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

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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- κ B $\rightarrow$ C/EBP α -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, stimulates 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

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against VDR did not alter genistein-mediated upregulation of CAMP. Taken together, genistein induces CAMP production *via* an ER- β →S1P→NF- κ B→C/EBP α -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- κ B-C/EBP α -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.

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 1 α , 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 μ M.

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

Quantitative real-time polymerase chain reaction (qRT-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 μ g 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

(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 siRNA for VDR or non-targeted, control siRNA (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)-d₅-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.

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 μ M). 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 μ M) (Fig. 3C). Together, these results suggest that genistein increased CAMP production through ER- β activation. Since genistein at concentrations $\geq$ 1 mM did not significantly alter cell viability (not shown), we employed genistein at concentrations of $\leq$ 50 μ M 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 μ M), 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 μ M) (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).

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, *qPCR* 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 tumorigenesis [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

endoplasmic reticulum stressed conditions by sphingosine-1-phosphate (S1P), which in turn initiates an NF- κ B→C/EBP α transcriptional mechanism of CAMP expression [5]. Importantly, it is possible to directly stimulate S1P→NF- κ B→C/EBP α 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- κ B 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- κ B 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 sufficient 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

the following mechanism; genistein activities ER- β , followed by induction of the S1P $\rightarrow$ NF- κ B $\rightarrow$ C/EBP α signaling pathway, leads to enhance CAMP production.

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Fig. 1.

Genistein increases estrogen receptors mRNA expression. Primary cultured KC (CHK) were incubated with genistein (50 μ M) 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.

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 $\pm$ 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).

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 μ M, 30 min), were incubated with genistein (50 μ M) or ER- β agonist (WAY, 1 μ M) 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 $p < 0.01$.

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).

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].

Table 1

Genistein increases Sphingosine-1-Phosphate contents

Treatment	Sphingosine-1-Phosphate (pmol/mg protein $\pm$ SD)
Vehicle	8.96 $\pm$ 0.11
Genistein	11.99 $\pm$ 0.38 ^{a)}

All value are mean $\pm$ SD.

^{a)}Significantly different from Vehicle. $P < 0.01$ (n=3).

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 $\pm$ SD)
Vehicle	1.00 $\pm$ 0.05 ^b	9.65 $\pm$ 0.30 ^b
SKI ¹⁾	1.11 $\pm$ 0.07 ^b	7.46 $\pm$ 0.26 ^c
Genistein	2.54 $\pm$ 0.12 ^a	13.83 $\pm$ 1.52 ^a
Genistein+SKI	2.46 $\pm$ 0.21 ^a	11.72 $\pm$ 0.66 ^a

All value are mean $\pm$ 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).

Table 3

S1P lyase activity was diminished in KC following genistein

Treatment	S1P lyase activity (pentadecanal pmol/mg protein/min $\pm$ SD)
Vehicle	13.63 $\pm$ 0.53
Genistein	11.51 $\pm$ 0.35 ^{a)}

All value are mean $\pm$ SD.

^{a)}Significantly different from Vehicle. $P < 0.01$ (n=3).