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High calcium enhances the expression of double-stranded RNA sensors and antiviral activity in epidermal keratinocytes

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Abbreviations

DAMPs damage-associated molecular patterns

dsRNA Double-stranded RNA

HBD2 Human beta-defensin-2

HSV-1 Wild-type herpes simplex virus type-1

MDA5 Melanoma differentiation-associated protein 5

NHEKs Normal human epidermal keratinocytes

PAMPs pathogen-associated molecular patterns

RIG-I Retinoic acid-inducible gene-I

TLR3 Toll-like receptor 3

Abstract

Double-stranded RNA (dsRNA) sensors including TLR3, MDA5, and RIG-I are expressed in epidermal keratinocytes, and play an important immunological role by enhancing various innate and adaptive immune responses. Although the role of elevated extracellular calcium concentration in keratinocyte differentiation is well understood, the effect of high calcium on dsRNA sensors is not well studied.

We investigated alterations in dsRNA sensor expression and antiviral activity induced by a high extracellular concentration of calcium in epidermal keratinocytes. Normal human epidermal keratinocytes (NHEKs) were stimulated with high calcium and/or synthetic dsRNA, poly (I:C). *TLR3*, *IFIH1* (MDA5), and *DDX58* (RIG-I) expression were measured via qPCR, and IFN-β and human beta defensin 2 (HBD2) levels were measured using ELISA. TLR3 localization was evaluated with immunocytofluorescence. Antiviral activity was quantified with virus plaque assays using herpes simplex virus type-1 (HSV-1). High calcium significantly upregulated mRNA expression of *TLR3*, *IFIH1*, and *DDX58* in NHEKs. In addition, high calcium significantly enhanced poly (I:C)-induced anti-HSV-1 activity in NHEKs. The anti-viral molecule, HBD2 but not IFN-β induction by poly (I:C) was enhanced by high calcium.

Our findings indicate that high levels of extracellular calcium enhance the expression of dsRNA sensors and augment antiviral activity in epidermal keratinocytes.

Keywords: keratinocytes; Toll-like receptor 3; calcium; RIG-I; MDA5; viral infection

1. Introduction

Toll-like receptors (TLRs) are a family of pattern-recognition receptors which recognize molecules derived from microbes (called pathogen-associated molecular patterns [PAMPs]) or from injured cells (damage-associated molecular patterns [DAMPs]) [1-3]. TLRs are thought to function by detecting PAMPs and DAMPs and subsequently activating several gene programs which promote various innate and adaptive immune responses[1-3]. As a critical part of the response to viral infection, TLR3 recognizes double-stranded RNA (dsRNA) and subsequently stimulates the production of cytokines, chemokines, and antimicrobial peptides[4,5].

Epidermal keratinocytes constantly interface with the external environment and are thus the first defense against invading pathogens[6]. Keratinocytes express functional TLRs, which enable the cells to play an integral role in cutaneous immune responses against microbial pathogens[7]. Our group previously showed that TLR3 signaling in keratinocytes is involved in the skin immune response against herpes simplex virus (HSV)[8]. Surasombatpattana et al. reported the involvement of TLR3 in Dengue viral infection in human keratinocytes[9]. Furthermore, TLR3 has been reported to recognize the double-stranded domains of self RNA released from injury- or ultraviolet radiation-damaged keratinocytes, an event which was observed to lead to immune responses against these danger signals[3,10]. In addition to TLR3, melanoma differentiation-associated gene 5 (MDA5) and retinoic acid-inducible gene-I (RIG-I) are also dsRNA sensors expressed in epidermal keratinocytes[11-14]. These receptors are located in the cytoplasm, and contribute to defenses against intracellular viral infection[1,11,15].

In light of these observations, it is important to understand the regulatory mechanisms behind the expression of dsRNA sensors in epidermal keratinocytes. Several groups have reported that inflammatory cytokines induce the expression of these sensors in cells[1]. For

example, Prens et al. reported that a type-I interferon, IFN-α, induces the expression of TLR3, MDA5, and RIG-I, and enhances poly (I:C) responses in human keratinocytes[16]. Similarly, Zhang et al. found that a type-III interferon, IL-29, enhances TLR3 expression in epidermal keratinocytes[17]. Our group has recently demonstrated that IFN-γ, a TH1 cytokine and type-II interferon, enhances TLR3 expression and antiviral activity in epidermal keratinocytes[8].

