Regulation of antimicrobial peptide gene expression by nutrients and byproducts of microbial metabolism

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Abstract

Background

Antimicrobial peptides (AMPs) are synthesized and secreted by immune and epithelial cells that are constantly exposed to environmental microbes. AMPs are essential for barrier defense and deficiencies lead to increased susceptibility to infection. In addition to their ability to disrupt the integrity of bacterial, viral and fungal membranes, AMPs bind lipopolysaccharides, act as chemoattractants for immune cells and bind to cellular receptors and modulate the expression of cytokines and chemokines. These additional biological activities may explain the role of AMPs in inflammatory diseases and cancer. Modulating the endogenous expression of AMPs offers potential therapeutic treatments for infection and disease.

Methods

The present review examines published data from both *in vitro* and *in vivo* studies reporting effects of nutrients and byproducts of microbial metabolism on the expression of antimicrobial peptide genes in order to highlight an emerging appreciation for the role of dietary compounds in modulating the innate immune response.

Results

Vitamins A and D, dietary histone deacetylases and byproducts of intestinal microbial metabolism (butyrate and secondary bile acids) have been found to regulate the expression of AMPs in humans. Vitamin D deficiency correlates with increased susceptibility to infection and supplementation studies indicate an improvement in defense against infection. Animal and

human clinical studies with butyrate indicate that increasing expression of AMPs in the colon protects against infection.

Conclusion

These findings suggest that diet and/or consumption of nutritional supplements may be used to improve and/or modulate immune function. In addition, byproducts ofgut microbe metabolism could be important for communicating with intestinal epithelial and immune cells, thus affecting the expression of AMPs. This interaction may help establish a mucosal barrier to prevent invasion of the intestinal epithelium by either mutualistic or pathogenic microorganisms.

Introduction

Cathelicidins, defensins and other antimicrobial peptides (AMPs) are an evolutionarily conserved component of the innate immune system and play an important role in combating infection [1-4]. AMPs have two or more positively charged residues in acidic environments and can bind to the negatively charged membranes of both gram (-) and gram (+) bacteria, mycobacteria, fungi and enveloped viruses which in turn kills the pathogens by disrupting their cell membranes [5, 6] .

The human cathelicidin antimicrobial peptide (CAMP) gene encodes a preproprotein with an ~30 amino acid long N-terminal signal sequence, a 94 amino acid cathelin domain and a 37 amino acid C-terminal cationic AMP domain (LL-37) [6]. The CAMP gene is primarily expressed in myeloid bone marrow cells and the pro-protein, hCAP18, is packaged in neutrophil specific granules [7]. CAMP is also expressed by epithelial cells in tissues exposed to environmental microbes and the protein is secreted in semen, saliva and sweat providing barrier

protection to mammals [8-10]. Mice and humans lacking CAMP are susceptible to bacterial infections in numerous tissues [4, 11, 12]. High circulating levels of hCAP18 are found in human plasma [13]. The hCAP18/LL37 peptide binds to and neutralizes LPS [14], thus preventing its interaction with the LPS-binding protein and subsequent activation of TLR-4 [15], NF-κB signaling and cytokine release from host cells [16]. LL-37 protects against LPS-induced sepsis in animals [17, 18] and kidney dialysis patients with low serum levels of hCAP18 are twice as likely to die from infectious disease or sepsis than patients with higher levels [19]. Therefore, circulating hCAP18 or LL-37 may protect against both bacterial infection and sepsis.

Like cathelicidin, defensins are expressed by immune cells and epithelial cells of tissues that are exposed to the environment [3]. Defensins have six highly conserved cysteines that form disulfide bonds [2]. Human defensins are classified into two families: α- and β-defensins [20]. All defensins are expressed as a biologically inactive preproproteins and activated by cleavage of a prosequence [20]. To date, six α-defensins have been identified with HNP-1-4 packaged in human neutrophil primary granules and HD-5 and -6 expressed by Paneth cells in the small intestine [3]; HD-5 is also expressed by epithelial cells of the female genitourinary tract [21]. The α-defensins have antimicrobial activity against a range of bacteria, viruses and fungi [20].

β-defensins are the most widely distributed of the antimicrobial peptides. They are typically 38-42 amino acids long [22] and expressed in monocytes, macrophages and dendritic cells (DCs) [23] as well as epithelial cells in the respiratory and urogenital tracts, skin and tonsil [20, 24]. Human β-defensin 1 (HBD-1) is constitutively expressed in many tissues while HBD-2, -3 and -4 expression is induced by inflammatory stimuli, such as bacterial infection, LPS, IL-1β, TNF- α or phorbol-myristate-acetate (PMA) [2, 3, 20, 22, 25]. β-defensins have shown activity against both gram (-) and gram (+) bacteria, fungi, viruses and parasites [26-28].

In this paper, we review the regulation of the CAMP gene and the defensin gene family by various nutritional compounds and/or microbial byproducts of metabolism and discuss the potential importance of this regulation for human innate immune function.

Nutritional regulation of AMP expression

Vitamin D

CAMP: There are two forms of vitamin D that humans can utilize: vitamin D_2 (ergocalciferol) from fungi and vitamin D_3 (cholecalciferol) from animal sources and synthesized in the skin [29]. Expression of the human CAMP gene is induced by $1,25(OH)_2 D_3$ and its analogs in various cell lines and primary cells [30-32]. Vitamin D induction of CAMP is solely a human and non-human primate phenomenon [33, 34] due to a vitamin D response element (VDRE) located within a primate-specific retro-transposable element (Alu-SINE) found in the upstream promoter region of the CAMP gene [34].

