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PTH/PTHrP and Vitamin D Control Antimicrobial Peptide Expression and Susceptibility to Bacterial Skin Infection

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Abstract

The production of antimicrobial peptides is essential for protection against a wide variety of microbial pathogens and plays an important role in the pathogenesis of several diseases. The mechanisms responsible for expression of antimicrobial peptides are incompletely understood, but a role for vitamin D as a transcriptional inducer of the antimicrobial peptide cathelicidin has been proposed. We show that 1,25-dihydroxyvitamin D_3 (1,25-D3) acts together with parathyroid hormone (PTH), or the shared amino-terminal domain of PTH-related peptide (PTHrP), to synergistically increase cathelicidin and immune defense. Administration of PTH to mouse skin decreased susceptibility to skin infection by group A *Streptococcus*. Mice on dietary vitamin D_3 restriction that responded with an elevation in PTH have an increased risk of infection if they lack 1,25-D3. These results identify PTH/PTHrP as a variable that serves to compensate for inadequate vitamin D during activation of antimicrobial peptide production.

INTRODUCTION

Antimicrobial peptides are a diverse group of molecules that prevent microbial invasion and infection in several tissues (1). In particular, cathelicidin and β-defensins are antimicrobial peptides that kill a broad spectrum of bacteria, fungi, and viruses (2). Antimicrobial peptides are produced by keratinocytes in the epidermis and can be further induced at sites of microbial entry to act as an impediment to infection. In mice, cathelicidin expression is essential for defense against invasive skin infection by group A *Streptococcus* (3), as well as

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SUPPLEMENTARY MATERIALS www.sciencetranslationalmedicine.org/cgi/content/full/4/135/135ra66/DC1
Materials and Methods
Fig. S1. PTH induces cathelicidin in Ca^{2+}-differentiated human keratinocytes and basal mouse keratinocytes but requires the presence of vitamin D_3 in basal human keratinocytes.
Fig. S2. Dietary vitamin D_3 restriction increases serum PTH and decreases 25-D3.
Reference

Author contributions: B.M., D.D.B., C.A., G.L.S., D.W.B., L.J.D., and R.L.G. designed the experiments; B.M. performed the mouse experiments and the experiments with cultured cells; C.A. performed the mouse experiments; G.L.S. performed the DNA methylation experiments; D.W.B. performed the transfection experiments with PTHrP; B.M., D.D.B., D.W.B., L.J.D., G.L.S., and R.L.G. analyzed the data; and B.M. and R.L.G. wrote the paper.

Competing interests: The authors declare that they have no competing interests.

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protection against a wide variety of other bacterial, viral, and fungal pathogens (1). In humans, the importance of antimicrobial peptides to resist infection is seen in atopic dermatitis, where the expression of cathelicidin and β-defensins is diminished in skin lesions compared to skin lesions from other inflammatory disorders (4). This defect in antimicrobial peptide production is associated with increased susceptibility to skin infections due to group A *Streptococcus, Staphylococcus aureus*, and some viruses. Other diseases, such as psoriasis and rosacea, have been associated with abnormally high expression of antimicrobial peptides (1). Thus, understanding mechanisms that control antimicrobial peptide expression will be useful for several human diseases.

The observation that vitamin D can directly induce the production of cathelicidin in monocytes and epithelial cells (5, 6) provided an explanation for the association between increased rates of tuberculosis in patient populations with inadequate endogenous vitamin D synthesis (7). Supporting this finding, laboratory studies have shown potential biological mechanisms for a role of vitamin D in the immune response (8). These observations include findings that the vitamin D receptor exists in high concentrations on immune cells (9, 10) and that 25-hydroxyvitamin D_3 (25-D3) 1α-hydroxylase (CYP27B1) is also located in these cells (7, 11). This enzyme represents a critical step in regulation of vitamin D function as it converts 25-D3 to 1,25-D3 (1,25-dihydroxyvitamin D_3), the most potent form that activates the vitamin D receptor and can influence both adaptive and innate immune functions in vitro (12–14). Further support for an immunological role of vitamin D has been suggested by animal models of autoimmunity and inflammatory bowel disease (15, 16), and by observations in humans that polymorphisms in the vitamin D receptor are associated with increased susceptibility to infection with *Mycobacterium tuberculosis* (17).

Despite supportive laboratory evidence, only a minority of human clinical trials have shown that vitamin D supplementation provides protection against bacterial infection (18). Noncalcemic actions for vitamin D such as immune regulation have not yet been consistently proven in observations comparing only vitamin D intake or serum levels of 25-D3 (19). This inconsistency between in vitro model systems and human clinical studies suggests that there is an incomplete understanding of the variables that affect interactions between vitamin D and the immune system. Additional model systems are needed to understand these complex interactions.

Parathyroid hormone (PTH) is an important compensatory response to vitamin D deficiency (20, 21). The PTH-related peptide (PTHrP) is expressed in skin and other tissues, and its N-terminal peptide can bind similar receptors to PTH. Here, we investigated how PTH/PTHrP may act with vitamin D to influence immune defense against skin infection. Results uncover an unexpected role for PTH/PTHrP for enhancing antimicrobial peptide expression and for helping to protect against infection.

