UCSF
UC San Francisco Previously Published Works

Title
The dietary ingredient, genistein, stimulates cathelicidin antimicrobial peptide expression through a novel S1P-dependent mechanism.

Permalink
https://escholarship.org/uc/item/9nj8k6x2

Journal
The Journal of Nutritional Biochemistry, 25(7)

Authors
Park, Kyungho
Kim, Young-Il
Shin, Kyong-Oh
et al.

Publication Date
2014-07-01

DOI
10.1016/j.jnutbio.2014.03.005

Peer reviewed

eScholarship.org Powered by the California Digital Library
University of California

Author Manuscript
Author Manuscript
Author Manuscript
Author Manuscript

HHS Public Access

Author manuscript

J Nutr Biochem. Author manuscript; available in PMC 2015 July 01.

Published in final edited form as:

J Nutr Biochem. 2014 July ; 25(7): 734–740. doi:10.1016/j.jnutbio.2014.03.005.

The dietary ingredient, genistein, stimulates cathelicidin antimicrobial peptide expression through a novel S1P-dependent mechanism

Kyungho Park[a,b,c], **Young-Il Kim**[a,b,c], **Kyong-Oh Shin**[d], **Ho Seong Seo**[e], **Jong Youl Kim**[b,c,f], **Taj Mann**[a,b,c], **Yuko Oda**[b,c,f], **Yong-Moon Lee**[d], **Walter M. Holleran**[a,b,c], **Peter M. Elias**[a,b,c], and **Yoshikazu Uchida**[a,b,c,*]

[a]Department of Dermatology, School of Medicine, University of California, San Francisco, San Francisco, CA 94121, USA

[b]Department of Veterans Affairs Medical Center, San Francisco, CA 94121, USA

[c]Northern California Institute for Research and Education, San Francisco, CA 94121, USA

[d]College of Pharmacy, Chungbuk National University, Cheongju 361-763, South Korea

[e]Radiation Research Division, Korea Atomic Energy Research Institute, Jeongeup 580-185, South Korea

[f]Department of Medicine and Endocrinology, School of Medicine, University of California, San Francisco, San Francisco, CA 94121, USA

Abstract

We recently discovered that a signaling lipid, sphingosine-1-phosphate (S1P), generated by sphingosine kinase 1, regulates a major epidermal antimicrobial peptide's [cathelicidin antimicrobial peptide (CAMP)] expression *via* an NF-κB→C/EBPα-dependent pathway, independent of vitamin D receptor (VDR) in epithelial cells. Activation of estrogen receptors (ER) by either estrogens or phytoestrogens also is known to stimulate S1P production, but it is unknown whether ER activation increases CAMP production. We investigated whether a phytoestrogen, genistein, simulates CAMP expression in keratinocytes, a model of epithelial cells, by either a S1P-dependent mechanism(s) or the alternate VDR-regulated pathway. Exogenous genistein, as well as a ER-β ligand, WAY-200070, increased CAMP mRNA and protein expression in cultured human keratinocytes, while ER-β antagonist, ICI182780, attenuated the expected genistein- and WAY-200070-induced increase in CAMP mRNA/protein expression. Genistein treatment increased acidic and alkaline ceramidase expression and cellular S1P levels in parallel with increased S1P lyase inhibition, accounting for increased CAMP production. In contrast, *si*RNA

© 2014 Elsevier Inc. All rights reserved.

*Corresponding author: Yoshikazu Uchida, Ph.D., Dermatology Service (190), Veterans Affairs Medical Center, 1700 Owens Street, Room 326, San Francisco, California 94158, U.S.A., Tel: (415) 575-0524, Fax: (415) 750-2106, uchiday@derm.ucsf.edu.

The authors have declared no conflict of interest.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Author Manuscript
Author Manuscript
Author Manuscript
Author Manuscript

against VDR did not alter genistein-mediated upregulation of CAMP. Taken together, genistein induces CAMP production *via* an ER-β→S1P→NF-κB→C/EBPα-rather than a VDR-dependent mechanism, illuminating a new role for estrogens in the regulation of epithelial innate immunity and pointing to potential additional benefits of dietary genistein in enhancing cutaneous antimicrobial defense.

Keywords

Cathelicidin antimicrobial peptide; Estrogen receptor β; Genistein; Keratinocytes; Innate immunity

1. Introduction

Antimicrobial peptides (AMP) are innate immune elements that protect their host against pathogenic microbes [1]. A major epithelial AMP, cathelicidin antimicrobial peptide (CAMP), displays antimicrobial activity against a broad range of pathogens, including Gram-negative and Gram-positive bacteria, fungi, and certain viruses. CAMP displays not only antimicrobial activities, but it also modulates multiple cellular functions; *e.g.*, cytokine expression/production, angiogenesis, wound healing, and adaptive immune responses [2]. CAMP is an inducible AMP whose levels increase at both a transcriptional and post-transcriptional level in response to external perturbations; *e.g.*, pathogen exposure, wounding, UV irradiation, and barrier abrogation [3] that in turn increase endoplasmic reticulum stress [4].

We recently discovered that a signaling lipid, sphingosine-1-phosphate (S1P), upregulates a NF-κB-C/EBPα-dependent, (but VDR-independent) transcriptional pathway for CAMP in epithelial cells, in mouse epidermis, human epidermal keratinocytes, and HeLa cells [4, 5]. Importantly, this mechanism operates only after cells are exposed to external perturbations that induce endoplasmic reticulum stress, while VDR transactivity instead is suppressed under these conditions [4], suggesting that these two mechanisms adjust CAMP levels in epithelial tissues under circumstances that predominate under stress *vs.* basal conditions. Moreover, we more recently demonstrated a dietary plant-derived stilbenoid, resveratrol, stimulates CAMP production through our identified S1P-mediated pathway. Yet, the effects of dietary compounds on innate immunity (in particular AMP, including CAMP) are incompletely understood.

Estrogens and a phytoestrogen, the soy-derived isoflavone, genistein, alter the expression of certain genes, leading to diverse alterations in cellular functions, including increased proliferation, differentiation, apoptosis, and innate immunity [6]. The genomic mechanism classically involves ligand binding to one or both of two estrogen receptors, estrogen receptor-α (ER-α) and estrogen receptor-β (ER-β), forming homo- or hetero-dimers, followed by their translocation and binding to an ER binding element (ERE) in nuclear DNA [7, 8]. Estrogen and estradiol preferentially bind to ER-α and ER-β, respectively, while estradiol exhibits equivalent affinity towards both receptors [7, 8].