Nonetheless, the skin's immune response is unlikely to be regulated solely by inflammatory cytokines. While high calcium levels can induce differentiation in epidermal keratinocytes, calcium is thought to also affect innate immunity in the skin[18]. Our group previously reported that high calcium augments the expression and function of kallikreins, a group of serine proteases, and also regulates antimicrobial activity by processing cathelicidin in keratinocytes[19]. Hau et al. demonstrated that high calcium augments cytokine and chemokine production by β-glucan-stimulated NHEKs[20]. However, the effects of calcium on the expression of dsRNA sensors in keratinocytes are not well studied.

In this study, we report that high calcium induces the expression of TLR3, MDA5, and RIG-I, and enhances antimicrobial activity in human epidermal keratinocytes. Our results provide new evidence that high calcium contributes to the skin's innate immunity against viral infection.

2. Materials and methods

2.1. Cell culture and stimuli

Normal human epidermal keratinocytes (NHEKs) were obtained from Cascade Biologics/Invitrogen (Portland, OR) and grown in serum-free EpiLife cell culture medium containing 0.06 mM Ca^{2+} (Cascade Biologics/Invitrogen), 1× EpiLife Defined Growth Supplement (EDGS; Cascade Biologics/Invitrogen), 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B. Cells were grown at 37 °C with 5% CO_2 under

standard tissue culture conditions. NHEKs were stimulated with 0.5-2 mM calcium and/or 0.1-10 μg/ml poly (I:C) (InvivoGen, San Diego, CA) in 24-well flat-bottom plates for 24 - 96 hours. After stimulation, cell medium supernatants were collected and stored at -20 °C until analysis. After supernatant collection, RNA was extracted from cells using TRIzol Reagent (Invitrogen, Carlsbad, CA).

2.2. Quantitative real-time-PCR (qPCR)

Complementary DNA was synthesized from RNA with the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA) as described by the manufacturer's protocol. TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA) were used to analyze the expression of *IFNB1* (assay ID: Hs01077958_s1), *TLR1* (assay ID: Hs00413978_m1), *TLR2* (assay ID: Hs00610101_m1), *TLR3* (assay ID: Hs00152933_m1), *IFIH1* (assay ID: Hs01070332_m1), *DDX58* (assay ID: Hs00204833_m1), *KRT10* (assay ID: Hs00166289_m1), *DEFB4* (assay ID: Hs00823638_m1) , *MARCO* (assay ID: Hs00198937_m1) , *IFNA2* (assay ID: Hs00265051_s1), *DEFB103* (assay ID: Hs00218678_m1), and *IL6* (assay ID: Hs00174131_m1) as described by the manufacturer's protocol. *GAPDH* mRNA was used as an internal control for each sample, and was detected by the probe, VIC-CATCCATGACAACTTTGGTA-MGB, and following primer set: 5'-CTTAGCACCCCTGGCCAAG-3'; and 5'-TGGTCATGAGTCCTTCCACG-3'. Each sample's mRNA expression was calculated as its relative expression to *GAPDH* mRNA, and all data are presented as the fold change against the control value (mean of non-stimulated cells).

2.3. Immunocytofluorescence

NHEKs were cultured on chamber slides for immunocytofluorescence. Cells were

stimulated with high Ca^{2+} (2 mM) or the vehicle for 24 hours. After removing the culture medium, cells were fixed with 1:1 methanol/acetone, blocked with 10% normal goat serum, and then incubated with rabbit polyclonal anti-TLR3 antibody (LifeSpan Biosciences, Inc., Seattle, WA) at 4 °C overnight. After washing with PBS, the cells were incubated with Alexa Fluor 488-conjugated goat anti-rabbit IgG antibody (Molecular Probes/Invitrogen, Eugene, OR). Slides were mounted with ProLong Gold Antifade Mountant with DAPI (Molecular Probes/Life Technologies). Images were obtained by KEYENCE fluorescent microscope BZ-X700 (Keyence Corp, Osaka, Japan).

2.4. Enzyme-linked immunosorbent assay (ELISA)

Levels of IFN-β in NHEK culture supernatants were measured with the VeriKine™ Human IFN-β ELISA Kit (PBL Assay Science, Piscataway, NJ) according to the manufacturer's instructions.

