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Kallikrein Expression and Cathelicidin Processing Are Independently Controlled in Keratinocytes by Calcium, Vitamin D_3, and Retinoic Acid

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Abstract

Cathelicidin has dual functions in the skin, acting as an innate antibiotic and as an immunomodulator in diseases such as rosacea and psoriasis. The serine proteases kallikrein 5 (KLK5) and kallikrein 7 (KLK7) control enzymatic processing of cathelicidin precursor in the skin and regulate the eventual function of the final forms of these peptides. We analyzed factors that control expression of KLK5 and KLK7 in normal human epidermal keratinocytes to better understand how these may influence cathelicidin processing and function. Increased extracellular calcium-induced KLK5 and KLK7 mRNA expression and protein release in a time-dependent manner that is similar to induction of differentiation markers such as keratin 10 and involucrin. However, 1,25$(OH)_2$ vitamin D_3, 9-cis retinoic acid (RA), and 13-cisRA also induced the KLKs, but the timing and pattern of KLK induction for each were different and distinct from changes in differentiation markers. Increased protease activity and differential processing of cathelicidin accompanied increased KLK expression. These findings show that the expression and activity of KLK are under fine control and can be distinctly influenced by variables such as differentiation, calcium, vitamin D, and RA. Thus, these variables may further control the functions of antimicrobial peptides in the skin.

INTRODUCTION

Cathelicidin is one of several antimicrobial peptides in mammals and is best known for its capacity to protect against infection by microbial pathogens in skin and other organ systems (Zanetti *et al*., 2002). Peptides derived from the cathelicidin gene *Camp* have an integral role in innate immunity by directly killing pathogenic microorganisms such as Gram-positive (Nizet *et al*., 2001) and Gram-negative bacteria (Rosenberger *et al*., 2004; Iimura *et al*., 2005), fungi (Lopez-Garcia *et al*., 2005) as well as some viruses (Howell *et al*., 2004). LL-37, the carboxy-terminal peptide fragment derived from the human cathelicidin precursor protein hCAP18, not only has the capacity to kill a wide variety of microbes, but also can modify host immune and growth responses (Lai and Gallo, 2009). These host responses to LL-37 are complex and dependent on the system evaluated. Observations for LL-37 action have included those showing proinflammatory activity (Braff *et al*., 2005b;

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CONFLICT OF INTEREST

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Yamasaki *et al*., 2007), anti-inflammatory activity (Mookherjee *et al*., 2006a, b; Morioka *et al*., 2008), promoting chemotaxis (De *et al*., 2000) and angiogenesis (Koczulla *et al*., 2003), and enhancing wound repair (Tokumaru *et al*., 2005). Owing to these and other functions, LL-37 has been implicated in a wide variety of inflammatory skin diseases including psoriasis, atopic dermatitis, and rosacea (Ong *et al*., 2002; Lande *et al*., 2007; Yamasaki *et al*., 2007). Importantly, the activity of cathelicidin in humans is controlled by enzymatic processing to LL-37 or smaller peptides. Recently, our group showed that serine proteases kallikrein 5 (KLK5) and kallikrein 7 (KLK7) participate in the enzymatic processing of hCAP18 in the skin (Yamasaki *et al*., 2006).

Human tissue KLKs consist of 15 trypsin-like or chymotrypsin-like secreted serine proteases and their genes form a cluster at the telomeres of the long arm of chromosome 19, in the cytogenic region q13.3–4 (Lundwall and Brattsand, 2008). In the skin, KLK5 and KLK7, known as the major serine proteases, degrade corneodesmosome proteins, leading to desquamation (Caubet *et al*., 2004; Lundwall and Brattsand, 2008). Aberrant KLK expression or function has been reported to be involved in skin diseases such as Netherton syndrome and rosacea (Descargues *et al*., 2005; Yamasaki *et al*., 2007). Overexpression of human KLK7 in mouse epidermis results in chronic itchy dermatitis (Hansson *et al*., 2002).

Despite the importance of KLKs to multiple biological processes, the regulation of KLK has been incompletely studied. Expression in some cell lines, such as a prostate carcinoma cell line LNCaP and a breast carcinoma cell line BT-474, has been reported, but the factors that regulate KLK5 and KLK7 expression in keratinocytes remain largely unknown (Yousef and Diamandis, 2001; Shaw and Diamandis, 2008). Therefore, we studied here several factors that influence keratinocyte function to examine how these might influence KLK expression and eventual processing of cathelicidin. Our findings suggest that Ca^{2+}, $1,25(OH)_2$ vitamin D_3 ($1,25(OH)_2VD_3$), and retinoic acid (RA) can modulate innate antimicrobial peptide function in the epidermis through modulation of the final balance of KLK enzymatic activity in the skin.

RESULTS

KLK 5 and 7 expression is inducible in epidermal keratinocytes during differentiation

To analyze the regulation of KLK5 and KLK7 expression in keratinocytes, an array of potential stimuli for keratinocytes was examined, including hormones, biological stimuli, cytokines, growth factors, and microbial products. Of these stimuli, we found that increased Ca^{2+} in the medium to 2 mM, $1,25(OH)_2VD_3$, 9-*cis* RA, and 13-*cis* RA each increased KLK5 and KLK7 mRNA expression in cultured normal human epidermal keratinocytes (NHEKs) (Figure 1). It is interesting to observe that several molecules with potent effects on keratinocyte growth or cytokine release, including dexamethasone, epidermal growth factor, poly(I:C), and MALP-2, had little effect on KLK gene expression.

KLK5 and KLK7 are constitutively expressed in greatest abundance in the granular layer of the epidermis, (Ekholm and Egelrud, 2000). To partially induce this phenotype, keratinocytes have been cultured in high concentrations of Ca^{2+}, a manipulation that is known to induce differentiation of keratinocytes and thought to mimic the Ca^{2+} gradient observed in normal skin (Mauro *et al*., 1998). We therefore examined if the increase observed in KLK5 and KLK7 that is induced by increasing the concentration of Ca^{2+} in the culture medium also induces markers of differentiation in parallel, or if the acquisition of a differentiated phenotype could be distinguished from induction of KLK expression. NHEKs in basal growth conditions at 30% confluence were stimulated with 2 mM Ca^{2+} for various times and quantitative real-time-PCR performed to analyze the time course of KLK5 and KLK7 mRNA expression. Ca^{2+} induced KLK5 and KLK7 mRNA expression in

keratinocytes after 24 hours, and the increase in KLK7 mRNA (10,000-fold at 96 hours) was greater than that in KLK5 (25-fold at 96 hours) (Figure 2a and b). It is noted, however, that the basal expression of KLK5 mRNA was abundant, whereas that of KLK7 was very low in nonstimulated conditions. Thus, after Ca^{2+} stimulation, although the relative expression of the *KLK7* gene was exponentially increased, this does not imply a greater expression of KLK7. Consistent with these observations, protein abundance for KLK5 and KLK7 increased after elevated Ca^{2+} in a time-dependent manner, and the total amount of KLK7 remained less than KLK5 (Figure 2c and d). Analysis of the timing for expression of keratin 10 (KRT10) and involucrin (IVL) mRNAs, evaluated as markers of differentiation for epidermal keratinocytes, showed that NHEKs cultured in high-concentration Ca^{2+} had higher KRT10 expression than those in basal media, and this pattern was similar to that of KLK7 expression (Figure 2e and g). Induction of IVL, similar to KLK5, was also dependent on the presence of high concentration of Ca^{2+} in media and occurred over a similar time as KLK5 and KLK7 (Figure 2f and h).

