) and non-lipid raft (fraction number 11) fractions were subjected to HPLC analysis for measurement of strictinin. The retention time for strictinin was confirmed using the strictinin standard. (C) DND39 cells were treated or untreated with hrIL-4 before lysis. Cell lysates were subjected to sucrose gradient centrifugation and fractions were collected and separated by 8% SDS–PAGE. Localization of IL-4Rα and JAK3 was analyzed by immunoblotting.

(Fig. 2C). These data indicate that strictinin specifically inhibits IL-4R inducing signaling.

Given these findings, we hypothesized that the target of strictinin may exist upstream of IL-4R signaling. Activation of JAKs by IL-4 stimuli results in phosphorylation of the cytoplasmic tyrosines in IL-4Rα, leading to the recruitment of STAT6 to the receptor, followed by STAT6 phosphorylation and activation [17]. Therefore, we examined the effect of strictinin on the IL-4-induced phosphor-

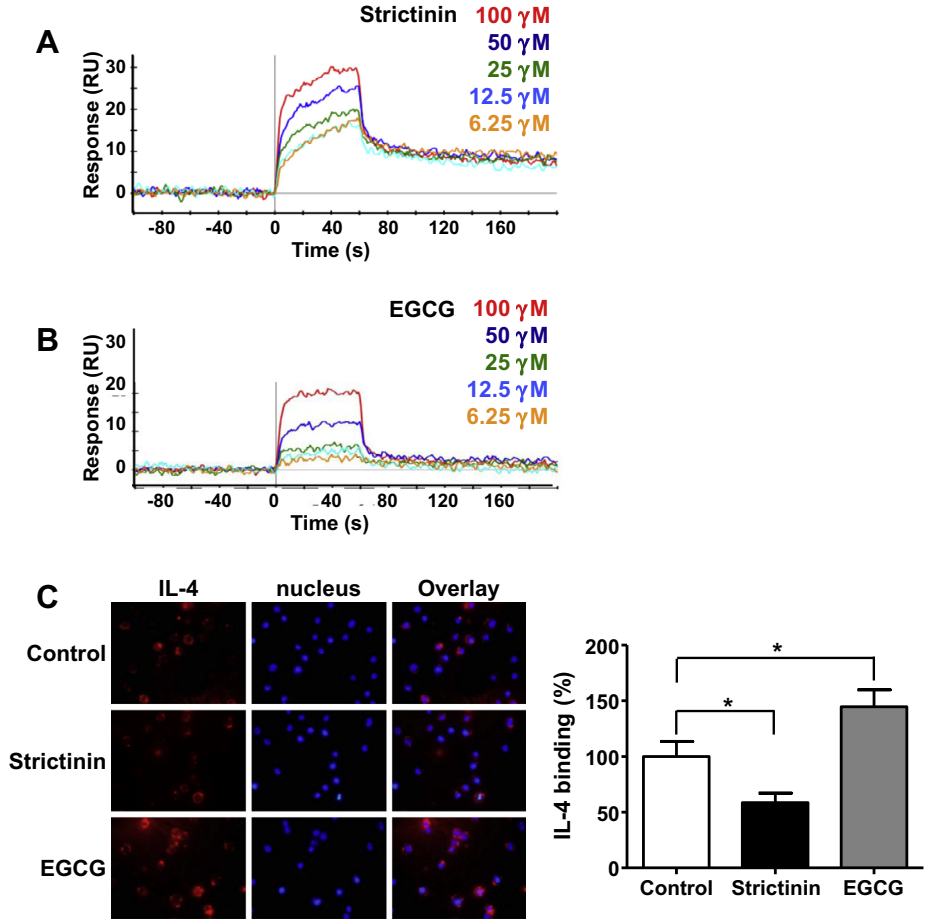

Fig. 4. Strictinin binds to IL-4Rα and inhibits binding of IL-4 to the cell surface. (A and B) Kinetic affinity analysis of IL-4Rα-binding strictinin or EGCG (0, 6.25, 12.5, 25, 50, and 100 μM) as recorded by SPR analysis with human recombinant IL-4Rα protein immobilized on the surface. (C) DND39 cells were treated with biotinylated anti-human IL-4 after treatment with strictinin or EGCG. Then, cells were mixed with streptavidin-conjugated Q dot 605 and were detected by fluorescence microscopy. Each data value is expressed as mean ± S.E.M ($n = 17$). Asterisks denote significant differences from the control at each condition at $^{*}P < 0.05$.

ylation of IL-4Rα and JAK3 in DND39 cells. As shown in Fig. 2D and E, strictinin inhibited the phosphorylation of IL-4Rα and JAK3 induced by IL-4. These results demonstrate that strictinin suppresses IL-4- or IL-13-induced STAT6 phosphorylation by attenuating the activation of IL-4Rα.