Estrogens can also modulate cell functions by non-genomic mechanisms through binding to the G protein-coupled receptor, GPR30, which is widely distributed on cellular membranes.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

This mechanism then modulates cellular functions through the MAP kinase pathway [9, 10], among others. Though genistein exhibits a higher binding affinity for ER-β than for ER-α [11], it also binds to GPR30 [12]. Of potential pertinence to CAMP expression, genistein increases CYP27B1 expression, the enzyme that generates 1α, 25-dihydroxy vitamin D3 [13], the natural ligand of the VDR. But recent studies in breast cancer cells show that genistein also induces both acidic ceramidase expression, resulting in increased catalytic activity, and sphingosine kinase (SPHK) 1 mRNA expression, which likely increases S1P production (ceramide→sphingosine→S1P) [14]. Although S1P stimulates tumor growth in breast cancer cells [14], the impact of S1P appears to depend upon cell and tissue types. In keratinocytes, S1P stimulates differentiation rather than increasing cell proliferation [15].

Because either an estrogen receptor and/or GPR30 activation could increase VDR ligand production, and increased ceramidase expression/activation could also lead to enhanced S1P production, genistein could upregulate CAMP expression *via* either a VDR- and/or S1P-dependent mechanism(s). Our present studies indicate that genistein stimulates CAMP production *via* ER-β activated (but not stimulated by GPR activation) stimulation of S1P signal, and does not involve a VDR-dependent mechanism. The insights from these studies illuminate a previously unidentified role for this phytoestrogen in regulating epithelial innate immunity.

2. Materials and methods

2.1. Cell culture

Immortalized, nontransformed, low-passage number (HaCaT) keratinocytes (KC) derived from human epidermis were a gift from Dr. N. Fusenig (Heidelberg, Germany). HaCaT KC were maintained, as described previously [16]. Primary cultured human KC were isolated from human neonatal foreskins by a modification of the method of Pittelkow and Scott [17, 18] under an Institutional Review Board-approval protocol (University of California, San Francisco). Cells were grown in a keratinocyte growth medium as above. Culture medium was switched to serum-free keratinocyte growth medium 154CF containing 0.07 mM calcium chloride and growth supplements (Invitrogen, Carlsbad, CA) one day prior to genistein treatment. Keratinocytes grown at 60% to 70% confluency were exposed to genistein, which did not induce cell death at 50 μM.

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

Quantitative real-time polymerase chain reaction (*q*RT-PCR) was performed using 30 ng of cDNA prepared from mRNA fraction of cell lysates, specific primer sets (final concentration, 250 nM) (previously reported [4, 19, 20]), and SYBR green reagents, as described previously [19].

2.3. Western immunoblot analysis

Western immunoblot analysis was performed as described previously [4]. Cell lysates (25 μg protein per lane), prepared in RIPA buffer, were resolved by electrophoresis on 4-12% Bis-Tris Gel (Invitrogen, Carlsbad, CA). Resultant bands blotted onto nitrocellulose membranes, were probed with anti-CAMP (LifeSpan BioSciences, Seattle, WA), anti-human β-actin

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

(Abcam, Cambridge, MA), and detected using enhanced chemiluminescence (Thermo Scientific, Waltham, MA).

2.4. ELISA for CAMP/LL-37 quantification

CAMP/LL-37 content of cell lysates and conditioned medium of KC previously incubated with genistein are determined by ELISA kit (Hycult Biotech Inc., Plymouth Meeting, PA) in accordance with the manufacturer's instructions.

2.5 Immunohistochemistry

Immunohistochemistry was performed, as described previously [21]. KC were treated with genistein or vehicle for 24 h. CAMP distribution was assessed using anti-CAMP (LifeSpan BioSciences) and anti-rabbit IgG conjugated with fluorescein isothiocyanate (Invitrogen). Cells were counterstained with the nuclear marker 4′,6-diaminido-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA) and images were viewed under a fluorescence microscope (Carl Zeiss, Thornwood, NY).

2.6. siRNA and transfections

KC were transfected with 20 nM *si*RNA for VDR or non-targeted, control *si*RNA (Dharmacon, Lafayette, CO), using siLentFect (Bio-Rad, Hercules, CA), as previously described [22].

2.7. Measurement of intracellular levels of sphingosine-1-phosphate

To assess the levels of cellular S1P, cultured human keratinocyte (CHK) were pretreated with or without SPHK1 inhibitor, SKI, were incubated with genistein and washed with phosphate-buffered saline followed by extraction of total S1P, as we reported previously [20]. S1P was derivatized with o-phthalaldehyde (OPA) reagent and then quantitated using an HPLC system equipped with a fluorometrical detector system (JASCO, Tokyo, Japan), as described previously [23]. S1P levels were expressed as pmol per mg protein.

2.8. Enzyme activity assay for sphingosine-1-phosphate lyase

To determine the activity of S1P lyase, CHK were treated with genistein followed by washing with phosphate-buffered saline. Cell lysates were prepared in lysis buffer, as reported previously [24]. Cell lysates (50 μg) were incubated with 10 nmol S1P for 20 min. The reaction was stopped by lipid extraction with the addition of 100 pmol of (2E)-d_5-hexadecenal as the internal standard. Total lipid extracts were derivatized with 5 mM semicarbazide hydrochloride in methanol containing 5% formic acid at 40°C for 2 h and analyzed by ESI-LC/MS/MS (Thermo Scientific, Waltham, MA), as described previously [24]. The activity of S1P lyase is expressed as pentadecanal pmol per mg protein per min.