$1,25(OH)_2VD_3$ increases KLK expression independently of differentiation

We next examined the kinetics of KLK5, KLK7, KRT10, and IVL expression when keratinocytes were cultured in the presence of $1,25(OH)_2VD_3$, a culture condition that can also influence keratinocyte differentiation, but in a manner different from Ca^{2+} (Bikle, 2004). As in the growth conditions tested with Ca^{2+}, NHEKs at 30% confluence were stimulated with $1,25(OH)_2VD_3$ for various times. A peak increase in KLK5 and KLK7 mRNA expression was observed at 24 hours after $1,25(OH)_2VD_3$ addition, which gradually decreased afterward (Figure 3a and b). $1,25(OH)_2VD_3$ induced KLK5 and KLK7 in a dose-dependent manner between 10^{-9} M (1 nM) and 10^{-7} M (100 nM) (Figure 3c and d). KLK5 and KLK7 protein in the media were both induced by $1,25(OH)_2VD_3$ in a time-dependent manner (Figure 3e and f). Similar to Ca^{2+} stimulation, the relative increase in KLK7 protein was greater than that for KLK5 protein. However, in contrast to the induction of KRT10 and IVL expression by Ca^{2+}, $1,25(OH)_2VD_3$ suppressed KRT10 mRNA expression in NHEKs at 30% confluence (Figure 3g), and IVL mRNA expression was not affected under the conditions observed for induction of the KLKs (Figure 3h). Protein levels of KRT10 and IVL showed a similar trend to mRNA expression (Figure 3i and j). Thus, unlike Ca^{2+}, $1,25(OH)_2VD_3$ can increase the expression and release of KLK5 and KLK7 from keratinocytes in a manner that is not dependent on induction of differentiation markers KRT10 and IVL.

KLK and cathelicidin colocalizes in keratinocytes stimulated with $1,25(OH)_2VD_3$

$1,25(OH)_2VD_3$ is also known to greatly induce cathelicidin expression in keratinocytes (Schauber *et al*., 2006). Next, to further confirm results of Figure 3 and to determine the relative localization of KLK5 and cathelicidin in keratinocytes stimulated with $1,25(OH)_2VD_3$, immunostaining for KLK5 and cathelicidin was performed. Enhanced expression of both KLK5 and cathelicidin protein was observed in NHEKs stimulated with $1,25(OH)_2VD_3$, and these antigens were colocalized in a peri-nuclear pattern consistent with ER or cis-Golgi (Figure 4). This pattern of expression was consistent with previous reports of basal expression of KLK7 and KLK5 in lamellar granules (Ishida-Yamamoto *et al*., 2005), and the expression of cathelicidin also matched that previously reported (Braff *et al*., 2005a). These data therefore support the conclusion that the localization of KLK7 in NHEKs stimulated with $1,25(OH)_2VD_3$ was similar to KLK5, and both colocalize with cathelicidin.

RAs increase KLK expression independently of keratinocyte differentiation

RA is known to modulate keratinocyte differentiation, and it has been reported that all-*trans* RA upregulates IVL expression but downregulates KRT10 expression (Poumay *et al*.,

1999). We therefore examined the kinetics of KLK5, KLK7, KRT10, and IVL expression by RA in keratinocytes to further establish that KLK expression can be regulated independently of differentiation. Both 9-*cis* RA and 13-*cis* RA increased KLK5 and KLK7 mRNA expression with the peak at 72 hours (Figure 5a and b). Both 9-*cis* RA and 13-*cis* RA induced KLK5 and KLK7 in a dose-dependent manner between 10^{-9} M (1 nM) and 10^{-7} M (100 nM) (Figure 5c and d). Similar to $1,25(OH)_2VD_3$, both 9-*cis* RA and 13-*cis* RA also increased KLK5 and KLK7 protein in the media (Figure 5e and f). In contrast, both 9-*cis* RA and 13-*cis* RA decreased KRT10 mRNA expression compared with the control in NHEKs at 30% confluence (Figure 5g), consistent with the previous reports (Poumay *et al*., 1999). IVL mRNA expression was not significantly changed by RA (Figure 5h). Therefore, similar to the observations with $1,25(OH)_2VD_3$, KLK5 and KLK7 could be induced in cultured keratinocytes without inducing an increase in IVL and in a relative kinetic pattern different from the induction of KRT10.

Increasing KLK expression promotes differential processing of cathelicidin in keratinocytes

Next, we analyzed whether the increase in KLK expression could affect the protease activity of keratinocytes and final capacity to process endogenous cathelicidin precursor protein. Protease activity was significantly increased by culturing in 2 mM Ca^{2+} (Figure 6a), a finding consistent with earlier findings of an increase in mRNA and protein abundance. Importantly, $1,25(OH)_2VD_3$ and RA had an additive effect on protease activity in high calcium conditions (Figure 6a). We also observed an increase in protease activity in the culture media of cells maintained in 0.06 mM calcium and then treated with $1,25(OH)_2VD_3$ or RA, although the increase was not as large as that observed in NHEKs cultured in 2 mM calcium media (data not shown).

To examine whether the increase in protease activity by these stimuli could modify the processing of cathelicidin, active cathelicidin peptides in NHEKs were analyzed by surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS). To examine the intrinsic cathelicidin processing by calcium gradient, we cultured NHEKs in 1 mM calcium because (1) cathelicidin is gradually synthesized by calcium gradient, and culturing NHEKs in 1-mM calcium media is more efficient to induce cathelicidin than in 2-mM calcium media (data not shown), (2) 1 mM and 2 mM calcium has a similar effect on KLK induction in 48 hours (data not shown). $1,25(OH)_2VD_3$ is a primary factor of cathelicidin induction in NHEKs (Schauber *et al*., 2006), and we detected intrinsic cathelicidin processing. RAs, however, barely induce cathelicidin (Schauber *et al*., 2006), and we could not observe the cathelicidin processing in this assay. The mass of cathelicidin peptides that was detected in the Ca^{2+}-stimulated or $1,25(OH)_2VD_3$-stimulated NHEKs was different from each other but both were abundantly detected compared with the lack of detectable processed peptides in non-treated NHEKs (Figure 6b). Thus, induction of KLKs correlated with the appearance of processed cathelicidin peptides, but the mass distribution of these peptides was dependent on the nature of the stimulus used to increase KLK expression.