3.3. Strictinin interacts with non-lipid rafts regions localized IL-4Rα

To elucidate which interactions of strictinin with the cells are responsible for its inhibitory effect on IL-4R signaling, we examined the cell-surface binding of strictinin to DND39 cells using a SPR biosensor. As shown in Fig. 3A, the SPR signal was significantly increased by the injection of strictinin in a dose-dependent manner (Fig. 3A).

Next, we investigated where strictinin was located on the cell membrane. Lipid raft fractions or non-lipid raft fractions were prepared from strictinin-treated cells by sucrose gradient fractionation. The level of strictinin in a lipid raft fraction (faction 2) or a non-lipid raft fraction (fraction 11) was determined by HPLC. Interestingly, the strictinin peak was not detected in the lipid raft fraction and was just detected in the non-lipid raft fraction (Fig. 3B). These results indicate that strictinin specifically localizes on the non-lipid raft region of cell membrane, where it may exert its inhibitory effect on IL-4R signaling.

Next, we evaluated the location of IL-4Rα and JAK3, possible candidates target molecules of strictinin. Interestingly, IL-4Rα and JAK3 were also observed in the non-lipid raft region of the cell membrane (Fig. 3C). Following treatment with IL-4, there were no changes in localization.

3.4. Kinetic analysis of strictinin binding to IL-4Rα

We examined whether strictinin directly binds to IL-4Rα. The binding affinities of various concentration of strictinin and EGCG toward human recombinant IL-4Rα protein were measured using SPR (Fig. 4A and B). The K_D value of strictinin binding to IL-4Rα was determined as 4.53 μM while that of EGCG, which was mainly located in the lipid raft region of the cell surface [21], was 155 μM by equilibrium analysis (Fig. S1). These data suggest that strictinin directly interacts with IL-4Rα.

3.5. Strictinin inhibits binding IL-4 to IL-4Rα

Finally, we examined whether strictinin inhibits binding IL-4 to IL-4Rα using fluorescent immunostaining. Strictinin significantly inhibited cell surface binding of IL-4, but EGCG did not (Fig. 4C). These data indicate that the inhibitory effect of strictinin on IL-4R-induced signaling may be due to the inhibition of IL-4 binding to IL-4Rα.

4. Discussion

This is the first report to reveal the target molecule of strictinin, an ellagitannin in green tea, on the cell membrane to be STAT6 phosphorylation, which leads to IgE class switching. We present direct evidence showing that IL-4Rα localized to the non-lipid raft region is the first target molecule of strictinin to inhibit STAT6 activation induced by IL-4R signaling.

Strictinin is one of the hydrolysable tannins that can be described as esters of gallic acid with a polyol (typically β-D-glucose). It can be subject to oxidation reactions that form linkages between suitably orientated galloyl residues to yield hexahydroxydiphenoyl (HHDP) moieties, thus giving rise to the second subclass of hydrolysable tannins known as ellagitannins [22]. In a previous study, we reported that pentagalloylglucose (PGG), a representative hydrolysable tannin and a fully galloylated glucose derivative, also inhibits IgE production through suppression of IL-4R-inducing STAT6 activation [23]. On the other hands, EGCG, a major green tea polyphenol with galloyl group like strictinin, has a low affinity (K_D = 155 μM) against IL-4Rα while the K_D value of EGCG is 39.9 nM against a 67LR [24]. This is consistent with the finding that EGCG has no effect on the cell surface binding of IL-4 and IL-4-induced STAT6 phosphorylation. These facts suggest that galloyl glucose moiety may be an important structure for the inhibitory effect of strictinin.

There are many kinds of ellagitannin present in various fruits and nuts such as pomegranate, black raspberries, raspberries, strawberries, walnuts, and almonds [25,26]. Although there have been no studies investigating whether other ellagitannins besides strictinin have an inhibitory effect on IL-4R signaling, our results present the possibility that ellagitannins with a galloyl glucose moiety have an inhibitory effect on IL-4R signaling, although this needs to be explored in future studies.

We found that strictinin localizes in non-lipid raft fractions while EGCG is located in lipid raft fractions [21]. In addition, we found that IL-4Rα is localized on the non-lipid raft region whereas 67 LR is located on lipid raft region [21,27]. These observations suggest that the localization of polyphenol on the cell surface is dependent on the localization of the target molecules of polyphenols.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgment

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.06.069.

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