RESULTS

PTHrP is induced by bacterial products

The expression of PTHrP was found to be inducible in keratinocytes by bacterial products. Stimulation of primary cultures of normal human keratinocytes with the Toll-like receptor 2 (TLR2) agonist lipoteichoic acid (LTA) enhanced PTHrP mRNA expression (Fig. 1A). PTH and PTHrP act through a common receptor [PTH receptor type 1 (PTH1R)], but previous work has reported inconsistent results regarding the expression of PTH1R in keratinocytes (22, 23). Treatment of normal human keratinocytes with 1,25-D3 (10^{-7} M) for 24 hours induced PTH1R mRNA (Fig. 1B) and abundant PTH1R protein expression (Fig. 1C).

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PTHrP and PTH cooperate with vitamin D_3 to induce cathelicidin expression

Given that keratinocytes were observed to produce PTHrP and its receptor under the influence of LTA and vitamin D, and that cathelicidin has previously been shown to also respond to these stimuli, we sought to determine whether the endogenous production of PTHrP could induce the cathelicidin gene (*CAMP*). PTHrP exists as three isoforms: PTHrP(1–139), PTHrP(1–141), and PTHrP(1–173) (24). Transfection of the human keratinocyte line HaCaT with constructs of each isoform increased *CAMP* expression, but transfection with vector alone, or constructs of each isoform that lack the N-terminal PTHrP 1 to 32 amino acids, did not (Fig. 2A). Transfection of HaCaT with PTHrP small interfering RNA (siRNA) was successful in suppressing endogenous PTHrP by 89% and simultaneously decreased the expression of *CAMP* mRNA (Fig. 2B).

To investigate whether exogenous PTH could also influence cathelicidin expression, we directly added PTH to the culture medium. This experiment was conducted with and without simultaneous addition of 25-D3 to induce PTH1R. Pilot experiments with normal human epidermal keratinocytes were performed in medium containing several Ca^{2+} concentrations to induce differentiation. *CAMP* was significantly induced at all Ca^{2+} concentrations in the presence of both low-dose 25-D3 and PTH (fig. S1A). Normal human epidermal keratinocytes under basal conditions were chosen for further experiments. At a 25-D3 concentration of 50 nM, costimulation with physiological doses of PTH (50 to 100 pg) for 24 hours resulted in a dose-dependent increase in *CAMP* (Fig. 2C). The action of PTH to induce *CAMP* was dependent on conversion of 25-D3 to 1,25-D3, because inhibition of keratinocyte endogenous hydroxylase activity with itraconazole blocked the response after addition of 25-D3 (Fig. 2D). The response to PTH and PTHrP by keratinocytes was dependent on the expression of PTH1R, because addition of PTH1R siRNA suppressed the cathelicidin response (Fig. 2E). PTH1R knockdown efficiency was 92%. In addition to *CAMP*, CD14 also responded to PTH and vitamin D (fig. S1, B and C). The murine cathelicidin gene *Camp* was also induced by PTH, but the addition of 1,25-D3 had no additional effect (fig. S1D).

Antimicrobial peptide expression is mediated by DNA methylation and protein kinase C

It is unclear how PTH/PTHrP enhances *CAMP* expression. Previously, PTH has been shown to control gene expression by demethylation (25), so we set out to determine whether methylation might also influence *CAMP* expression. Genome-wide methylation analysis of basal human keratinocytes using methylated DNA immunoprecipitation (MeDIP) revealed three methylated DNA sites in the vicinity of the *CAMP* transcription start site (TSS) (Fig. 3A). Treatment of keratinocytes with the methylation inhibitor 5-azacytidine (5azaC) enhanced 1,25-D3–induced *CAMP* expression to a degree similar to that of PTH-enhanced 1,25-D3. 5azaC did not further increase the combination of PTH and 1,25-D3 (Fig. 3B). In addition to methylation, PTH1R signaling involves protein kinase C (PKC) (25). Analysis of the effect of the PKC inhibitor GF109203X demonstrated that this abrogated the expression of *CAMP* induced by PTH (Fig. 3C).

PTH and vitamin D_3 protect against invasive bacterial skin infection in vivo

We next tested the functional relevance of PTH/PTHrP in enhancing protection against skin infection in vivo. Subcutaneous administration of mouse PTH (mPTH) 24 hours before challenge with group A *Streptococcus* protected mice against infection as observed by a modest reduction in group A *Streptococcus* recovered from the skin after 72 hours (Fig. 4A) and a significant reduction in the visible lesion size (Fig. 4, B and C). To test the co-dependence of mPTH and 1,25-D3 seen in Fig. 2, we also evaluated CYP27B1$^{-/-}$ mice lacking CYP27B1 and subsequently without 1,25-vitamin D_3 after injection of mPTH. In

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contrast to wild-type mice, administration of mPTH before group A *Streptococcus* did not protect these mice against infection (Fig. 4, D and E).