DISCUSSION

Recent progress in understanding elements of the epidermis that participate in defense against pathogenic micro-organisms has identified antimicrobial peptides such as cathelicidins and β-defensins as peptides that have an important role in preventing infection and modulating inflammation in the skin (Schauber and Gallo, 2008). These and other antimicrobial molecules form a chemical shield that works with the physical barrier of the stratum corneum. KLK5 and KLK7, enzymes known for their role in controlling the physical barrier of the stratum corneum (Eissa and Diamandis, 2008), have now been shown

to also influence the capacity of cathelicidin to be activated to peptide forms that can provide an antimicrobial barrier. Therefore, the combination of effects by KLK5 and KLK7 on skin barrier defense makes the understanding of the systems that control these genes important. To our best knowledge, it is previously unreported that the regulation of KLK5 and KLK7 expression in keratinocytes is influenced by several factors that can also influence epidermal differentiation. These observations uncover the existence of an important but distinct regulatory system that influences cutaneous innate immunity.

KLK5 and KLK7 are constitutively expressed in the skin and are abundant in the granular layer, an observation that appeared to link their expression to differentiation (Ekholm and Egelrud, 2000). In this study, we have shown that increasing extracellular Ca^{2+} induced KLK5 and KLK7 expression, and that this manipulation also increased the expression of differentiation markers such as KRT10 and IVL. KLK7 has been previously reported to be induced at a transcriptional level by Ca^{2+}-induced differentiation (Seo *et al*., 2004), but we show now that KLK5 and KLK7 are both induced by high-concentration Ca^{2+} and confirm that this increase in transcript is accompanied by increased KLK protein and enzyme activity. The increase in KLK5 or KLK7 expression by Ca^{2+} was similar to that of IVL or KRT10 expression. These findings suggest that Ca^{2+} itself or the state of Ca^{2+}-induced differentiation in keratinocytes will influence expression and activity of KLKs.

Several factors that activate keratinocyte growth and immune function were shown from the data of Figure 1 to have relatively little effect on KLK expression. However, we found here that $1,25(OH)_2VD_3$, a relatively recently recognized important modulator of immune status (Bikle, 2008), will induce KLK5 and KLK7 in keratinocytes. Unexpectedly, $1,25(OH)_2VD_3$ suppressed KRT10 mRNA expression in NHEKs and also had little effect on IVL. These time course experiments were begun at 30% confluence to avoid the initial influence of spontaneous differentiation when they reach confluence. As variable differentiation responses to $1,25(OH)_2VD_3$ have been previously observed, it is conceivable that $1,25(OH)_2VD_3$ might differently affect KRT10 and IVL expression depending on confluence. At later time points, the $1,25(OH)_2VD_3$ added to culture medium at the start of the experiment may no longer be active, or vitamin D receptor expression registered a decrease. Either or both processes are one of several explanations for the drop in KLK5 and KLK7 expression observed after 24 hours. Clearly, however, these results further show that $1,25(OH)_2VD_3$ regulates the expression of KLKs independently from the expression of these differentiation markers. In addition, through the coincident induction of cathelicidin by $1,25(OH)_2VD_3$, it could be observed that the increased KLK5 colocalized with increased cathelicidin, an observation that predicted that the cathelicidin precursor protein hCAP18 (18 kDa in mass) would be observed to be processed into smaller peptide forms between 4 and 5 kDa as observed in Figure 6b.

In addition, but distinctly from $1,25(OH)_2VD_3$, both 9-*cis* RA and 13-*cis* RA also induced KLK5 and KLK7 expression, and this was also observed to occur independently of differentiation. 9-*cis* RA is known to bind to both RA receptors (RARs) and retinoid X receptors (RXRs) (Nagpal and Chandraratna, 2000). 13-*cis* RA does not bind to RARs and RXRs with high affinity, but rather is metabolized in the cell into other isomers including all-trans RA and 9-*cis* RA (Allenby *et al*., 1993; Agarwal *et al*., 1996; Blaner, 2001). RXRs form RXR homodimers and RAR–RXR heterodimers or can heterodimerize with other nuclear receptors, including the vitamin D receptor (Nagpal and Chandraratna, 2000). These receptors induce transcription by binding to response elements such as RA response element, retinoid X response element, and vitamin D response element. The human KLK10 promoter has been reported to have an RA response element and retinoid X response element in some studies (Zeng *et al*., 2006). Analysis of the human KLK5 and KLK7 promoters using the MatInspector program did not comment on these RA response element

and retinoid X response element sites (Dong *et al*., 2008), and neither did this program predict the vitamin D response/RXR element in the cathelicidin promoter region, in which a functional vitamin D response/RXR element in keratinocytes has been confirmed for the cathelicidin gene *CAMP* (Schauber *et al*., 2006). Despite this, a manual search of KLK5 and KLK7 5′UTR reveals several possible RXR/RAR-binding sites that require future analysis and confirmation. Such further analyses will enable better understanding of the mechanism of transcriptional and post-transcriptional regulation of KLK5 and KLK7.

Importantly, to our best knowledge, it is previously unreported that the increase in KLK expression in keratinocytes occurs in parallel to an increase in protease activity and in enzymatic processing of cathelicidin. Additional analysis will be necessary to report with confidence the exact mass and sequence of cathelicidin peptides produced by keratinocytes stimulated with $1,25(OH)_2VD_3$ or Ca^{2+}. However, it is apparent that extracts from NHEKs stimulated with high concentration Ca^{2+} showed different peptides than that of $1,25(OH)_2VD_3$-stimulated NHEKs. Furthermore, peptides significantly smaller than the mass predicted for the best known human cathelicidin peptide (LL-37) were observed in NHEKs stimulated with $1,25(OH)_2VD_3$. Complete understanding of the proteolytic balance necessary for predicting the steady-state peptide product is difficult and must also include an understanding of the action of endogenous protease inhibitors. Furthermore, KLKs are also known to require their own enzymatic processing for activation. Pro-KLK7 can be activated by KLK5, other serine proteases, and metalloproteases; however, the activation of pro-KLK5 is not fully understood (Borgono and Diamandis, 2004). As a Ca^{2+} gradient is observed in normal epidermis with the maximum concentration in the granular layer (Mauro *et al*., 1998), the present result might explain why cathelicidin peptides are more abundant in upper layers of the epidermis, which are more important for defense against pathogenic microbes, than basal layers that may be more important in communication to other elements of the skin immune system.