HBD2 ELISA were performed as follows; The wells of ninety-six-well EIA plates (Corning) were coated with mouse monoclonal anti-HBD-2 antibody (100 μg ml^{-1}, Peprotech, Rocky Hill, NJ) at 20-25 °C overnight. The wells were washed with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (washing buffer) and were blocked with PBS containing 1% bovine serum albumin (BSA) for 1 hour at 20-25 °C. The wells were then incubated with culture supernatants or recombinant HBD2 for 2 hours, and further with the detection antibody (100 μ ml^{-1}, Peprotech) for another 2 hours. Streptoavidin-conjugated horseradish peroxidase (Peprotech) and 3.3'5,5'-tetramethylbenzidine substrate (BD Biosciences, San Jose, CA) were added to the wells for colorimetric quantification, and the reaction was stopped by 0.2M sulfuric acid (Sigma-Aldrich).

The absorbance at 450 nm was monitored with SpectaMax Plus384 (Molecular Devices, Sunnyvale, CA), and the concentrations of the wells were calibrated by the standard curve of

recombinant HBD-2 or IFN-β using SoftMax Pro4.6 (Molecular Devices).

2.5. Virus plaque assays

Wild-type herpes simplex virus type-1 (HSV-1; KOS strain) was obtained from the Research Institute for Microbial Diseases, Osaka University. NHEKs were stimulated with poly (I:C) (0.1 μg/ml) for 24 hours, infected with HSV-1 (multiplicity of infection [MOI] = 0.001), and then incubated for 1 hour at 37 °C to allow virus adsorption. The culture medium was replaced with medium containing γ-globulin (5 mg/ml of Gammagard; Baxalta, Bannockburn, IL) and the cells were incubated for 48 hours at 37 °C. After this final incubation, viral plaque formation was visualized by crystal violet staining.

2.6. Statistical analysis

All statistical analyses were conducted with GraphPad Prism, version 6 (GraphPad, La Jolla, CA). One-way analysis of variance with Tukey's test was used to determine significance when comparing more than two groups. *P*-values < 0.05 were considered significant.

3. Results

3.1. High calcium induces the expression of dsRNA sensors in epidermal keratinocytes

Because the epidermis has a calcium gradient and the dsRNA sensor TLR3 is highly expressed in the epidermal granular layer[21], we first investigated whether high calcium could induce mRNA expression of dsRNA sensors TLR3, MDA5, and RIG-I in epidermal keratinocytes. The qPCR analysis revealed that mRNA levels of *TLR3*, *IFIH1* (MDA5), and *DDX58* (RIG-I) were upregulated in a dose-dependent manner in NHEKs (Fig. 1A-C). Levels of mRNA for *KRT10*, which encodes the differentiation marker keratin 10, were similarly

increased in a dose-dependent manner (Fig. 1D). In addition, the mRNA levels of other TLRs, including *TLR1* and *TLR2*, followed the same pattern of being upregulated by high calcium (Fig. 1E, F). Macrophage receptor with collagenous structure (MARCO) is the class A scavenger receptor and thought to be the entry receptor herpes simplex virus (HSV)-1[22]. High calcium also significantly increased *MARCO* expression in NHEKs (Fig. 1G). IFN-α, IFN-γ, and IL-29 are reported to induce TLR3 expression in epidermal keratinocytes[8,14,16], and *IFNA2* (IFN-α) was induced by high calcium in NHEKs (Fig. 1H), while IFN-γ and IL-29 were not constitutively detectable in NHEKs (data not shown).

We also examined the kinetics of *TLR3*, *IFIH1*, and *DDX58* expression induced by high calcium in epidermal keratinocytes. The qPCR analysis indicated that the induction of *TLR3*, *IFIH1*, and *DDX58* expression by high calcium was time-dependent in NHEKs, which was similar to the induction kinetics of *KRT10* expression (Fig. 2A-D).

3.2. Intracellular TLR3 expression is induced by high calcium in epidermal keratinocytes

We next investigated the calcium-induced expression pattern of TLR3 protein in NHEKs with immunocytofluorescence. TLR3 expression appeared to increase in the cytoplasm of calcium-stimulated NHEKs compared with non-stimulated cells (Fig. 3), indicating that both TLR3 mRNA and protein are upregulated by high calcium in epidermal keratinocytes.