Dietary vitamin D_3 restriction in CYP27B1$^{-/-}$ mice increases susceptibility to group A *Streptococcus* infection

To understand the effect of vitamin D deficiency on defense against invasive bacterial infection of the skin, we maintained CYP27B1$^{-/-}$ or wild-type mice for 4 weeks on a diet not containing any source of vitamin D. These mice were then challenged by subcutaneous injection with group A *Streptococcus*. Dietary restriction of vitamin D_3 in CYP27B1$^{-/-}$ mice increased susceptibility to invasive group A *Streptococcus* infection (Fig. 5, A to C). This group also had higher group A *Streptococcus* in skin and spleen (Fig. 5, D and E). However, no significant increase in susceptibility to infection was detectable in CYP27B1$^{-/-}$ mice maintained on a control diet or wild-type mice on a vitamin D–restricted diet.

Evaluation of serum PTH, calcium, 25-D3, and 1,25-D3 showed that dietary vitamin D_3 restriction resulted in an expected compensatory increase in serum PTH and decrease in serum 25-D3. CYP27B1$^{-/-}$ mice lacked 1,25-D3 (fig. S2). As predicted by earlier in vitro analysis, skin *Camp* expression showed an increase that correlated with elevated PTH in wild-type mice if 1,25-D3 was present, but this response did not occur in CYP27B1$^{-/-}$ mice (Fig. 5E). Elevated mouse β-defensin 4 (mBD4) was found in CYP27B1$^{-/-}$ mice on the rescue diet (Fig. 5F). Increased group A *Streptococcus* infection was only observed in those mice that lacked an increase in *Camp* and mBD4.

DISCUSSION

The current study demonstrates that PTH/PTHrP and vitamin D both influence the expression of cathelicidin antimicrobial peptide and the capacity of mice to resist infection by group A *Streptococcus*. Previously, cell culture has suggested a link between infection and vitamin D (26, 27), but animal model data have been less compelling. A lack of a useful mouse model for dietary vitamin D deficiency has been explained by the difficulty in generating severe vitamin D deficiency in this species (15). Here, the action of PTH, which compensates for vitamin D deficiency by maintaining calcium homeostasis, was found to be a potent mechanism for inducing the expression of cathelicidin in both mice and human cells. These observations advance our understanding of how vitamin D_3 influences immune defense by suggesting that the elevation of PTH in response to dietary vitamin D_3 restriction may compensate for the loss of vitamin D and its role in immune functions.

1,25-D3 and PTH/PTHrP appear to act together in human keratinocytes. Several interrelationships exist in the innate immune response between metabolic activation of vitamin D and regulation of PTH/PTHrP. An overview of the interactions between PTH/PTHrP, PTH1R, vitamin D, and cathelicidin expression is shown in Fig. 6. We found that 1,25-D3 induced PTH1R expression, thus enabling a response to PTH/PTHrP. 25-D3 enabled keratinocytes to respond to PTH/PTHrP because of the endogenous capacity of keratinocytes to hydroxylate 25-D3 to 1,25-D3. Inhibition of this activity by either addition of itraconazole in culture or targeted deletion of CYP27B1 in mice blocked the function of PTH/PTHrP. Furthermore, in the absence of 1,25-D3, the expression of PTH1R was low. Therefore, this result supported previous reports that 1,25-D3 acts as a stimulus of *CAMP* expression (5–7) and also suggests that it enables action of PTH/PTHrP. PTH can also increase 1,25-D3 levels in keratinocytes (28), suggesting a positive feedback system. We also observed that PTH/PTHrP and PTH1R also had an effect on keratinocyte *CAMP* expression in the absence of exogenous sources of vitamin D, suggesting that the PTH system has the capacity to act by itself or amplify other stimuli that regulate cathelicidin

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expression. Alternative pathways that induce cathelicidin expression exist in the mouse, where a vitamin D receptor element is absent from the *Camp* promoter. Therefore, the PTH/PTHrP enhancer system is active in the control of cathelicidin in mice even if the mouse promoter lacks a vitamin D receptor element.

Our current findings provide evidence that DNA methylation is involved in the control of cathelicidin. Previous studies have shown that transcriptional suppression through DNA methylation is derepressed upon PTH1R activation (25). We find that the cathelicidin promoter in human keratinocytes is methylated and that chemical inhibition of methylation can increase *CAMP* expression to a degree similar to that induced by PTH/PTHrP. These associations, as well as the role of PKC, support the notion that activation of PTH1R can induce *CAMP*. Future work is needed to investigate this system and expand on the hypothesis that PTH1R acts on *CAMP* through epigenetic mechanisms involving DNA methylation.

The exact functions of PTH/PTHrP in skin are poorly understood. PTHrP may have a role in the hair growth cycle (29), angiogenesis (30), epidermal cell proliferation, and differentiation (31). PTHrP modulates intracellular calcium in a number of cell types, and calcium has been implicated as an early signal in the differentiation of keratinocytes (32). Given that keratinocytes under high-calcium conditions show enhanced expression of skin antimicrobial peptides and their processing enzymes (33, 34), this may be an alternate mechanism of action. Furthermore, a recent study has shown that expression of *CAMP* can be induced by CREB (cyclic adenosine monophosphate response element–binding protein) (35), a downstream transcriptional regulator activated by engagement of the PTH1R (36). Thus, several cellular signaling mechanisms may enable PTH/PTHrP to influence innate immunity.