In summary, we show here that Ca^{2+}, $1,25(OH)_2VD_3$, and RA will each induce the processing enzymes of cathelicidin, KLKs, in keratinocytes. Further studies to understand the significance of the effect of these systems on KLK activation may clarify the clinical effects of these compounds on skin barrier function and lead to unique applications relevant to the manipulation of cutaneous innate immunity.

MATERIALS AND METHODS

Cell culture and stimuli

NHEKs were obtained from Cascade Biologics/Invitrogen. (catalog number: C-001-5C, Portland, OR), and grown in serum-free EpiLife cell culture media (Cascade Biologics/Invitrogen) containing 0.06 mM Ca^{2+} and 1× EpiLife Defined Growth Supplement (EDGS, Cascade Biologics/Invitrogen) at 37 °C under standard tissue culture conditions. Cultures were maintained for up to eight passages in this media with the addition of 100 Uml^{-1} penicillin and 50 μgml^{-1} streptomycin. Cells were stimulated with $1,25(OH)_2VD_3$ (100 nM; Sigma-Aldrich, St Louis, MO), 9-*cis* RA (100 nM; Sigma-Aldrich), 13-*cis* RA (100 nM; Sigma-Aldrich), dexamethasone, (1 μM; Sigma-Aldrich), 17β-estradiol (100 nM; Sigma-Aldrich) Ca^{2+} (2 mM), epidermal growth factor (20 ng ml^{-1}; R&D Systems, Minneapolis, MN), tumor necrosis factor-α (20 ng ml^{-1}; Chemicon, Temecula, CA), IFN-α(200 Uml^{-1}, Chemicon), transforming growth factor β_1 (10 $ngml^{-1}$, R&D Systems), IL-6 (50 $ngml^{-1}$; R&D Systems), IL-8 (50 ng ml^{-1}; R&D Systems), Pam3CSK4 (1 μgml^{-1}; Invivogen, San Diego, CA), lipoteichoic acid (10 μgml^{-1}; Sigma-Aldrich), poly(I:C) (10 μgml^{-1}; Invivogen), lipopolysaccharide (1 μgml^{-1}; Sigma-Aldrich), flagellin (50 ng ml^{-1}; Alexis Biochemicals, Carlsbad, CA), MALP-2 (100 ng ml^{-1}; Alexis Biochemicals), imiquimod (10 μgml^{-1}; Invivogen), CL075 (5 μgml^{-1}; Invivogen), or ODN M362 (10 μgml^{-1}; Invivogen)

in 24-well flat bottom plates (Corning Incorporated Life Sciences, Lowell, MA) for up to 96 hours. After cell stimulation, total cell media were stored at −20 °C until analysis. RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) after supernatant collection. RNA was stored at −80°C.

Quantitative real-time-PCR

Complementary DNA was synthesized from RNA by the iScript cDNA Synthesis Kit (BioRad, Hercules, CA) as described by the manufacturer's protocol. TaqMan Gene Expression Assays (Applied Biosystems ABI, Foster City, CA) were used to analyze expressions of human *KLK5* (assay ID: Hs00202752_m1), human *KLK7* (assay ID: Hs00192503_m1), human *KRT10* (assay ID: Hs00166289_m1), human *IVL* (assay ID: Hs00846307_s1) as described by the manufacturer's instructions (the user bulletin #2 by Applied Biosystems). *GAPDH* mRNA was detected by probe: VIC-CATCCATGACAACTTTGGTA-MGB; primers: 5′-CTTAGCACCCCTGGCCAAG-3′; and 5′-TGGTCATGAGTCCTTCCACG-3, and was used as an internal control to validate RNA for each sample. Each mRNA expression was calculated as the relative expression to *GAPDH* mRNA, and all data are presented as fold change against each control (mean of non-stimulated cells).

ELISA

KLK5 or KLK7 protein in NHEK cultured media was measured by ELISA. 96-well EIA plates (Corning) were coated with mouse monoclonal anti-KLK5 or KLK7 antibody (1 $\mu g ml^{-1}$ in phosphate-buffered saline (PBS), catalog number: MAB1108 or MAB2624, respectively, R&D Systems) at 20–25 °C overnight. After washing with PBS containing 0.05% Tween 20 (washing buffer), the wells were blocked with PBS containing 1% BSA for 1 hour at 20–25 °C. After incubation with the cultured media or recombinant KLK5 or KLK7 (R&D Systems) as standards for 2 hours, biotinylated goat anti-KLK5 or KLK7 antibody (0.1 $\mu g ml^{-1}$, catalog number: BAF1108 or BAF2624, respectively, R&D Systems) was used as detection antibody. Streptoavidin-conjugated horseradish peroxidase (R&D Systems) and 3,3′5,5′-tetramethylbenzidine substrate (BD Biosciences, San Jose, CA) were used for colorimetric quantification, and reactions were stopped by 0.2 M sulfuric acid (Sigma-Aldrich). The absorbance at 450 nm was monitored with SpectraMax Plus384 (Molecular Devices Corp., Sunnyvale, CA), and concentrations in the samples were calibrated from the standard curve of recombinant KLK5 or KLK7 using SoftMax Pro4.6 (Molecular Devices Corp.).

In-cell western assay

KRT10 and IVL protein expression was examined by In-Cell Western Assay (LI-COR Biosciences, Lincoln, NE). NHEKs were cultured in 96-well plates and stimulated with high calcium (2 mM), $1,25(OH)_2VD_3$ (10^{-9} or 10^{-7} M), 9-*cis* RA (10^{-9} or 10^{-7} M), or 13-*cis* RA (10^{-9} or 10^{-7} M) for 24 or 48 hours. After the media were removed, cells were fixed with 3.7% paraformaldehyde for 20 minutes at room temperature (20–25 °C), permeabilized by 0.1% Triton X-100 containing PBS. Mouse monoclonal anti-IVL antibody (clone SY5, Sigma-Aldrich) or rabbit polyclonal anti-KRT10 antibody (Covance, Emeryville, CA) was used for primary antibodies, and IRDye 800 CW-labeled secondary antibodies were used to detect the signals. Cells were counterstained with DRAQ5 and Sapphire 700 by following the manufacturer's instructions. The signals from IRDye 800 CW were normalized by the signals from DRAQ5 and Sapphire 700, and the % response was calculated each time against vehicle-treated control as 100%. The data represent means and SEM in graphs.