2.9. Statistical Analyses

The differences among treatments were determined by either the unpaired Student *t* Test or the one-way ANOVA coupled with Duncan's multiple comparison test. Results from one (each) experiment are triplicate. We also performed separate experiments using different batches of cells. The p values were <0.01 in all cases except where indicated.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

3. Results

3.1. Genistein stimulates ER-β and CAMP expression in cultured human keratinocytes (KC)

Prior studies have shown that estrogen receptor activation induces feed-forward upregulation of bound estrogen receptor isoforms [25]. Therefore, we first ascertained whether genistein activates estrogen receptor expression in KC. Because genistein is a known activator of ER-β, but not ER-α, *q*RT-PCR analysis showed the expected increase in ER-β, but not ER-α mRNA expression in KC following genistein treatment (Fig. 1). We next investigated whether exogenous genistein stimulates CAMP mRNA expression in KC. *q*RT-PCR analysis revealed a significant dose-dependent increase in CAMP mRNA expression in normal human KC (Fig. 2A and Fig. 3A), following treatment with exogenous genistein (0.1-50 μM). Genistein also increases CAMP mRNA expression in immortalized, nontransformed HaCaT KC (Fig. 3B). Consistent with increased levels of CAMP mRNA, Western immunoblot analysis showed that CAMP protein levels also increase in both types of KC following incubation with genistein (50 μM) (Fig. 3C). Together, these results suggest that genistein increased CAMP production through ER-β activation. Since genistein at concentrations 1 mM did not significantly alter cell viability (not shown), we employed genistein at concentrations of 50 μM in all of the subsequent studies described below.

3.2. Genistein upregulates CAMP expression by ER-β, but not by GPR30 activation

We next investigated whether ER-β and/or GPR30, both of which are known to be activated by genistein, is (are) required for genistein-stimulated CAMP upregulation. Not only genistein, but also the specific ER-β agonist, WAY-200070 (1 μM), significantly increased CAMP expression in both HaCaT KC and KC (Fig. 3A and B, respectively). In contrast, blockade of ER-β-induced activation of GPR30, using a specific antagonist of ER-α/-β, ICI182780, which also serves as specific agonist of GRP30 [26], significantly attenuated the genistein-induced increase in CAMP mRNA expression in both HaCaT KC and KC co-treated with genistein (Fig. 3A and B). Consistent with changes in CAMP mRNA levels, Western immunoblot and immunohistochemical analyses revealed decreased CAMP protein expression in CHK co-treated with ICI182780 (1 μM) (Fig. 3C and D, respectively). Together, these results suggest that genistein likely modulates CAMP expression by an ER-β, but not by a GPR30-dependent mechanism.

3.3. Mechanisms responsible for genistein-mediated stimulation of CAMP expression

To investigate the downstream mechanism(s) responsible for genistein-induced CAMP upregulation, we first assessed the potential involvement of the VDR-dependent pathway. *q*PCR results showed that VDR expression was not altered following treatment of KC with genistein (Fig. 4A). Instead, genistein suppressed mRNA expression for the endogenous VDR ligand-deactivating enzyme, 24-hydroxylase (CYP24A1), which downregulates VDR-mediated transcriptional regulation (Fig. 4B), suggesting that VDR pathway is attenuated by genistein treatment. Next, we directly assessed whether silencing of VDR, using *si*RNA, alters genistein-mediated upregulation of CAMP. In initial studies, basal both VDR and CAMP expressions were not altered by control *si*RNA, while these expressions significantly declined in cells treated with *si*RNA against VDR (Fig. 4C), but CAMP expression was still elevated by genistein (Fig. 4D).

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Next, we investigated whether genistein instead regulates CAMP by stimulating the recently identified S1P-dependent mechanism [5]. We inhibit sphingosine kinase 1, which generates S1P required for the S1P-induced stimulation of CAMP expression using a sufficient concentration of specific pharmacological inhibitor, SKI, significantly decreases basal S1P levels [5], while S1P levels increased significantly following genistein treatment of KC (Table 1 and 2). However, blockade of S1P synthesis by SKI affects genistein-mediated upregulation of neither CAMP mRNA expression, nor S1P contents (Table 2). Alternatively, *q*PCR analysis demonstrated that acidic and alkaline, but not neutral ceramidase, expression, increased significantly in KC following genistein treatment (Fig. 5A). Pertinently, genistein significantly suppressed SPHK 2 mRNA expression, a negative regulator of S1P signaling of CAMP expression in KC [5], while SPHK 1 expression was not altered (Fig. 5B). Thus, genistein appears to stimulate hydrolysis of ceramide to sphingosine, a necessary first step in S1P production, in parallel with suppression of a negative regulator of S1P signal to stimulate CAMP production.

Our prior studies demonstrated that blockade of S1P lyase activity/expression increases cellular S1P levels, thereby stimulating CAMP production in both CHK and murine epidermis [5]. Therefore, we next investigated whether genistein decreases S1P hydrolysis by inhibiting S1P lyase expression, thereby contributing to increased S1P levels. Indeed, genistein treatment modestly (albeit statistically significantly) decreased both S1P lyase expression (Fig. 5C) and S1P lyase activity in KC (Table 3). Together, these results indicate that an S1P-dependent, rather than a VDR-dependent mechanism likely accounts for the genistein-induced stimulation of CAMP expression.

4. Discussion

Estrogen regulates cellular functions through ER-α and/or ER-β, which are highly expressed in a diverse range of epithelial tissues, such as lung alveoli, ovary, mammary gland, and vagina [27] as well as skin [28]. Inactivation of either ER-α and/or ER-β caused by menopause-related estrogen deficiency leads to numerous epithelial dysfunctions, *e.g*., reduced alveolar regeneration [29], ovary follicular immaturation [30], failed terminal differentiation of mammary epithelial cells [31], vaginal atrophy [32]. In addition, deficiency in estrogens alters numerous cutaneous functions, producing delays in wound healing, as well as accelerating features of cutaneous aging, such as reduced elasticity and dehydration due to alterations in connective tissue and hyaluronan metabolism [33]. Accordingly, estrogen replacement therapy has been employed for the treatment of post-menopausal symptoms, and topical estrogens have been widely deployed as anti-skin aging agents [34. 35]. Because synthetic administration of estrogen has raised concerns about enhanced tumorgenesis [36], replacement therapy has shifted to plant-derived compounds, *e.g.,* genistein, daidzein, biochanin A, or coumestrol, which all show estrogenic activity [37, 38]. Importantly, in contrast to estrogens, certain of these phytoestrogens, including genistein, show anti-tumor activity due in part to their antioxidant activities [39].