The relevance of PTH/PTHrP in vivo was demonstrated by observations that mice injected with PTH increase their resistance to invasive group A *Streptococcus* infection. Although the decrease induced by PTH in the absolute number of group A *Streptococcus* colony-forming units (CFU) measured in the skin was relatively modest, a large decrease in absolute lesion size was observed, therefore suggesting that a local increase in PTH is important to enhance resistance. Furthermore, the potential consequences of diet were illustrated in mice on dietary vitamin D restriction. Mice with intact 1,25-D3 levels but restricted dietary vitamin D had elevated PTH and *Camp*, and maintained normal protection against infection. On the basis of our results in culture, it is tempting to speculate that the increase in *Camp* was a result of the increase in PTH. Supporting this were additional observations that mice lacking CYP27B1 expression did not increase *Camp* despite elevated PTH. Only this group showed increased susceptibility to infection by group A *Streptococcus*. This is consistent with our findings in culture that response to PTH is optimal in the presence of 1,25-D3 and that mice injected with mPTH do not increase resistance to infection if they lack CYP27B1.

The antimicrobial responses of mice on vitamin D restriction support the notion of a role for PTH in innate immunity. Wild-type mice on dietary vitamin D_3 restriction had a 600-fold increase in cathelicidin that accompanied the rise in PTH. We believe that this increase may have been induced by the elevated PTH and protected these mice against increased infection. The unexpected response in this system was that CYP27B1$^{-/-}$ mice on a normal diet showed a large increase in mBD4 and also did not have enhanced skin infection. This finding suggests that 25-D3, in the absence of 1,25-D3, has other influences on innate immunity beyond cathelicidin. Given that vitamin D_3 and PTH induce CD14, one may speculate that enhanced pattern recognition through CD14 may result in enhanced mBD4

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and thus offer protection in the absence of cathelicidin. Future work is required to better understand this potential alternate system.

We have shown that either PTH or PTHrP can induce an antimicrobial peptide response and enhance immune defense. The current study also shows a role of dietary vitamin D_3 in the prevention of group A *Streptococcus* skin infection. We find that multiple factors can cooperate with dietary vitamin D_3 to influence innate immune responses in the skin: vitamin D_3 metabolism, PTH1R expression, and pattern receptor activation. These observations provide a new understanding of the relationship between vitamin D_3 status and infection.

MATERIALS AND METHODS

Mice

Mice heterozygous for the CYP27B1 null mutation, CYP27B1$^{-/+}$, outbred to C57BL/6 as previously described (37), were bred to provide wild-type (CYP27B1$^{+/+}$) and homozygous mutant CYP27B1$^{-/-}$ mice. All animal experiments were approved by the University of California, San Diego, Institutional Animal Care and Use Committee. CYP27B1$^{-/-}$ mice and their wild-type littermates were used at the age of 12 weeks and were raised on a "rescue diet" (TD.96348, Teklad) containing 2.2 IU of vitamin D_3 per gram of diet, 2% calcium, 1.25% phosphorus, and 20% lactose (38). The rescue diet was the control diet in all experiments. For dietary vitamin D restriction, mice were switched to a vitamin D–deficient diet (TD.87095, Teklad) 4 weeks before starting the experiments. The vitamin D–deficient diet differs from the rescue diet only in that it does not contain any source of vitamin D.

Bacterial skin infection

Skin infection experiments with group A *Streptococcus* NZ131 were done as described before (26). In brief, group A *Streptococcus* was grown to mid-log phase in Todd Hewitt broth (Sigma). Group A *Streptococcus* (10^7 CFU) in 100 μl of phosphate-buffered saline (PBS) was injected subcutaneously into mouse back skin. Lesion size was assessed after 24, 48, and 72 hours. After 72 hours, a 5-mm skin punch biopsy comprising the center of infection was harvested, homogenized with 2-mm zirconia beads in a Mini-Beadbeater (BioSpec Products Inc.), serially diluted, plated onto Todd Hewitt agar, and enumerated after 18 hours to quantify the CFU per milliliter.

Cell culture

Normal human keratinocytes (Life Technologies Inc.) were cultured in EpiLife medium (Cascade Biologics) supplemented with 0.06 mM calcium, EpiLife Defined Growth Supplement (EDGS) (Cascade Biologics), penicillin (50 U/ml), and streptomycin (50 mg/ml) (Cellgro Mediatech). HaCaT keratinocytes were grown in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich). Cells at 70 to 80% confluence were stimulated with 25-D3, 1,25-D3 (10^{-10} to 10^{-7} M; Sigma), or control [0.1% ethanol, 15% (v/v) trypticase soy broth], human PTH [hPTH(1–34), 12.5 to 100 pg/ml or 10^{-11} M; Sigma], mPTH(1–34) (10^{-11} M; Sigma), or LTA from *S. aureus* (10 μg/ml; Sigma) for 24 hours. In addition, normal human epidermal keratinocytes were pretreated with the CYP27B1 enzyme inhibitor itraconazole (10^{-7} M) (5), the vitamin D receptor antagonist VAZ (ZK159222; provided by U. Zügel from Schering AG; 10^{-7} M), DNA methyltransferase inhibitor 5azaC (10^{-6} M; Sigma), or PKC inhibitor GF109203X (5 μM; Sigma) 1 hour before stimulation with vitamin D_3. Furthermore, normal human epidermal keratinocytes were differentiated in high calcium (0.5 to 2.0 mM) for 48 hours before stimulation with PTH and 25-D3.