3.3. High calcium enhances antiviral activity in epidermal keratinocytes

We then evaluated the functional significance of dsRNA sensors in epidermal keratinocytes by performing virus plaque assays with HSV-1. A synthetic dsRNA, poly (I:C), is well known to enhance antiviral activities in keratinocytes[7]. As expected, high calcium significantly enhanced the antiviral activity of NHEKs stimulated with poly (I:C) (Fig. 4A). Furthermore, we focused on the antiviral molecules in epidermal keratinocytes. The type I interferon,

IFN-β is thought to be the major factor responsible for anti-viral activity in human keratinocytes[23,24]. We measured IFN-β expression in our culture system, but unexpectedly, we found that high extracellular calcium significantly suppressed poly (I:C)-induced IFN-β expression (Fig. 4B, C). The similar suppressive effect was also observed in the expression of IL-6, a representative proinflammatory cytokine (Fig. 4D). On the other hand, the antimicrobial peptide, human β-defensin 2 (HBD2) is also reported to have capacity of antiviral molecules[25]. In contrast to IFN-β suppression, high calcium significantly enhanced poly (I:C)-induced HBD2 expression in NHEKs (Fig 4E, F). Human β-defensin 3 (*DEFB103*) also showed upregulation in poly (I:C)-induced RNA expression under high extracellular calcium (Fig. G).

4. Discussion

Calcium is a widely known component of mechanisms regulating epidermal keratinocyte differentiation, and is also suggested to play an important role in the immune system[19,26]. In general, the elevation of extracellular calcium increases intracellular free calcium via the calcium receptor, and subsequently induces keratinocyte differentiation[18]. The increase in intracellular calcium activates protein kinase C, which in turn activates several transcription factors such as activator protein 1 which induce the expression of keratinocyte differentiation markers, keratin 10, filaggrin, and involucrin[18]. While these observations highlight the importance of high calcium in keratinocyte differentiation, our group observed that high calcium also induces kallikrein 5 (KLK5) and KLK7 expression, and modulates processing of the antimicrobial peptide cathelicidin[19]. These findings suggest that high calcium influences cutaneous innate immunity in epidermal keratinocytes.

Here, we report that the expression of TLR3 is induced by high calcium in a dose- and time-dependent manner in epidermal keratinocytes. TLR3 is a dsRNA sensor located in the

endosome[1], and is capable of recognizing the extracellular synthetic dsRNA poly (I:C) in epidermal keratinocytes. On the other hand, MDA5 and RIG-I are located in cytoplasm and recognize transfected poly (I;C)[12,15]. These receptors are considered to play important roles in the skin's response to viral infections[14]. However, in our experiment system, poly (I;C) was simply added to the media without transfection. Therefore, TLR3 seems to be the most important dsRNA-sensor in the system. We further revealed that IFN-α is also induced by high calcium in NHEKs. Therefore, IFN-α might be involved in the increase of TLR3 expression in epidermal keratinocytes. Furthermore, we showed that high calcium enhances antiviral activity against HSV-1 in NHEKs. IFN-β is thought to be a major factor responsible for antiviral functions in human epidermal keratinocytes[27]. In the present study, high extracellular calcium in NHEKs significantly downregulated poly (I:C)-induced IFN-β and IL-6 expression. On the other hand, high extracellular calcium significantly upregulated the induction of the antimicrobial peptides, HBD2 and HBD3, by poly (I:C)-stimulation. Since HBD2 and HBD3 also have anti-viral activity[25,28], our result of viral plaque assay might be due to the increase in these molecules. The mechanism which makes the difference between the upregulation and downregulation is unknown. Further investigation is required to figure it out.

In addition, consistent with previous studies, this enhancement by calcium seems to occur via increase in sensor levels[8,11,17]. Notably, high calcium induced a similar increase in the expression of dsRNA sensors, keratin 10, and MARCO. Since the increase in MARCO seems to make the entry of HSV easier, the enhancement of the antiviral activity does not seem to be associated with MARCO increase. Either high calcium itself or the state of high calcium-induced differentiation in keratinocytes might influence antiviral activity through the increase of TLR3 expression in the cells. Intriguingly, we also showed that high calcium induces TLR1 and TLR2 expression in NHEKs. Antibacterial activity may therefore be

enhanced by high calcium through both the augmentation of serine protease activity and a TLR1-/TLR2-mediated increase in sensitivity for bacterial lipopeptides.