We have demonstrated a novel mechanism whereby genistein upregulates epithelial innate immunity by increasing production of the antimicrobial peptide, CAMP. CAMP is regulated by both VDR-dependent and VDR-independent mechanisms, the latter signaled under

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

endoplasmic reticulum stressed conditions by sphingosine-1-phosphate (S1P), which in turn initiates an NFκ-B→C/EBPα transcriptional mechanism of CAMP expression [5]. Importantly, it is possible to directly stimulate S1P→NF-κB→C/EBPα signal with certain exogenous natural-occurring compounds, such as resveratrol [21]; and the phytoestrogen, genistein, provides another example, though it operates *via* other downstream mechanisms than resveratrol. Additionally, curcumin is a naturally occurring polyphenolic compound that has a weak phytoestrogen activity [40], as well as potent antioxidant, anti-inflammatory, anticancer, antiviral and antifungal activities [41, 42, 43]. Recent study showed that curcumin stimulates CAMP expression in human monocytic U937 cells and human colonic epithelial HT-29 cells independent of both VDR- and ER stress-mediated mechanisms [44]. Yet, the mechanism of curcumin-mediated CAMP expression remains unknown. Curcumin suppresses NF-κB activation [45]. Therefore, our prior studies support the finding that curcumin stimulates CAMP expression by ER stress-independent mechanism, because ER stress-mediated upregulation of CAMP requires NF-κB activation [4].

While several consensus transcription factor-binding sequences, *e.g.,* Sp1, C/EBP, PU.1, as well as VDRE, are present on the promoter sequence of the CAMP gene, no putative estrogen receptor binding element (ERE) has been identified on the respective promoter [46]. We demonstrated here that it is the recently characterized S1P, rather than the VDR pathway that accounts for genistein-mediated transcriptional regulation of CAMP. Genistein appears to regulate this innate immune element by the former (VDR-independent) mechanism, because we showed here that 1) genistein does not alter VDR expression, and even suppresses the expression of CYP24A1 that deactivates the VDR pathway by decreased endogenous ligand levels; 2) VDR silencing does not affect genistein-induced CAMP expression; 3) genistein increases cellular S1P levels by increasing in genistein-mediated stimulation of ceramide hydrolysis by acidic and alkaline ceramidase activation, along with a corresponding decline in S1P lyase activity; and 4) genistein stimulates C/EBP mRNA expression mechanism that we previously showed to regulate CAMP gene expression. Importantly, genistein also suppressed SPHK 2 mRNA expression, which we showed recently to be a negative regulator of CAMP expression [5]. Yet, blockade of sphingosine kinase 1 using a specific inhibitor (SKI) did not attenuate increased CAMP expression and cellular S1P levels by genistein. Hence, since we treated cells with a sufficent concentration of sphingosine kinase 1 inhibitor [5], multiple other activities, *i.e.,* both an increase in S1P levels by S1P lyase inhibition and in ceramide hydrolysis by ceramidase activation, as well as suppression of SPHK 2 expression, suffice to stimulate CAMP expression in CHK following genistein treatment.

Although recent studies showed that genistein induces acidic ceramidase gene expression through a GPR30-dependent mechanism in breast cancer cells, suggesting that increasing ceramide catabolism could lead to increased S1P production [14], we showed here that genistein increases CAMP expression by ER-β, but not GPR30 activation. In addition, genistein stimulated *acidic* and *alkaline* ceramidase, rather than *neutral* ceramidase in CHK.

In summary, we demonstrated here that genistein improves innate immunity through enhanced production of the multifunctional antimicrobial peptide, CAMP. We show further

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

the following mechanism; genistein activities ER-β, followed by induction of the S1P→NF-κB→C/EBPα signaling pathway, leads to enhance CAMP production.

Acknowledgments

Funding sources: This study was supported by grants from the National Rosacea Society and the National Institutes of Health (AR051077 and AR062025 [the National Institute of Arthritis and Musculoskeletal and Skin Diseases] to YU).

The authors thank to Ms. Sally Pennypacker for technical support in cell culture. We thank Ms. Joan Wakefield for superb editorial assistance.

References

1. Nijnik A, Hancock RE. The roles of cathelicidin LL-37 in immune defences and novel clinical applications. Curr Opin Hematol. 2009; 16:41–7. [PubMed: 19068548]
2. Lai Y, Cogen AL, Radek KA, Park HJ, Macleod DT, Leichtle A, et al. Activation of TLR2 by a small molecule produced by Staphylococcus epidermidis increases antimicrobial defense against bacterial skin infections. J Invest Dermatol. 2010; 130:2211–21. [PubMed: 20463690]
3. Schauber J, Gallo RL. Antimicrobial peptides and the skin immune defense system. J Allergy Clin Immunol. 2009; 124:R13–8. [PubMed: 19720207]
4. Park K, Elias PM, Oda Y, Mackenzie D, Mauro T, Holleran WM, et al. Regulation of Cathelicidin Antimicrobial Peptide Expression by an Endoplasmic Reticulum (ER) Stress Signaling, Vitamin D Receptor-independent Pathway. J Biol Chem. 2011; 286:34121–30. [PubMed: 21832078]
5. Park K, Elias PM, Shin KO, Lee YM, Hupe M, Borkowski AW, et al. A novel role of a lipid species, sphingosine-1-phosphate, in epithelial innate immunity. Mol Cell Biol. 2013; 33:752–62. [PubMed: 23230267]
6. Barnes S. The biochemistry, chemistry and physiology of the isoflavones in soybeans and their food products. Lymphat Res Biol. 2010; 8:89–98. [PubMed: 20235891]
7. Spelsberg TC, Subramaniam M, Riggs BL, Khosla S. The actions and interactions of sex steroids and growth factors/cytokines on the skeleton. Mol Endocrinol. 1999; 13:819–28. [PubMed: 10379881]
8. Klinge CM. Estrogen receptor interaction with estrogen response elements. Nucleic Acids Res. 2001; 29:2905–19. [PubMed: 11452016]
9. Prossnitz ER, Arterburn JB, Smith HO, Oprea TI, Sklar LA, Hathaway HJ. Estrogen signaling through the transmembrane G protein-coupled receptor GPR30. Annu Rev Physiol. 2008; 70:165–90. [PubMed: 18271749]
10. Prossnitz ER, Maggiolini M. Mechanisms of estrogen signaling and gene expression via GPR30. Mol Cell Endocrinol. 2009; 308:32–8. [PubMed: 19464786]
11. Kuiper GG, Lemmen JG, Carlsson B, Corton JC, Safe SH, van der Saag PT, et al. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. Endocrinology. 1998; 139:4252–63. [PubMed: 9751507]
12. Thomas P, Dong J. Binding and activation of the seven-transmembrane estrogen receptor GPR30 by environmental estrogens: a potential novel mechanism of endocrine disruption. J Steroid Biochem Mol Biol. 2006; 102:175–9. [PubMed: 17088055]
13. Somjen D, Weisman Y, Kohen F, Gayer B, Limor R, Sharon O, et al. 25-hydroxyvitamin D3-1alpha-hydroxylase is expressed in human vascular smooth muscle cells and is upregulated by parathyroid hormone and estrogenic compounds. Circulation. 2005; 111:1666–71. [PubMed: 15795327]
14. Lucki NC, Sewer MB. Genistein stimulates MCF-7 breast cancer cell growth by inducing acid ceramidase (ASAH1) gene expression. J Biol Chem. 2011; 286:19399–409. [PubMed: 21493710]
15. Geilen CC, Barz S, Bektas M. Sphingolipid signaling in epidermal homeostasis. Current knowledge and new therapeutic approaches in dermatology. Skin Pharmacol Appl Skin Physiol. 2001; 14:261–71. [PubMed: 11586067]