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Quantitative real-time polymerase chain reaction

Total RNA was isolated with TRIzol reagent (Invitrogen), and 1 μg of RNA was reverse-transcribed with iScript (Bio-Rad). Expression of the cathelicidin gene in human (*CAMP*) and murine (*Camp*) cells was evaluated with the FAM-CAGAGGATTGTGACTTCA-MGB probe with primers 5′-CTTCACCAGCCCGTCCTTC-3′ and 5′-CCAGGACGACACAGCAGTCA-3′. TaqMan gene expression assays detecting human CD14 (assay ID: Hs00169122_g1), PTH1R (Hs00174895_m1), PTHrP (Hs00174969_m1), and mBD4 (Mm00731768_m1) were purchased from Applied Biosystems. Gene expression was normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH). For GAPDH expression, a VIC-CATCCATGACCACCCCTGGCCAAGMGB probe with primers 5′-CTTAGCACCCCTGGCCAAG-3′ and 5′-TGGTCATGAGTCCTTCCACG-3′ was used. The quantitative real-time polymerase chain reaction (qPCR) assays were performed in triplicate, and assays with mouse skin RNA were used at least five mice per group and were repeated twice.

MeDIP and DNA methylation profiling

MeDIP was performed as published before (39). In brief, genomic DNA was isolated from normal human epidermal keratinocytes with the DNeasy kit (Qiagen), sonicated to produce fragments from 300 to 1000 base pairs (bp), and subjected to immunoprecipitation with a monoclonal antibody against 5-methylcytosine (Eurogentec). DNA enriched for 5-methylcytosine was subjected to amplification with the Whole Genome Amplification kit (Sigma), labeled, and hybridized to Nimblegen's HG18 tiled promoter array (Roche). The *CAMP* gene promoter was tiled 5000 bp upstream and 500 bp downstream. The one-sided Kolmogorov-Smirnov test was applied to determine significant intensity ratios.

Knockdown of PTH1R by siRNA in normal human epidermal keratinocytes

Normal human epidermal keratinocytes (5×10^6) were trypsinized and nucleofected with 1 nmol of ON-TARGETplus SMARTpool siRNA (Dharmacon, Thermo Scientific) for PTH1R or control siRNA with the Amaxa Human Keratinocyte Nucleofector Kit and an Amaxa Nucleofector II electroporator (Lonza) according to the manufacturers' protocols. Assessment of knockdown efficiency and subsequent experiments were performed 72 hours after nucleofection.

Immunostaining

For immunocytofluorescence staining, keratinocytes were grown on chamber slides, fixed in 4% paraformaldehyde, blocked with 3% bovine serum albumin (BSA) in PBS, and incubated with mouse monoclonal anti-PTH1R antibody (3D1.1) or normal mouse immunoglobulin G (IgG) control (Santa Cruz Biotechnology) at 1:200 dilution at 4°C overnight. Fluorescein isothiocyanate (FITC)–conjugated goat anti-mouse IgG antibody (Sigma) was used as secondary antibody. Sections were mounted in ProLong Gold Antifade reagent with 4′-6-diamidino-2-phenylindole (Molecular Probes/Invitrogen). Images were obtained with an Olympus BX41 fluorescence microscope (Scientific Instrument Company).

Transfections

HaCaT cells were transfected with 1 μg of plasmid per well with Lipofectamine 2000 (Life Technologies Corp.). Human prepro *PTHrP(1–139), PTHrP(1–141)*, and *PTHrP(1–173)* and the respective 1 to 32 deletions were directionally subcloned into the pCi-neo mammalian expression vector (Promega Corp.) as previously described (40). Individual G418-resistant colonies were isolated and selected for expansion on the basis of detection of elevated immunoassay levels of PTHrP in conditioned media. HaCaT cells were then transfected with 30 pmol of siRNA duplexes per well (Qiagen) targeted to PTHrP(16–22) or nonsilencing

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control siRNA duplexes targeted to a nonmammalian protein from *Thermotoga maritimia* with Lipofectamine RNAiMAX (Life Technologies), according to the manufacturer's recommendations.

PTHrP immunoassays

To evaluate PTHrP levels, we used one- and two-site immunoassays and assayed the samples in multiple dilutions that paralleled the corresponding PTHrP standard curves (41). Both immunoassays measured all three forms of PTHrP(1–139), PTHrP(1–141), and PTHrP(1–173), but only the one-site immunoassay was able to measure also the respective PTHrP forms with deletions 1 to 32.