In normal epidermis, the calcium gradient reaches its maximum concentration in the granular layer[29]. Our results may therefore account for the observation that, compared to basal layers, TLR3 levels are increased in the upper layers of the epidermis, which are more important for defense against pathogenic viruses. Our observations also indicate that further understanding of the regulation of dsRNA sensors and antiviral activity in epidermal keratinocytes might provide new therapeutic strategies against intractable viral infections in the skin.

Acknowledgments

Dr. Yamamura mainly performed the research and wrote the paper. Dr. Morizane designed the research study and contributed to all the work. Dr. Yamamoto, Dr. Wada, and Dr. Iwatsuki analyzed the data and discussed the results.

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Figure legends

Fig 1. High extracellular calcium induces the expression of dsRNA sensors in keratinocytes. NHEKs were cultured in the presence of 0.06 (control), 0.5, 1, and 2 mM Ca^{2+} for 24 hours. The relative mRNA expression of TLR3 *(*encoded by *TLR3)* (A), MDA5 *(IFIH1)* (B), RIG-I *(DDX58)* (C), keratin 10 *(KRT10)* (D), TLR1 *(TLR1)* (E), TLR2 *(TLR2)* (F), MARCO (*MARCO*) (G), and IFN-α (*IFNA2*) (H) to *GAPDH* were measured by quantitative real-time PCR. Data represent the mean ± SEM of triplicate determinants from a single experiment representative of three independent experiments. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$).

Fig 2. High extracellular calcium induces the expression of dsRNA sensor time-dependently in proportion to keratinocyte differentiation.

NHEKs initially at 30% confluence were cultured in the presence of 0.06 (control) or 2.0 mM Ca^{2+} for , 48, 72, and 96 hours. Relative mRNA levels of keratin 10 *(*encoded by *KRT10)* (A), TLR3 *(TLR3)* (B), MDA5 *(IFIH1)* (C), and RIG-I *(DDX58)* (D) to *GAPDH* were measured by quantitative real-time PCR. Data represent the mean ± SEM of triplicate determinants from a single experiment representative of three independent experiments (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Fig 3. High extracellular calcium induces intracellular TLR3 in epidermal keratinocytes.

NHEKs were cultured on chamber slides and then stimulated with PBS (vehicle) (A) or high Ca^{2+} (2 mM) (B, C) for 24 hours. The cells were treated with anti-TLR3 antibody (A, B) or the negative control (C). Immunocytofluorescence was used to visualize the expression pattern of TLR3 (green); nuclei were visualized with DAPI (blue). Scale bar = 20 µm.

Fig 4. Antiviral activity is enhanced in epidermal keratinocytes with high extracellular calcium.

（A）NHEKs were incubated with 1.0 mM Ca^{2+} for 48 hours and 1.6 mM Ca^{2+} for another 48 hours (High Ca^{2+}). Cells were then stimulated with poly (I:C) for 24 hours, and incubated with HSV-1 (MOI = 0.001) for 48 hours. The number of viral plaques were counted. Data represent the mean ± SEM of triplicate determinants from a single experiment representative of three independent experiments.

(B-G) NHEKs were incubated with 1.0 mM Ca^{2+} for 48 hours and 1.6 mM Ca^{2+} for another 48 hours (High Ca^{2+}). Cells were then stimulated with poly (I:C) for 24 hours. The relative mRNA expression of *IFNB1* (B), *IL-6* (D), *DEFB4B* (E), and *DEFB103* (G) to *GAPDH* were measured by quantitative real-time PCR. IFN-β (C) and HBD2 (F) protein levels in culture supernatants were measured by ELISA. Data represent the mean ± SEM of triplicate determinants from a single experiment representative of three independent experiments (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$).