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

16. Uchida Y, Murata S, Schmuth M, Behne MJ, Lee JD, Ichikawa S, et al. Glucosylceramide synthesis and synthase expression protect against ceramide-induced stress. J Lipid Res. 2002; 43:1293–302. [PubMed: 12177173]
17. Pittelkow MR, Scott RE. New techniques for the in vitro culture of human skin keratinocytes and perspectives on their use for grafting of patients with extensive burns. Mayo Clin Proc. 1986; 61:771–7. [PubMed: 2427897]
18. Holleran WM, Williams ML, Gao WN, Elias PM. Serine-palmitoyl transferase activity in cultured human keratinocytes. J Lipid Res. 1990; 31:1655–61. [PubMed: 2246616]
19. Houben E, Holleran WM, Yaginuma T, Mao C, Obeid LM, Rogiers V, et al. Differentiation-associated expression of ceramidase isoforms in cultured keratinocytes and epidermis. J Lipid Res. 2006; 47:1063–70. [PubMed: 16477081]
20. Uchida Y, Houben E, Park K, Douangpanya S, Lee YM, Wu BX, et al. Hydrolytic pathway protects against ceramide-induced apoptosis in keratinocytes exposed to UVB. J Invest Dermatol. 2010; 130:2472–80. [PubMed: 20520628]
21. Park K, Elias PM, Hupe M, Borkowski AW, Gallo RL, Shin KO, et al. Resveratrol stimulates sphingosine-1-phosphate signaling of cathelicidin production. J Invest Dermatol. 2013; 133:1942–9. [PubMed: 23856934]
22. Schauber J, Oda Y, Büchau AS, Yun QC, Steinmeyer A, Zügel U, et al. Histone acetylation in keratinocytes enables control of the expression of cathelicidin and CD14 by 1,25-dihydroxyvitamin D3. J Invest Dermatol. 2008; 128:816–24. [PubMed: 17943182]
23. Shin HW, Kim D, Lee Y, Yoo HS, Lee BJ, Kim JS, et al. Alteration of sphingolipid metabolism and pSTAT3 expression by dietary cholesterol in the gallbladder of hamsters. Arch Pharm Res. 2009; 32:1253–62. [PubMed: 19784582]
24. Berdyshev EV, Goya J, Gorshkova I, Prestwich GD, Byun HS, Bittman R, et al. Characterization of sphingosine-1-phosphate lyase activity by electrospray ionization-liquid chromatography/tandem mass spectrometry quantitation of (2E)-hexadecenal. Anal Biochem. 2011; 408:12–8. [PubMed: 20804717]
25. Staar S, Richter DU, Makovitzky J, Briese V, Bergemann C. Stimulation of endometrial glandular cells with genistein and daidzein and their effects on ERalpha- and ERbeta-mRNA and protein expresion. Anticancer res. 2005; 25:1713–8. [PubMed: 16033089]
26. Thomas P, Pang Y, Filardo EJ, Dong J. Identity of an estrogen membrane receptor coupled to a G protein in human breast cancer cells. Endocrinology. 2005; 146:624–32. [PubMed: 15539556]
27. Morani A, Warner M, Gustafsson JA. Biological functions and clinical implications of oestrogen receptors alfa and beta in epithelial tissues. J Intern Med. 2008; 264:128–42. [PubMed: 18513343]
28. Hasselquist MB, Goldberg N, Schroeter A, Spelsberg TC. Isolation and characterization of the estrogen receptor in human skin. J Clin Endocrinol Metab. 1980; 50:76–82. [PubMed: 7350189]
29. Massaro D, Clerch LB, Massaro GD. Estrogen receptor-alpha regulates pulmonary alveolar loss and regeneration in female mice: morphometric and gene expression studies. Am J Physiol Lung Cell Mol Physiol. 2007; 293:L222–8. [PubMed: 17449797]
30. Couse JF, Yates MM, Deroo BJ, Korach KS. Estrogen receptor-beta is critical to granulosa cell differentiation and the ovulatory response to gonadotropins. Endocrinology. 2005; 146:3247–62. [PubMed: 15831568]
31. Förster C, Mäkela S, Wärri A, Kietz S, Becker D, Hultenby K, et al. Involvement of estrogen receptor beta in terminal differentiation of mammary gland epithelium. Proc Natl Acad Sci USA. 2002; 99:15578–83. [PubMed: 12438700]
32. Yumru AE, Bozkurt M, Inci Co kun E, Baykan G. The use of local 17beta-oestradiol treatment for improving vaginal symptoms associated with post-menopausal oestrogen deficiency. J Int Med Res. 2009; 37:198–204. [PubMed: 19215691]
33. Emmerson E, Hardman MJ. The role of estrogen deficiency in skin ageing and wound healing. Biogerontology. 2012; 13:3–20. [PubMed: 21369728]
34. Verdier-Sévrain S, Bonté F, Gilchrest B. Biology of estrogens in skin: implications for skin aging. Exp Dermatol. 2006; 15:83–94. [PubMed: 16433679]
35. Shu YY, Maibach HI. Estrogen and skin: therapeutic options. Am J Clin Dermatol. 2011; 12:297–311. [PubMed: 21714580]