Immunostaining

For immunocytofluorescence, NHEKs were grown on chamber slides. Cells were stimulated with $1,25(OH)_2VD_3$ for 24 hours. After removing the culture media, cells were fixed with 4% paraformaldehyde, blocked with 3% BSA in PBS, and incubated with mouse monoclonal anti-KLK5 antibody or anti-KLK7 antibody and rabbit anti-LL-37 antibody at 4 °C overnight. After washing with PBS, FITC-conjugated goat anti-rabbit IgG antibody (Jackson Immuno Research Laboratories, West Grove, PA) and AlexaFluor568-conjugated goat anti-mouse IgG antibody (Molecular Probes/Invitrogen, Eugene, OR) were used as the second antibody. Sections were mounted in the ProLong Gold Anti-Fade reagent with DAPI (Molecular Probes/Invitrogen). Images were obtained by Zeiss LSM700 laser scanning confocal microscope (Carl Zeiss MicroImaging, Thornwood, NY) or by Olympus BX41 fluorescent microscope (Scientific Instrument Company, Temecula, CA).

Protease assay

Protease activity in cultured media was monitored with EnzChek Protease Assay Kit green fluorescence (Molecular Probes/Invitrogen) according to the manufacturer's instructions. Briefly, 100 μl of the cultured media was mixed with 100 μl of BODIPY FL casein substrate in 10 mM Tris-HCl, pH 7.8, and incubated at 37 °C for 48 hours. Protease activity was monitored as increased fluorescence with SpectraMax GEMINI EM (Molecular Devices Corp.).

SELDI-TOF-MS

NHEKs were stimulated with $1,25(OH)_2VD_3$ (10^{-7} M) or Ca^{2+} (1 mM) for 48 hours. Cells were dissolved in 100 ml of radioimmunoprecipitation assay (RIPA) buffer (50 mM HEPES, 150 mM NaCl, 0.05% SDS, 0.25% deoxycholate, and 0.5% NP-40; pH 7.4) containing protease inhibitors (Roche Applied Science, Indianapolis, IN). Samples were sonicated for 3 minutes and centrifuged for 10 minutes at 12,000 *g*. We transferred the supernatant to new tubes and kept it at −20 °C until SELDI-TOF-MS analysis. Protein chips (RS-100 protein chip array; Ciphergen Biosystems, Fremont, CA) were coated with 4 μl of rabbit antibody to LL-37 for 2 hours at room temperature, and then blocked with 0.5 M ethanolamine in PBS (pH 8.0). After three washes with 0.5% Triton-X in PBS, protein chips were assembled in the Bioprocessor reservoir, 50 μl of eluted sample was applied, and the chips were incubated for 2 hours at room temperature. The protein chips were washed twice with RIPA buffer, once with PBS containing 0.5% Triton-X and three times with PBS, and then soaked in 10 mM HEPES buffer and air-dried. We applied 0.5 μl of the energy absorbance molecule (50% saturated a-cyano-4-hydroxy cinnamic acid in 50% acetonitrile plus 0.5% trifluoric acid) twice, and all spots were allowed to dry completely. Samples were analyzed on a surface-enhanced laser desorption/ionization mass analyzer PBS II with a linear time-of-flight mass spectrometer (Ciphergen Biosystems) using time-lag focusing. Synthetic LL-37 peptide was used as reference to calibrate the exact mass sizes.

Statistics

Results are expressed as the mean±SEM. Student's *t*-test was used to determine significance. $P<0.05$ was considered significant.

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Abbreviations

IVL	involucrin
KLK	kallikrein
KRT	keratin
NHEKs	normal human epidermal keratinocytes
qPCR	quantitative real-time PCR
RA	retinoic acid
RIPA	radioimmunoprecipitation assay
SELDI-TOF-MS	surface-enhanced laser desorption/ionization time-of-flight mass spectrometry
1, $25(OH)_2VD_3$	$1,25(OH)_2$vitamin D_3

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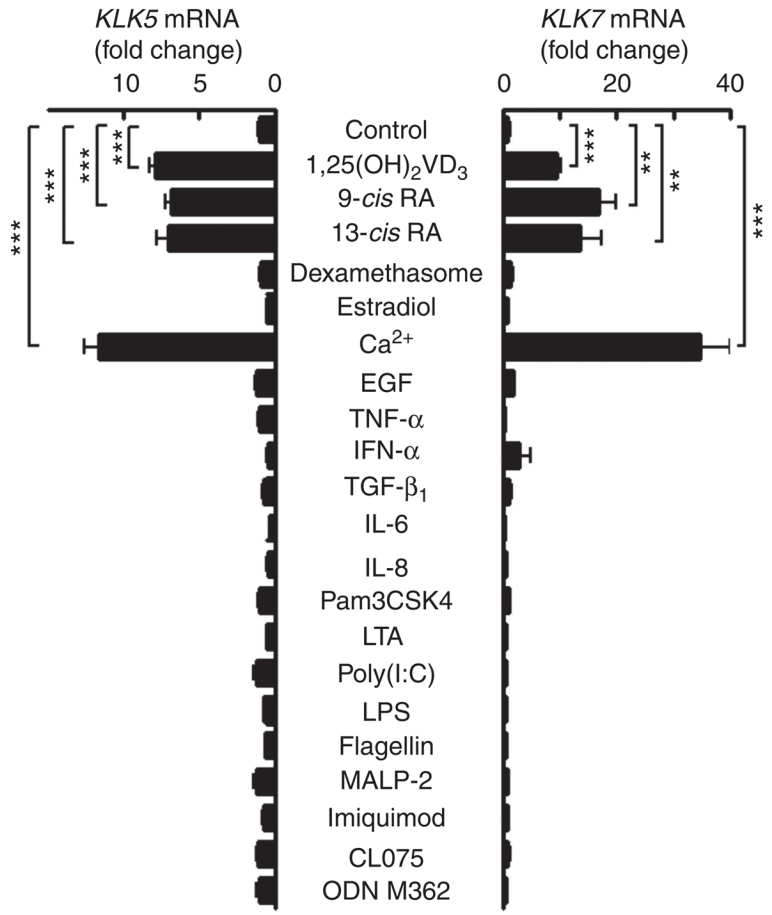

Figure 1. Regulation of KLK5 and KLK7 mRNA expression in keratinocytes

NHEKs (passage 7) at 80% confluence were stimulated with hormones, biological stimuli, cytokines, growth factors, and microbial products for 24 hours. The concentration used for each stimulus is shown in Materials and Methods. The expression of KLK5 and KLK7 mRNA was measured by quantitative real-time PCR. mRNA expression was calculated as relative to *GAPDH* mRNA, and all data are presented as fold change against vehicle-treated control. Data represent the mean±SEM of triplicate determinations from a single experiment representative of three independent experiments. ***P*<0.01, ****P*<0.001.