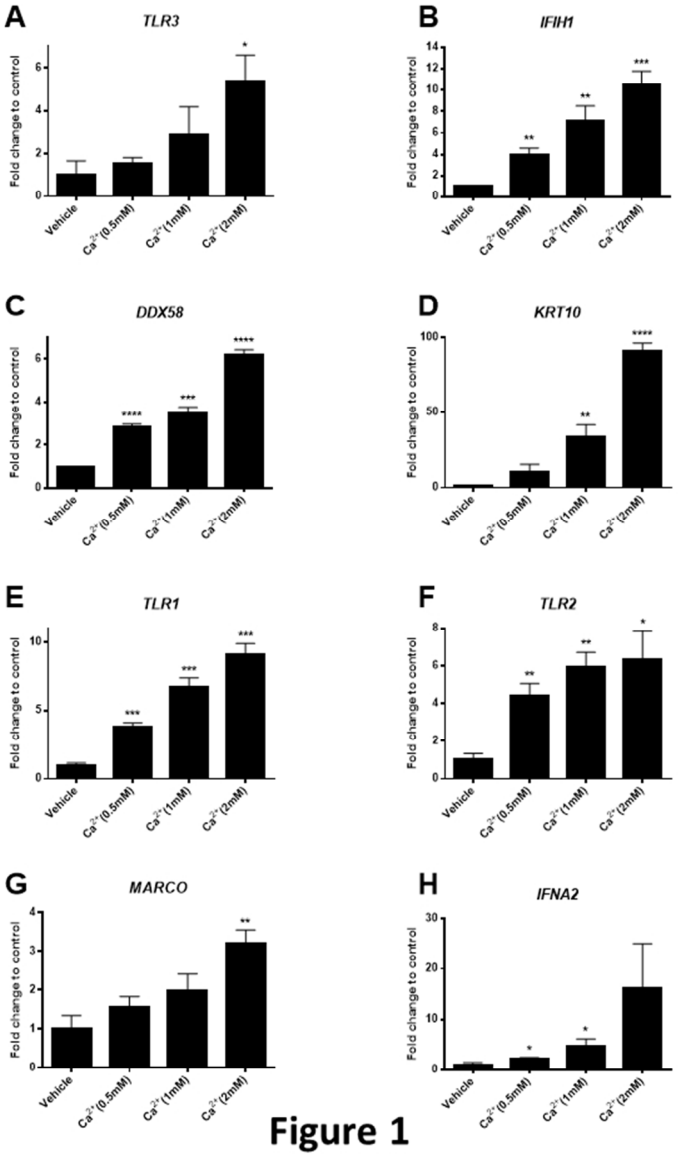

Figure 1

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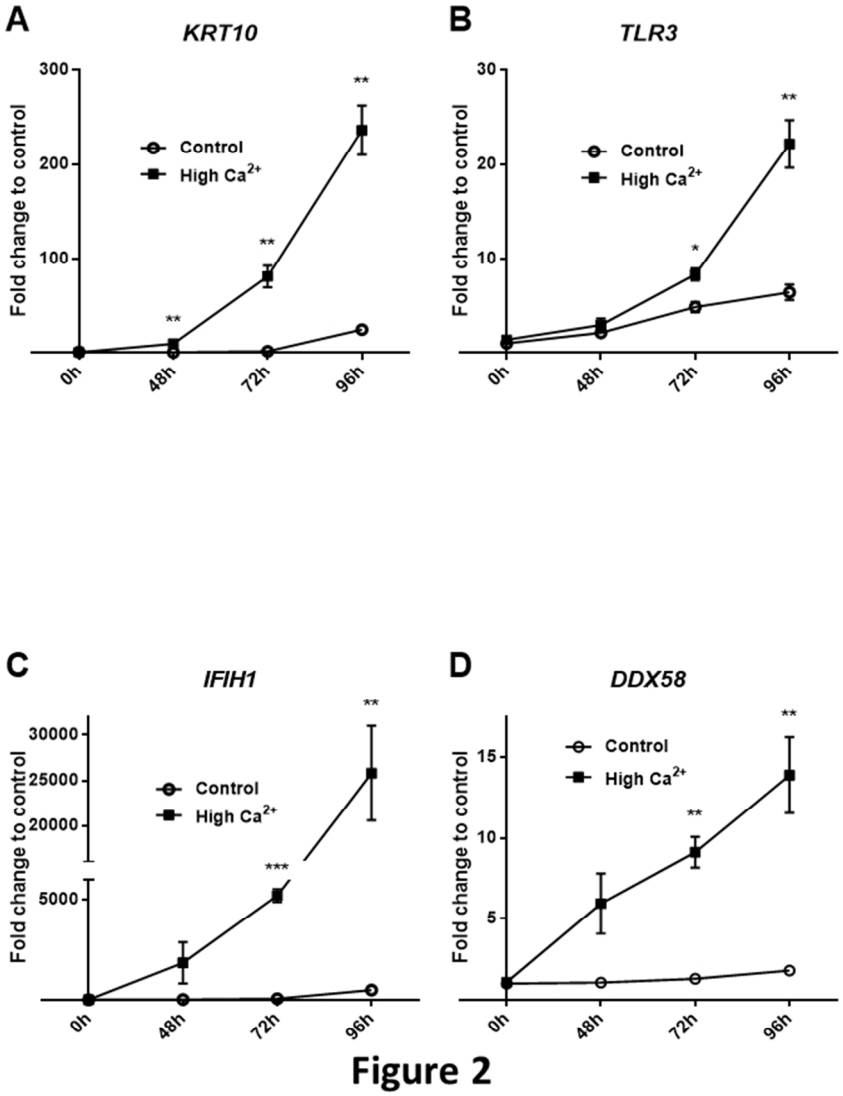