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

36. Grodstein F, Stampfer MJ, Colditz GA, Willett WC, Manson JE, Joffe M, et al. Postmenopausal hormone therapy and mortality. N Engl J Med. 1997; 336:1769–75. [PubMed: 9187066]
37. Keinan-Boker L, van Der Schouw YT, Grobbee DE, Peeters PH. Dietary phytoestrogens and breast cancer risk. Am J Clin Nutr. 2004; 79:282–8. [PubMed: 14749235]
38. Dornstauder E, Jisa E, Unterrieder I, Krenn L, Kubelka W, Jungbauer A. Estrogenic activity of two standardized red clover extracts (Menoflavon) intended for large scale use in hormone replacement therapy. J Steroid Biochem Mol Biol. 2001; 78:67–75. [PubMed: 11530286]
39. Wei H, Saladi R, Lu Y, Wang Y, Palep SR, Moore J, et al. Isoflavone genistein: photoprotection and clinical implications in dermatology. J Nutr. 2003; 133:3811S–9S. [PubMed: 14608119]
40. Bachmeier BE, Mirisola V, Romeo F, Generoso L, Esposito A, Dell'eva R, et al. Reference profile correlation reveals estrogen-like trancriptional activity of Curcumin. Cell Physiol Biochem. 2010; 26:471–82. [PubMed: 20798532]
41. Sharma OP. Antioxidant activity of curcumin and related compounds. Biochem Pharmacol. 1976; 25:1811–2. [PubMed: 942483]
42. Apisariyakul A, Vanittanakom N, Buddhasukh D. Antifungal activity of turmeric oil extracted from Curcuma longa (Zingiberaceae). J Ethnopharmacol. 1995; 49:163–9. [PubMed: 8824742]
43. Roth GN, Chandra A, Nair MG. Novel bioactivities of Curcuma longa constituents. J Nat Prod. 1998; 61:542–5. [PubMed: 9584408]
44. Guo C, Rosoha E, Lowry MB, Borregaard N, Gombart AF. Curcumin induces human cathelicidin antimicrobial peptide gene expression through a vitamin D receptor-independent pathway. J Nutr Biochem. 2013; 24:754–9. [PubMed: 22841393]
45. Singh S, Aggarwal BB. Activation of transcription factor NF-kappa B is suppressed by curcumin (diferuloylmethane) [corrected]. J Biol Chem. 1995; 270:24995–5000. [PubMed: 7559628]
46. Gombart AF, Borregaard N, Koeffler HP. Human cathelicidin antimicrobial peptide (CAMP) gene is a direct target of the vitamin D receptor and is strongly up-regulated in myeloid cells by 1,25-dihydroxyvitamin D3. Faseb J. 2005; 19:1067–77. [PubMed: 15985530]

Author Manuscript
Author Manuscript
Author Manuscript
Author Manuscript

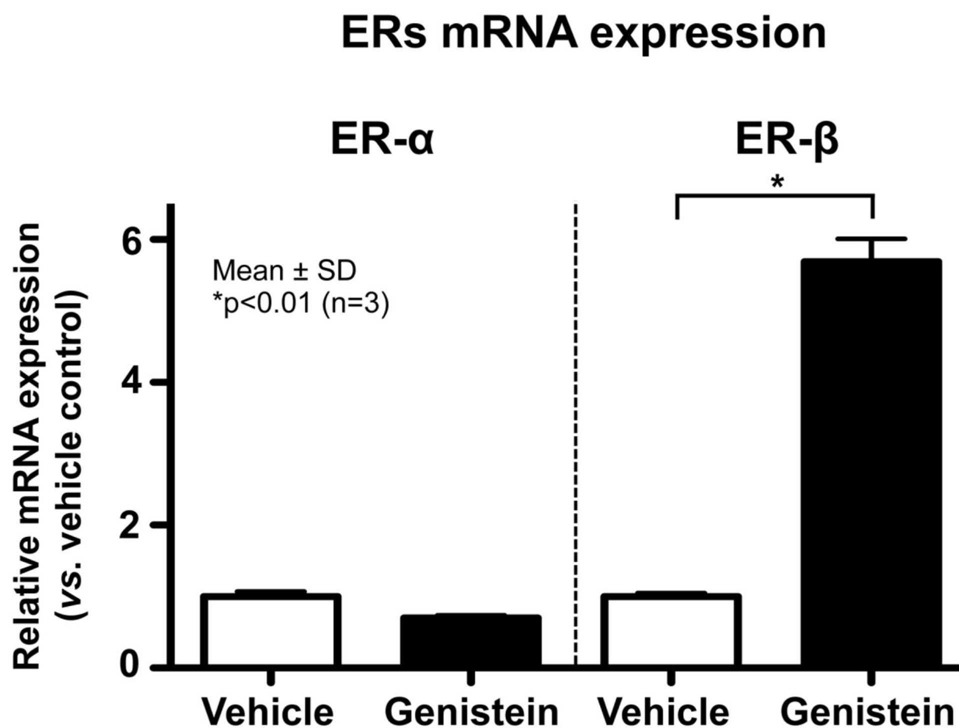

Fig. 1.

Genistein increases estrogen receptors mRNA expression. Primary cultured KC (CHK) were incubated with genistein (50 μM) for 24 h. ER-α and ER-β mRNA expression was determined by *q*RT-PCR. Similar results were obtained when the experiment was repeated (more than twice) using different cell preparations.

Author Manuscript
Author Manuscript
Author Manuscript
Author Manuscript

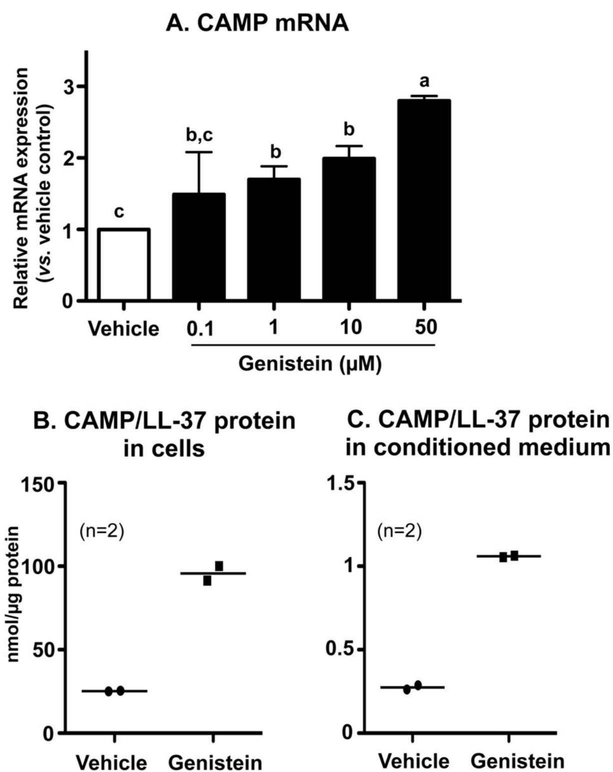

Fig. 2.