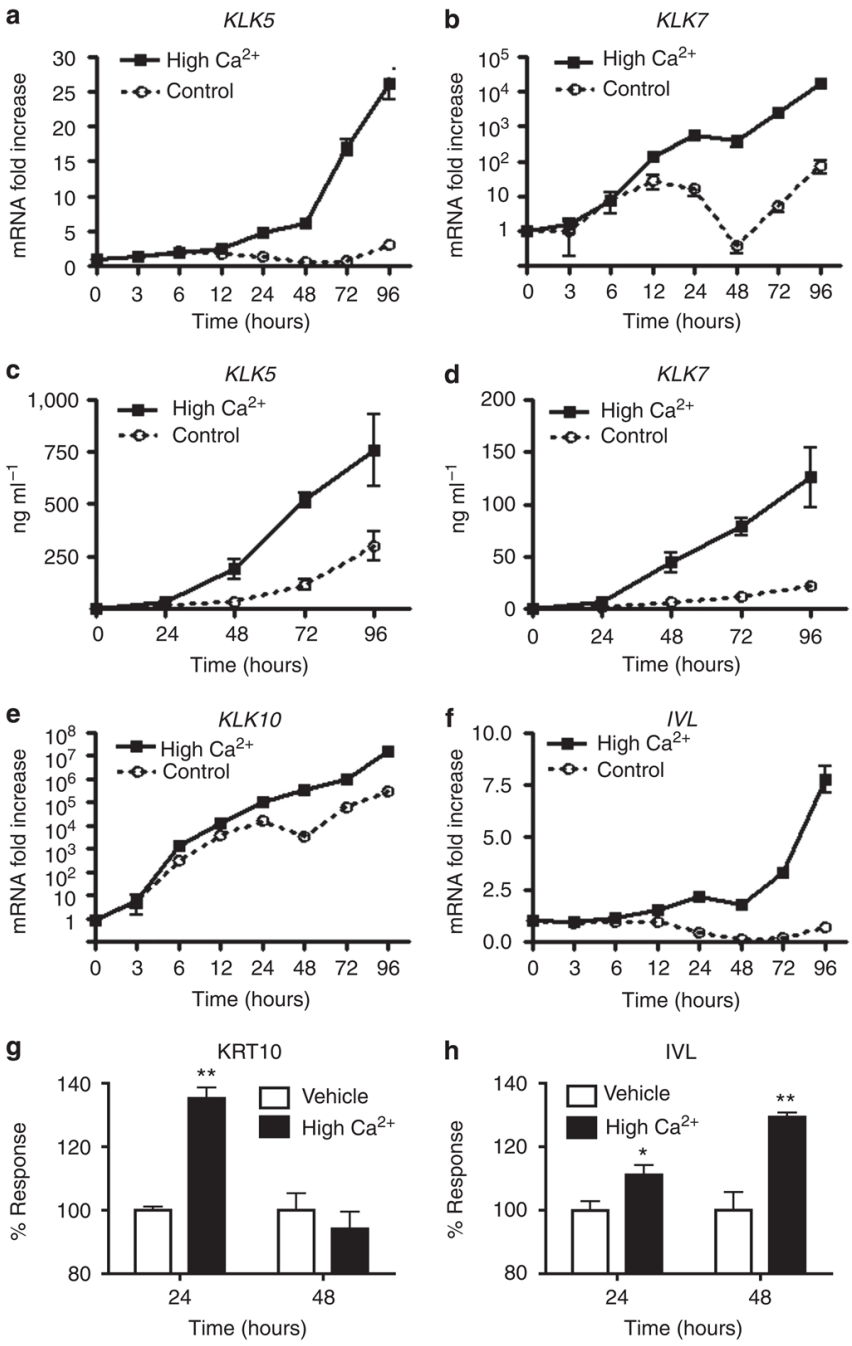

Figure 2. High Ca^{2+} induces KLK5 and KLK7 expression in keratinocytes

NHEKs (passage 5) initially at 30% confluence were cultured in the presence of 0.06 mM (control) or 2 mM Ca^{2+} for 3, 6, 12, 24, 48, 72, or 96 hours. The expression of KLK5 (**a**), KLK7 (**b**), KRT10 (**e**), and IVL (**f**) mRNA were measured by quantitative real-time PCR. KLK5 (**c**) and KLK7 (**d**) protein in media was measured by ELISA. mRNA expression was calculated as relative to *GAPDH* mRNA, and all data are presented as fold increase against 0 hour non-treated control. (**g, h**) KRT10 and IVL protein expression was examined in NHEKs (passage 6) cultured in 2 mM Ca^{2+} for 24 or 48 hours by In-Cell Western as described in Materials and Methods. The percent responses were calculated against vehicle-treated control at each time point. Data represent the mean±SEM of triplicate determinations

from a single experiment representative of three independent experiments. *P<0.05, **P<0.01.