Statistical analysis

Statistical analysis was performed with GraphPad Prism software v5.0 (GraphPad Software Inc.). The means and SEM were calculated for each data set. Data were analyzed by Mann-Whitney U test, an unpaired t test, or analysis of variance (ANOVA) with posttests when appropriate. $P < 0.05$ was considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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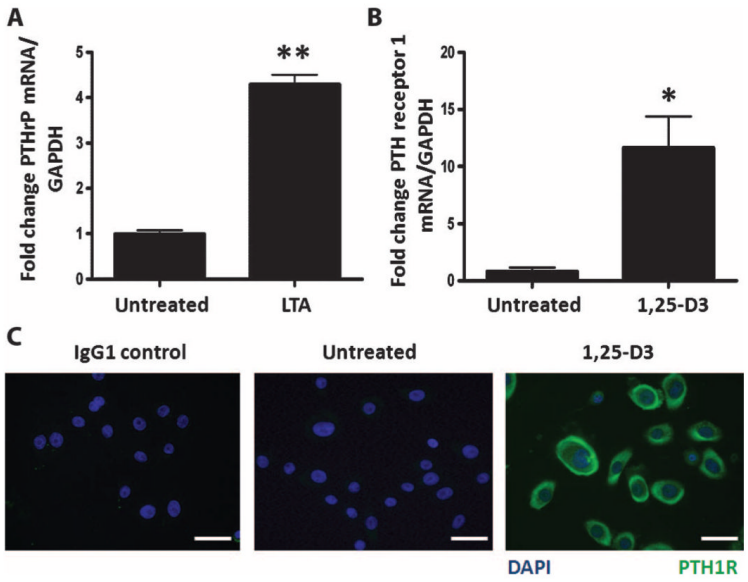

Fig. 1.

LTA induces PTHrP and 1,25-D3 induces its receptor, PTH1R, in keratinocytes. **(A)** Expression of PTHrP mRNA in normal human epidermal keratinocytes 24 hours after treatment with the TLR2 agonist LTA (10 μg/ml). **(B)** Expression of PTH1R mRNA in normal human epidermal keratinocytes treated with 1,25-D3 (10^{-7} M) for 24 hours. **(C)** Immunofluorescence staining of keratinocytes using IgG1 control antibody (left panel) or anti-PTH1R antibody (green) on untreated (middle panel) or 1,25-D3–treated cells (right panel). Blue, staining of nuclei with 4′,6-diamidino-2-phenylindole (DAPI). Scale bars, 20 μm. Data are means ± SEM of triplicate independent cultures and are representative of two independent experiments. $*P < 0.05$, $**P < 0.01$ by unpaired *t* test. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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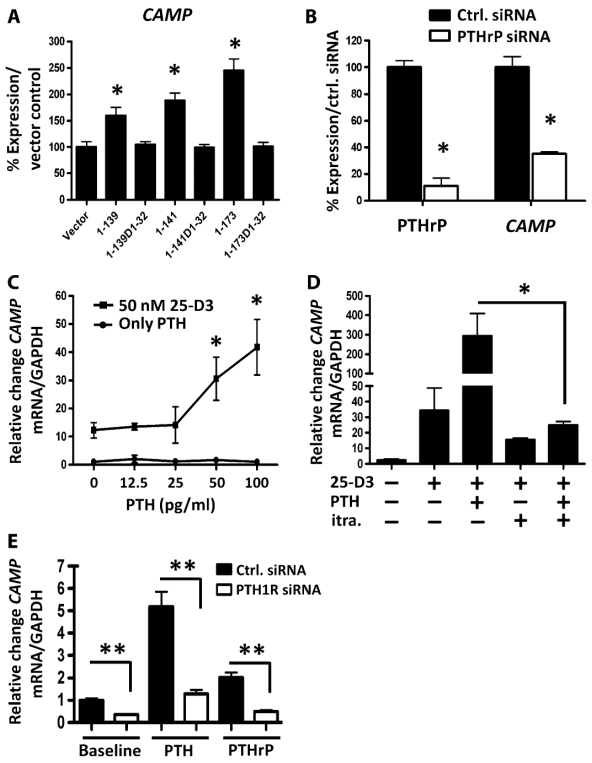

Fig. 2.

PTHrP and PTH cooperate with vitamin D_3 to induce cathelicidin antimicrobial peptide expression. (**A**) Cathelicidin (*CAMP*) mRNA expression in HaCaT keratinocytes transfected for 48 hours with different PTHrP expression plasmids. N-terminal amino acids encoded by each construct are designated. Constructs labeled D1-32 lack amino acids 1 to 32 of PTHrP and were inactive. Data are means ± SEM of triplicate independent cultures and are representative of two independent experiments. $*P < 0.05$ by unpaired *t* test. (**B**) HaCaT keratinocytes transfected with control siRNA (black bars) or PTHrP siRNA duplexes (white bars) and grown for 48 hours. PTHrP was measured in conditioned media by immunoassay, and cathelicidin (*CAMP*) mRNA was measured in cell extracts by qPCR. Data are means ± SEM of triplicate independent cultures and are representative of two independent experiments. $*P < 0.05$ versus vector control (unpaired *t* test). (**C**) *CAMP* mRNA expression in normal human epidermal keratinocytes after treatment with PTH (0 to 100 pg/ml) alone or cotreated with 25-D3 (50 nM). $*P < 0.05$ by Kruskal-Wallis with Dunn's post hoc nonparametric test. Data are means ± SEM of triplicate independent cultures and are representative of three independent experiments. (**D**) Normal human epidermal keratinocyte *CAMP* mRNA expression after pretreatment with the CYP27B1 inhibitor itraconazole (itra., 10^{-7} M) for 1 hour before pretreatment with 25-D3 (50 nM) for 24 hours and PTH (10^{-11} M) for another 24 hours. $*P < 0.05$ by unpaired *t* test. Data are means ± SEM of triplicate independent cultures and are representative of three independent experiments. (**E**) Normal human epidermal keratinocyte *CAMP* mRNA expression after knockdown using PTH1R siRNA (open bars) or control siRNA (Ctrl. siRNA, solid bars) at baseline (left) or after pretreatment for 24 hours with PTH (middle, 10^{-11} M) or PTHrP (right, 10^{-11} M). Data are means ± SEM of triplicate independent cultures and are representative of two independent experiments. $**P < 0.01$ by Mann-Whitney *U* test.