Increased expression of CAMP mRNA and protein in CHK following genistein. Primary cultured KC (CHK) were treated for 24 h with the indicated concentrations of genistein. CAMP mRNA expression was determined by *q*RT-PCR (A). CAMP and LL-37 (an active form of CAMP) protein/peptide levels quantified by ELISA (B and C). Similar results were obtained when the experiment was repeated (more than twice) using different cell preparations. Values are means ± SD (n=3). One-way ANOVA coupled with Duncan's multiple comparison test (A). Means with different letters are significantly different at $p < 0.01$ (B and C).

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

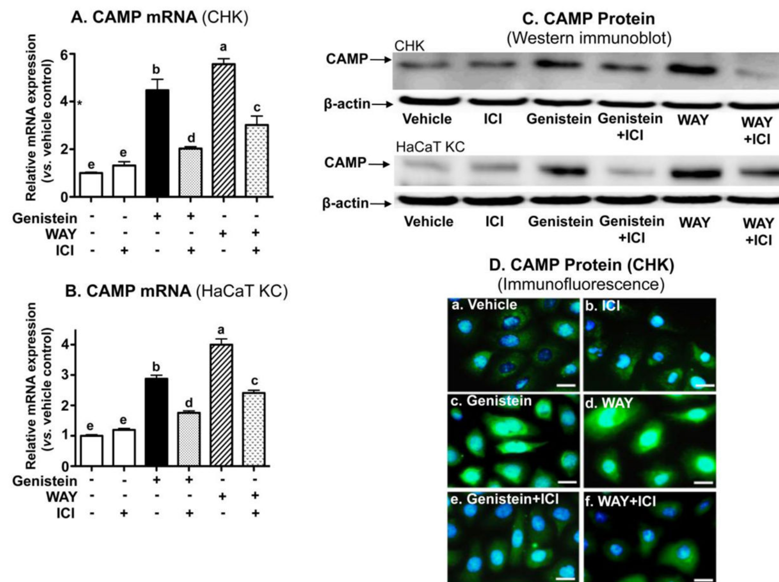

Fig. 3.

ER-β is required for genistein-induced upregulation of CAMP expression. Both Primary cultured KC (CHK) and HaCaT were pretreated with or without ER-β antagonist, ICI (1 μM, 30 min), were incubated with genistein (50 μM) or ER-β agonist (WAY, 1 μM) for 24 h. CAMP mRNA expression was assessed by *q*RT-PCR (A and B). Protein expression of CAMP was determined by western immunoblot analysis (C) and immunohistochemistry (D). Similar results were obtained when the experiment was repeated (in triplicate) using different cell preparations. Letters with different superscripts are significantly different at $p < 0.01$.

Author Manuscript
Author Manuscript
Author Manuscript
Author Manuscript

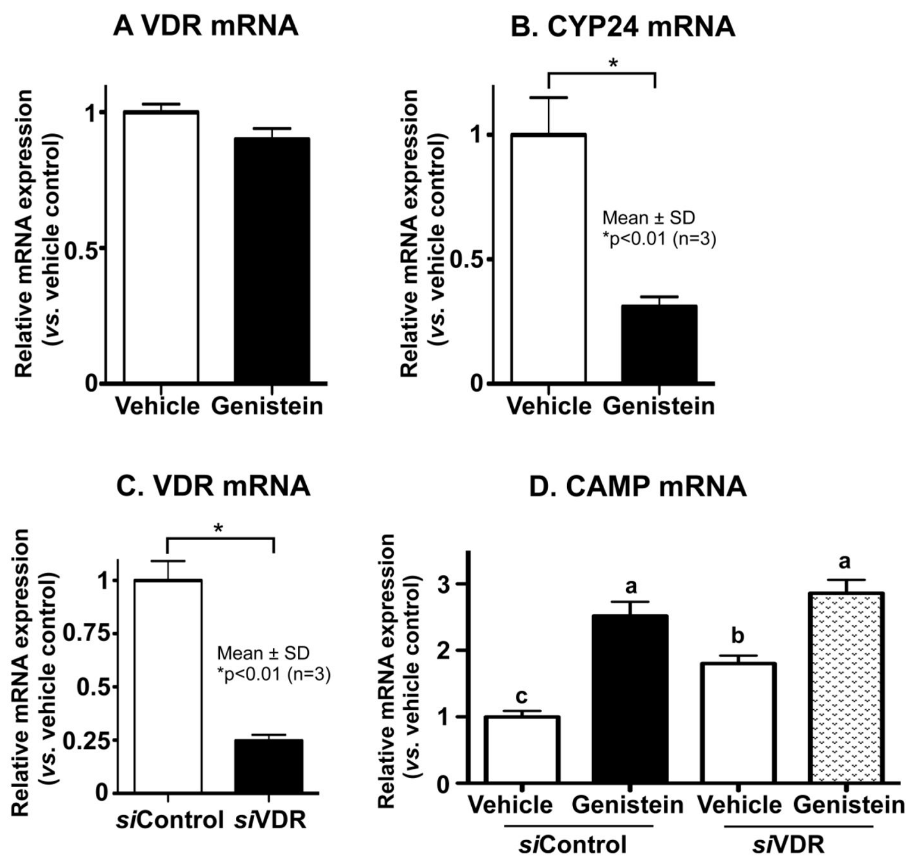

Fig. 4.

Genistein unlikely stimulates CAMP production through VDR mechanism. Primary cultured KC (CHK) were incubated as in Fig. 3. mRNA expressions were assessed by *q*RT-PCR. Similar results were obtained when the experiment was repeated (in triplicate) using different cell preparations. Letters with different superscripts are significantly different at $p < 0.01$ (D).