Fig 2. High extracellular calcium induces the expression of dsRNA sensor time-dependently in proportion to keratinocyte differentiation.
NHEKs initially at 30% confluence were cultured in the presence of 0.06 (control) or 2.0 mM Ca2+ for , 48, 72, and 96 hours. Relative mRNA levels of keratin 10 (encoded by KRT10) (A), TLR3 (TLR3) (B), MDA5 (IFIH1) (C), and RIG-I (DDX58) (D) to GAPDH were measured by quantitative real-time PCR. Data represent the mean ± SEM of triplicate determinants from a single experiment representative of three independent experiments (* P < 0.05, ** P < 0.01, *** P < 0.001).

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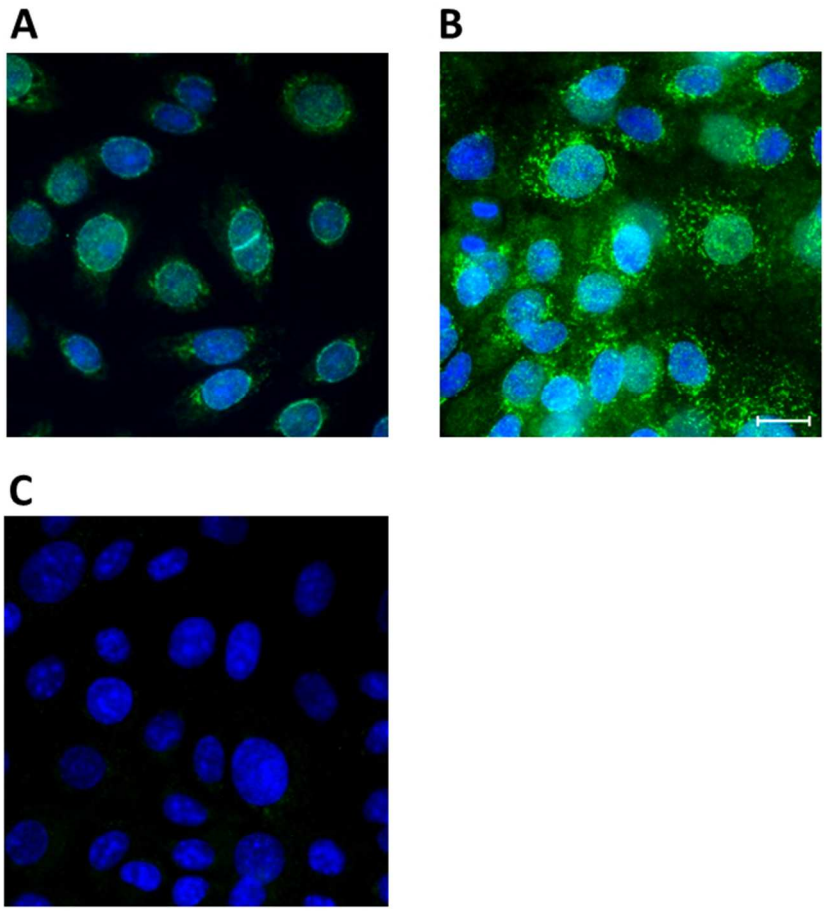