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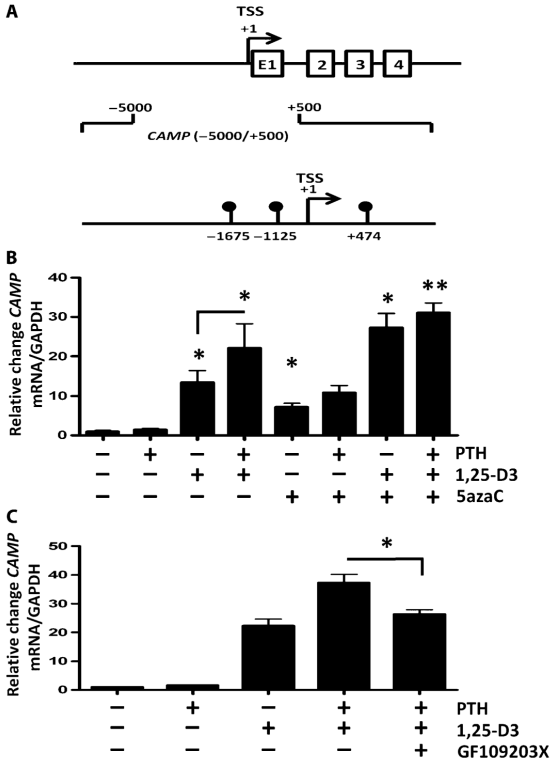

Fig. 3.

CAMP expression by PTH/PTHrP involves DNA methylation. (**A**) Whole-genome MeDIP of primary human keratinocytes revealed three methylated DNA sites within −5000/+500 bp of the *CAMP* gene TSS at −1675, −1125, and +474 bp. Shown are exons 1 to 4 (E1, 2, 3, 4) of the cathelicidin gene. (**B**) *CAMP* mRNA expression in normal human epidermal keratinocytes after treatment with PTH (10^{-11} M), 1,25-D3 (10^{-7} M), and the DNA methyltransferase inhibitor 5azaC (10^{-6} M). (**C**) *CAMP* mRNA expression after treatment with PTH (10^{-11} M), 1,25-D3 (10^{-7} M), and the PKC inhibitor GF109203X (5 μM) for 24 hours. Results are means ± SEM of triplicate independent cultures and are representative of three independent experiments. **P* < 0.05, ***P* < 0.01 by Mann-Whitney *U* test.

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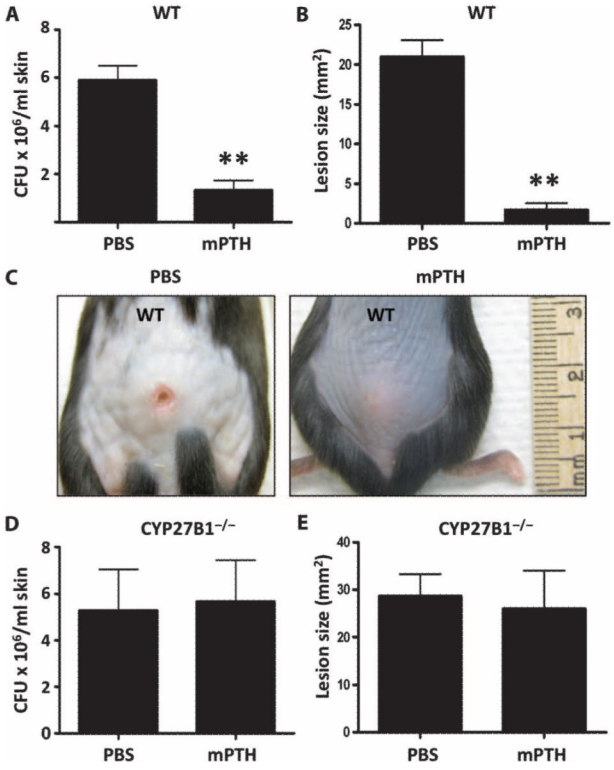

Fig. 4.

PTH in the presence of vitamin D_3 enhances protection against invasive bacterial skin infections in vivo. Wild-type (WT) mice and mice lacking the 1α-hydroxylase enzyme ($CYP27B1^{-/-}$) were injected subcutaneously with mPTH(1–34) peptide (80 μg/kg) or PBS 24 hours before challenge with 10^7 CFU of group A *Streptococcus* NZ131. (**A**) Number of group A *Streptococcus* CFU retrieved from homogenized infected WT mouse skin after 72 hours. (**B**) Group A *Streptococcus* lesion size (mm^2) in WT mice 72 hours after infection. $^{**}P < 0.01$ by unpaired *t* test. (**C**) A representative photograph of group A *Streptococcus* lesion size in WT mice injected with mPTH or PBS at 72 hours. (**D**) Number of group A *Streptococcus* CFU retrieved from homogenized infected $CYP27B1^{-/-}$ mouse skin after 72 hours. (**E**) Group A *Streptococcus* lesion size (mm^2) in $CYP27B1^{-/-}$ mice 72 hours after infection. All data are means ± SEM from six mice per group and are representative of three independent experiments.