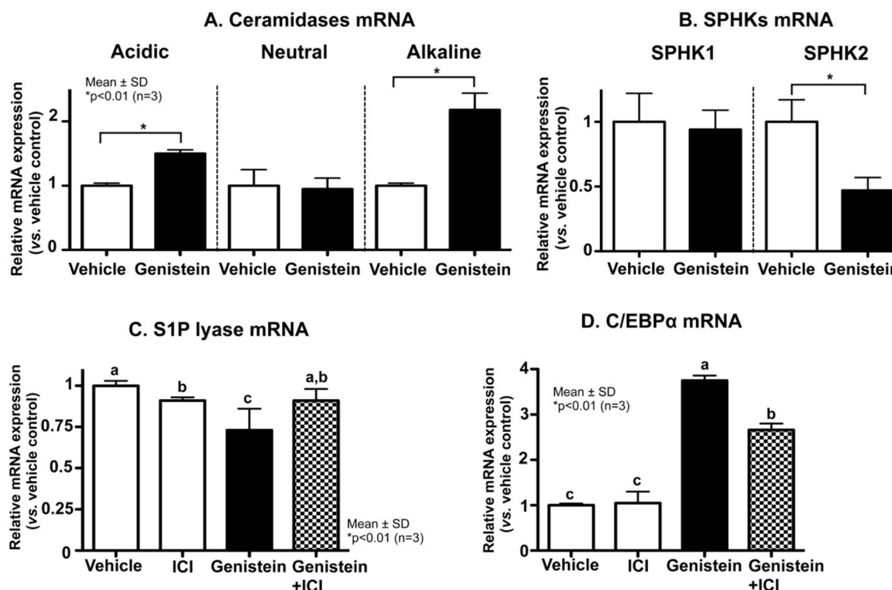

Fig. 5.

Genistein could alter S1P metabolism. Primary cultured KC were incubated as in Fig. 3. mRNA expressions were assessed by *q*RT-PCR. Similar results were obtained when the experiment was repeated (in triplicate) using different cell preparations. Letters with different superscripts are significantly different at $p < 0.01$ (C and D).

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Author Manuscript
Author Manuscript
Author Manuscript
Author Manuscript

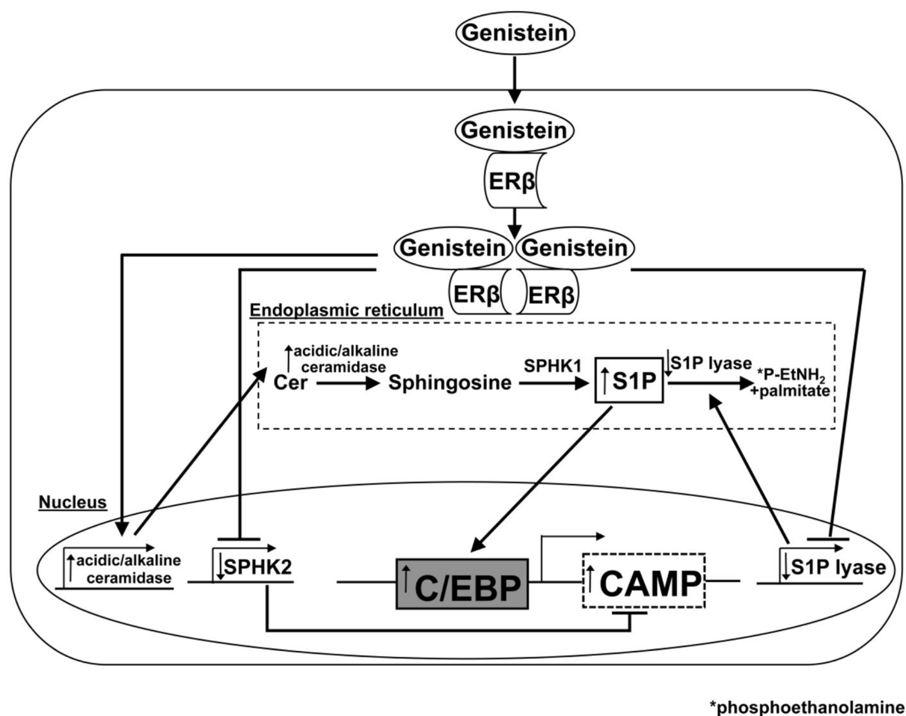

Fig. 6.

Proposed mechanism of genistein for CAMP expression. Genistein binds to estrogen receptor (ER) β to increase cellular S1P levels by upregulating acidic/alkaline ceramidase expression. Increased ceramidase leads to increased production of S1P. Simultaneously S1P lyase is inhibited to also decrease S1P hydrolysis, further stimulating the S1P signal, which increases CAMP production. Genistein also suppresses SPHK 2 expression, which decreases CAMP production [5]. The pool of S1P produced by SPHK 2 is hydrolyzed to sphingosine by S1P phosphatase [4] and sphingosine likely suppresses CAMP production [5].

Author Manuscript
Author Manuscript
Author Manuscript
Author Manuscript

Table 1

Genistein increases Sphingosine-1-Phosphate contents

Treatment	Sphingosine-1-Phosphate (pmol/mg protein ± SD)
Vehicle	8.96 ± 0.11
Genistein	11.99 ± 0.38[a)]

All value are mean ± SD.

[a)]Significantly different from Vehicle. P <0.01 (n=3).

Author Manuscript
Author Manuscript
Author Manuscript
Author Manuscript

Table 2

Blockade of sphingosine kinase 1 dose not alter genistein-induced CAMP expression and sphingosine-1-phosphate contents

Treatment	Relative CAMP mRNA expression (*vs*. vehicle control)	Sphingosine-1-Phosphate (pmol/mg protein ± SD)
Vehicle	1.00 ± 0.05[b]	9.65 ± 0.30[b]
SKI[1)]	1.11 ± 0.07[b]	7.46 ± 0.26[c]
Genistein	2.54 ± 0.12[a]	13.83 ± 1.52[a]
Genistein+SKI	2.46 ± 0.21[a]	11.72 ± 0.66[a]

All value are mean ± SD.

[1)] A specific pharmacological inhibitor of sphingosine kinase 1.

Letters with different superscripts in the same column are significantly different at $p < 0.01$ (one-way ANOVA coupled with Duncan's multiple comparison test).

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Table 3

S1P lyase activity was diminished in KC following genistein

Treatment	S1P lyase activity (pentadecanal pmol/mg protein/min ± SD)
Vehicle	13.63 ± 0.53
Genistein	11.51 ± 0.35 [a)]

All value are mean ± SD.

[a)] Significantly different from Vehicle. P <0.01 (n=3).