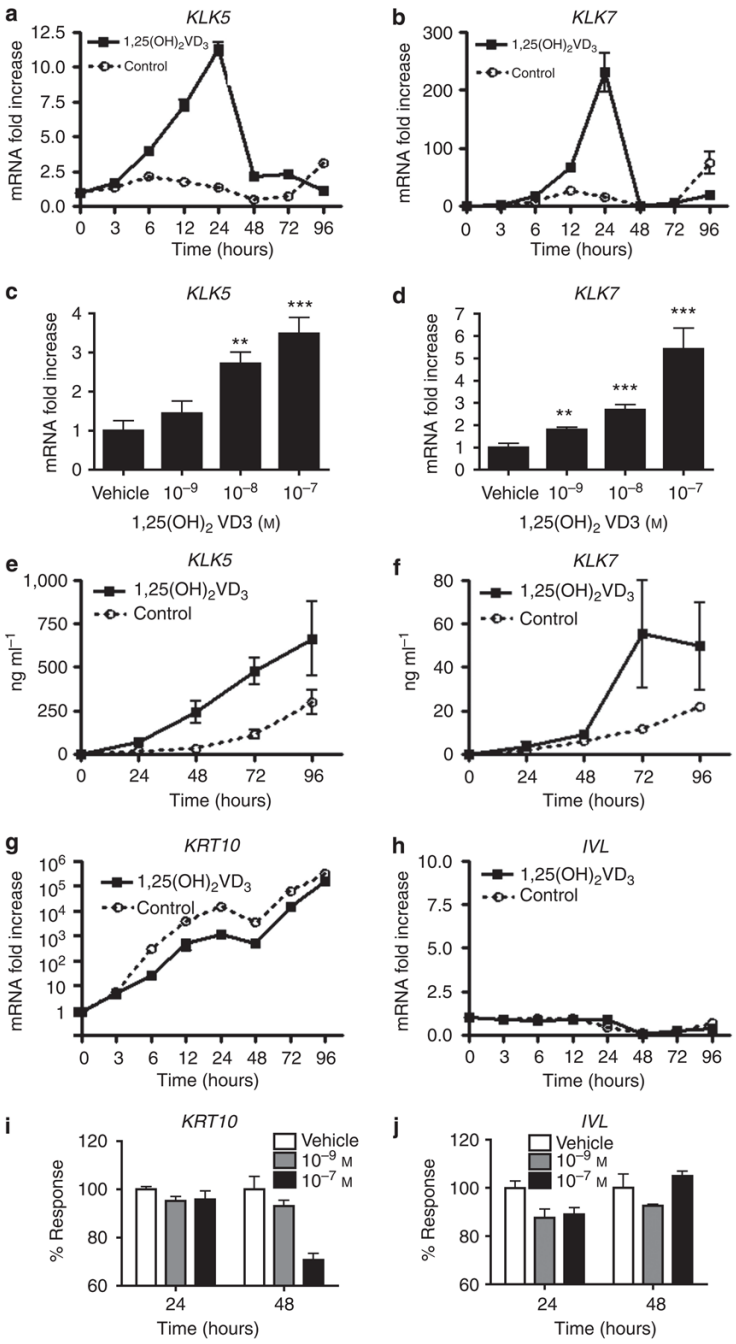

Figure 3. 1,25(OH)$_2$VD$_3$ induces KLK5 and KLK7 expression in keratinocytes

NHEKs (passage 5) initially at 30% confluence were stimulated with 1,25(OH)$_2$VD$_3$ (10^{-7} M) or the vehicle for 3, 6, 12, 24, 48, 72, or 96 hours. The expression of KLK5 (**a**), KLK7 (**b**), KRT10 (**g**), and IVL (**h**) mRNA was measured by quantitative real-time PCR (qPCR). (**c, d**) NHEKs (passage 5) were stimulated with 10^{-9} to 10^{-7} M of 1,25(OH)$_2$VD$_3$ for 24 hours, and the expression of KLK5 (**c**) and KLK7 (**d**) mRNA was measured by qPCR. mRNA expression was calculated as the relative expression to *GAPDH* mRNA, and all data are presented as fold increase against control. KLK5 (**e**) and KLK7 (**f**) protein in the culture media was measured by ELISA. (**i, j**) NHEKs (passage 6) were stimulated with 10^{-9} or 10^{-7} M of 1,25(OH)$_2$VD$_3$ for 24 or 48 hours, and KRT10 and IVL protein expression was

examined by In-Cell Western as described in Materials and Methods. The percent responses were calculated against vehicle-treated control at each time point. Data represent the mean ±SEM of triplicate determinations from a single experiment representative of three independent experiments. $^{**}P<0.01$, $^{***}P<0.001$.

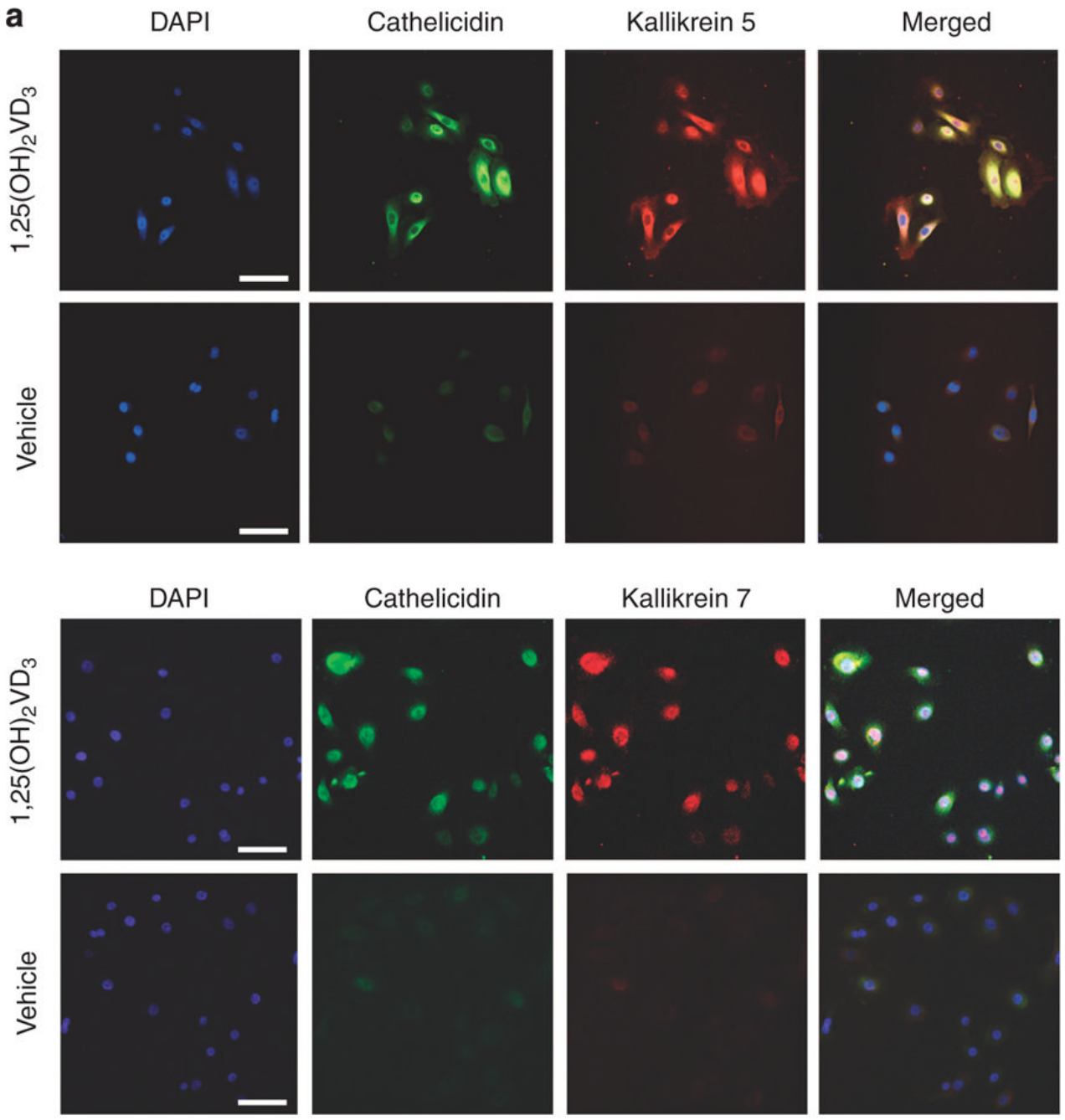

Figure 4. KLK5 and cathelicidin colocalize in keratinocytes stimulated with $1,25(OH)_2VD_3$

NHEKs (passage 6) were grown on chamber slides. Cells were stimulated with $1,25(OH)_2VD_3$ (10^{-7} M) or the vehicle for 24 hours. The expression of KLK5, KLK7, and cathelicidin was examined by immunocytofluorescence, and nuclei were visualized with DAPI. Scale bars=20 μm.