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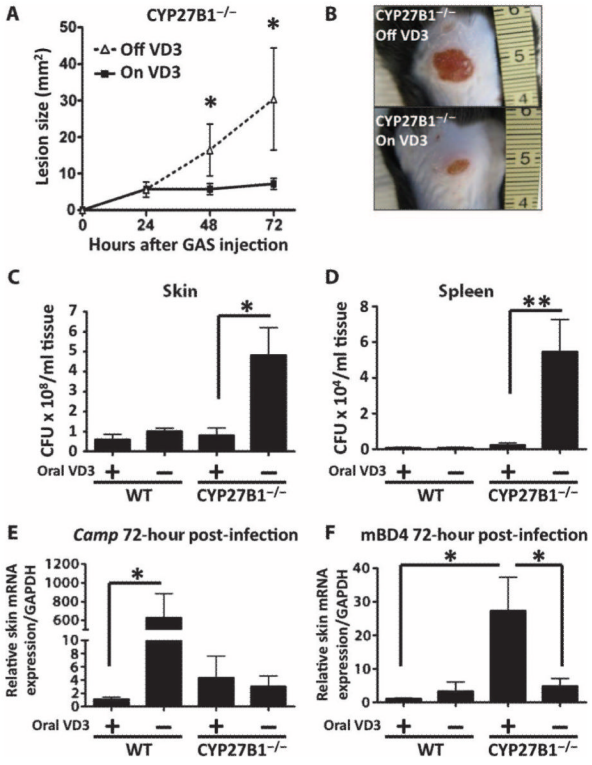

Fig. 5.

Dietary vitamin D_3 restriction in CYP27B1$^{-/-}$ mice increases susceptibility to group A *Streptococcus* infection. CYP27B1$^{-/-}$ mice were injected subcutaneously with 10^7 CFU of group A *Streptococcus* NZ131 after having been on a diet either containing vitamin D_3 (2.2 IU/g) ("On VD3" or "+") or not containing any vitamin D ("Off VD3" or "−") for 4 weeks. (**A**) Lesion size of group A *Streptococcus* (GAS) skin infections (mm^2) 24, 48, and 72 hours after injection. All data are means ± SEM from six mice per group and are representative of three independent experiments. *$P < 0.05$ by ANOVA with Bonferroni's posttest. (**B**) A representative photograph of group A *Streptococcus* lesion size at 72 hours is shown. (**C** and **D**) Number of group A *Streptococcus* NZ131 CFU retrieved from homogenized skin (C) and spleen (D) 72 hours after infection. (**E** and **F**) Cathelicidin (*Camp*) (E) and mBD4 (F) mRNA expression in mouse skin 72 hours after group A *Streptococcus* injection. *$P < 0.05$, **$P < 0.01$ by Mann-Whitney *U* test.

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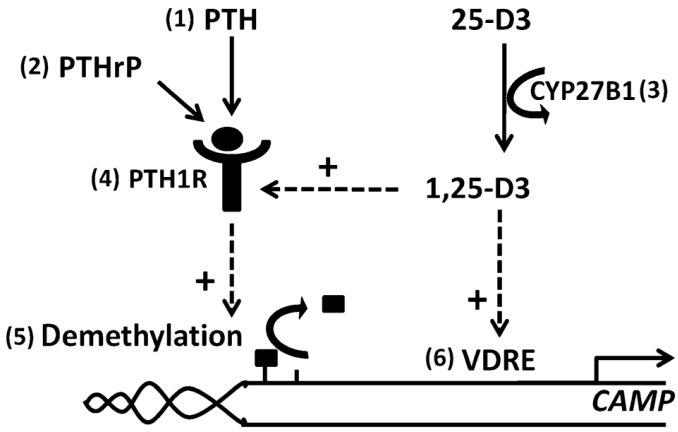

Fig. 6.

Schematic showing how cathelicidin expression, PTH/PTHrP, and vitamin D may be connected. The human cathelicidin gene *CAMP* is induced by vitamin D, and its expression is further enhanced by the action of PTH/PTHrP. (1) PTH levels are elevated in response to low 25-D3 such as occurs with limited vitamin D intake. (2) PTHrP is increased locally in keratinocytes by the TLR2 agonist LTA. (3) The most active metabolite of vitamin D, 1,25-D3, is increased when TLR2 activation induces CYP27B1 to enhance conversion of 25-D3 to 1,25-D3. (4) 1,25-D3 enhances expression of PTH1R. (5) Activation of PTH1R results in demethylation of the *CAMP* promoter, thus enhancing expression of cathelicidin. (6) *CAMP* contains a functional vitamin D response element that induces its expression. These interactions define a potential mechanism to compensate for low serum 25-D3 by increasing the amount of PTH available for activation of *CAMP*, and also show how recognition of bacterial products can amplify cathelicidin expression by increasing both local 1,25-D3 and PTH1R.