Figure 3

Fig 3. High extracellular calcium induces intracellular TLR3 in epidermal keratinocytes. NHEKs were cultured on chamber slides and then stimulated with PBS (vehicle) (A) or high Ca2+ (2 mM) (B, C) for 24 hours. The cells were treated with anti-TLR3 antibody (A, B) or the negative control (C). Immunocytofluorescence was used to visualize the expression pattern of TLR3 (green); nuclei were visualized with DAPI (blue). Scale bar = 20 μm.

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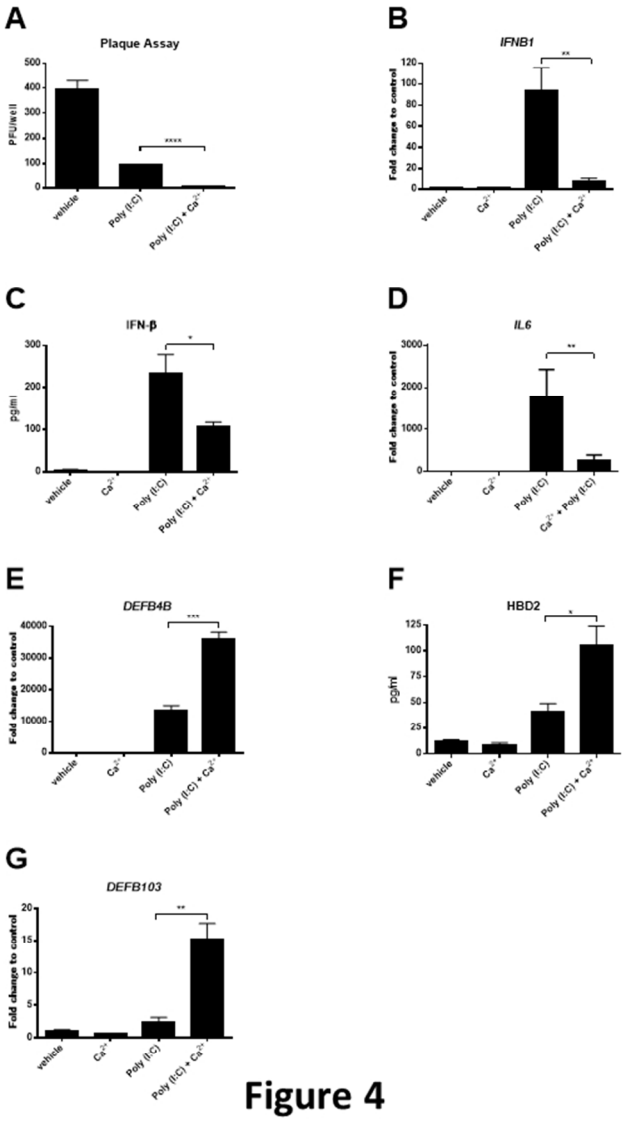

Figure 4

Fig 4. Antiviral activity is enhanced in epidermal keratinocytes with high extracellular calcium. (A) NHEKs were incubated with 1.0 mM Ca2+ for 48 hours and 1.6 mM Ca2+ for another 48 hours (High Ca2+). Cells were then stimulated with poly (I:C) for 24 hours, and incubated with HSV-1 (MOI = 0.001) for 48 hours. The number of viral plaques were counted. Data represent the mean ± SEM of triplicate determinants from a single experiment representative of three independent experiments. (B-G) NHEKs were incubated with 1.0 mM Ca2+ for 48 hours and 1.6 mM Ca2+ for another 48 hours (High Ca2+). Cells were then stimulated with poly (I:C) for 24 hours. The relative mRNA expression of IFNB1 (B), IL-6 (D), DEFB4B (E), and DEFB103 (G) to GAPDH were measured by quantitative real-time PCR. IFN-β (C) and HBD2 (F) protein levels in culture supernatants were measured by ELISA. Data represent the mean ± SEM of triplicate determinants from a single experiment representative of three independent experiments (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$).

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