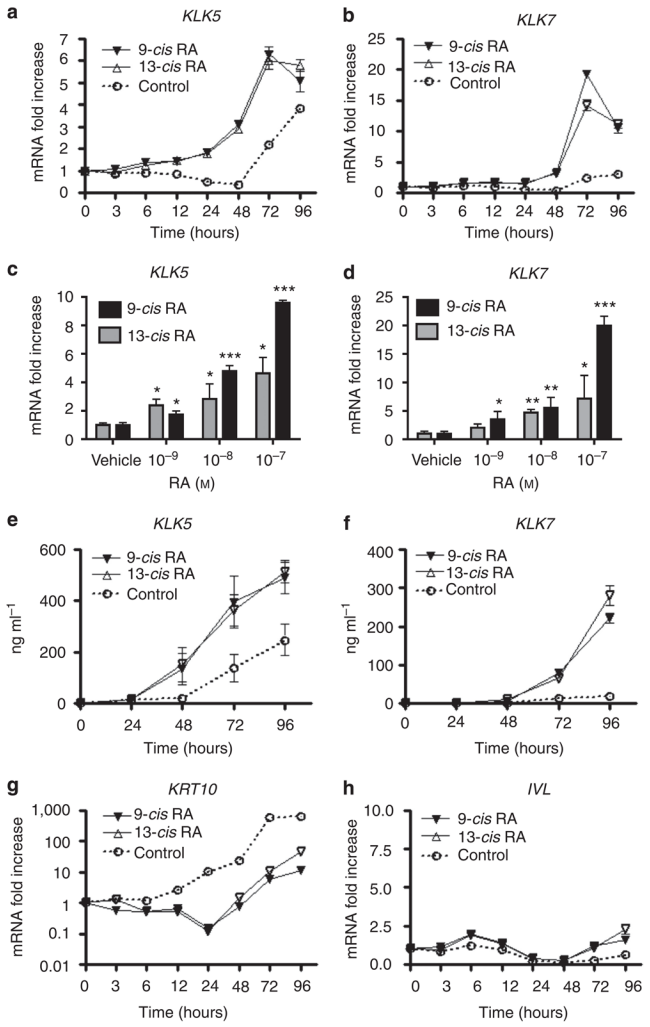

Figure 5. Retinoic acids induce KLK5 and KLK7 expression in keratinocytes

NHEKs (passage 3) initially at 30% confluence were stimulated with 9-*cis* RA (10^{-7} M) or 13-*cis* RA (10^{-7} M) for 3, 6, 12, 24, 48, 72, or 96 hours. The expression of KLK5 (**a**), KLK7 (**b**), KRT10 (**g**), and IVL (**h**) mRNA was measured by quantitative real-time PCR (qPCR). NHEKs (passage 4) were stimulated with 10^{-9} to 10^{-7} M of 9-*cis* or 13-*cis* RA for 24 hours, and the expression of KLK5 (**c**) and KLK7 (**d**) mRNA was measured by qPCR. mRNA expression was calculated as relative to expression of *GAPDH* mRNA, and is presented as fold increase against control. KLK5 (**e**) and KLK7 (**f**) proteins in culture media were measured by ELISA. Data represent the mean±SEM of triplicate determinations from a single experiment representative of two independent experiments. **P*<0.05, ***P*<0.01, ****P*<0.001.

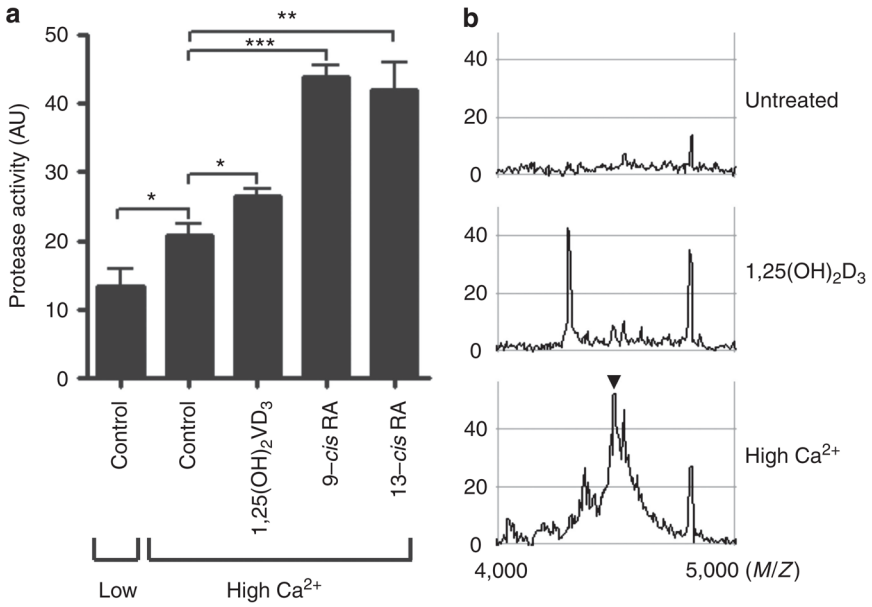

Figure 6. Increase in protease activity and processing of cathelicidin by factors that induce KLK5 and KLK7 in keratinocytes

(**a**) NHEKs (passage 8) were simulated with $1,25(OH)_2VD_3$ (10^{-7} M), 9-*cis* RA (10^{-7} M), or 13-*cis* RA (10^{-7} M) in the presence of 2 mM Ca^{2+} for 96 hours. Culture media were incubated with fluorescence-conjugated casein substrate for 48 hours, and protease activity was measured on the basis of generation of fluorescent product from this substrate as described in Material and Methods. **P*<0.05, ***P*<0.01, ****P*<0.001. Data represent the mean±SEM of triplicate determinations from a single experiment representative of three independent experiments. (**b**) NHEKs (passage 2) were stimulated with $1,25(OH)_2D_3$ (10^{-7} M) or 1 mM Ca^{2+} media for 48 hours, and peptides were extracted with radioimmunoprecipitation assay buffer. Cathelicidin peptide mass was determined by surface-enhanced laser desorption/ionization time-of-flight mass spectrometry. Arrowhead indicates the peak expected for LL-37 (4496 m/z).