In subsequent studies, it was demonstrated *in vitro* that toll like receptor (TLR) activation induced CAMP expression via a vitamin D-dependent pathway in macrophages [35]. In the proposed model, sufficient levels of circulating $25(OH)D_3$, the precursor to $1,25(OH)_2D_3$, are required for induction of CAMP gene expression and production of adequate LL-37 peptide levels to effectively combat infection (Fig. 1A) [35]. Cathelicidin expression is also increased in keratinocytes via a similar pathway after skin wounding [36].

Interestingly, the vitamin D-mediated induction of CAMP potentially boosts antimicrobial activity against pathogens through direct killing by LL-37 and enhancing phagosome maturation [37]. In THP-1 and human primary monocytes, $1,25(OH)_2D_3$ triggers the

formation of autophagosomes and autophagolysosomes via a hCAP18/LL-37-mediated pathway [37].

Defensins: The HBD-2 gene (DEFB4) was induced by $1,25(OH)_2D_3$ through a VDRE in the promoter, but the induction was not as robust as that observed for the CAMP gene [30]. Also, the induction of the HBD-2 gene in macrophages requires TLR activation and the convergence of the IL-1β and vitamin D pathways (Fig. 1B) [38]. *In vitro* studies demonstrated that activation of intracellular pattern recognition receptor nucleotide-binding oligomerization domain protein 2 (NOD2) by its ligand muramyl dipeptide (MDP), a lysosomal breakdown product of peptidoglycan from both gram-negative and gram-positive bacteria, induced the expression of the HBD-2 gene [39]. More recently, $1,25(OH)_2D_3$ was shown to strongly induce the expression of NOD2/CARD15/IBD1 in primary human monocytic and epithelial cells [40]. In the absence of $1,25(OH)_2D_3$, the activation of NOD2 by MDP activates NF-κB and there is a modest induction of the HBD-2 gene; however, pre-treatment with $1,25(OH)_2D_3$ followed by MDP leads to a robust, synergistic induction of the HBD-2 gene (Fig. 1B) [40]. Activation of the vitamin D pathway alone is not sufficient to induce robust expression of HBD-2 and additional signaling pathways are required [38, 40]. Treatment of normal human keratinocytes with $1,25(OH)_2D_3$ increased HBD-3 mRNA levels in a dose-dependent manner [41]. Interestingly, treatment of lesional psoriatic plaques with the vitamin D analog calcipotriol increased IκB-α protein levels inhibiting the NF-κB signaling pathway and blocked IL-17A-induced expression of HBD-2 in the plaques [42]. Down-regulation of HBD-3 was also observed in the lesional psoriatic plaques after treatment with calcipotriol [42].

Primary and secondary bile acids

Bile acids play an important role in digestion and absorption of dietary fat and nutrients by the digestive system. They bind to and activate many nuclear receptors and thus modulate metabolism. In humans, the two major primary bile acids are cholic acid (CA) and chenodeoxycholic acid (CDCA) [43]. CDCA binds to farnesoid X receptor (FXR) [44] and up-regulates cathelicidin expression (Fig. 1C) [45]. This regulation may contribute to sterility of the bile duct.

Lithocholic acid (LCA), a toxic secondary bile acid, is a byproduct of CDCA metabolism by bacteria in the colon. It is a low affinity ligand for FXR, pregnane X receptor (PXR) as well as the VDR[46-48]. LCA binds to the VDR and significantly increases both human CAMP transcript and protein levels in human primary keratinocytes *(NHEK)* in a time-and dose-dependent manner [49]. In the human colonic epithelial cell line HT-29, LCA and butyrate act synergistically to induce human CAMP gene expression [50]. The LCA derivatives LCA acetate and LCA propionate induced expression of human CAMP more efficiently than LCA itself and were less toxic in tissue culture cells and mice, suggesting they are more potent VDR agonists and might be safer potential therapeutic agents than LCA [51]. Unlike LCA, the secondary bile acid ursodeoxycholic acid (UDCA) is not a ligand for the VDR, but rather increases the levels of VDR in the nucleus that, in turn, increases CAMP expression (Fig. 1C) [45].

Butyrate

CAMP: Butyrate is a short chain fatty acid produced in the colon by fermentation of dietary fiber by anaerobic bacteria. It is found in many foods, such as butter and cheeses and is a histone deacetylase inhibitor (HDACi) [52]. *Shigella* infections down-regulate LL37 and β-defensin-1 levels in the colon of adult patients [53]. and rabbit CAP18 levels in colon surface epithelium

are reduced during *Shigella* infection; however CAP18 can be restored after oral administration of sodium butyrate [54].

Because of its unpleasant smell, butyrate is rarely used in clinical trials and instead 4-phenylbutyrate (PBA), an odorless and palatable derivative of butyrate is used clinically. In several human cell lines PBA up-regulates CAMP gene expression more potently than butyrate [55]. It was recently shown that PBA also counteracts the down-regulation of cathelicidin in both the colon and lung by *Shigella* in rabbits [56]. Furthermore, butyrate and its derivatives up-regulate transcription of cathelicidin in human colon epithelial cells [57] and synergistically induces human CAMP mRNA levels with $1,25(OH)_2D_3$ in lung epithelial and myeloid cells [32, 55]. A chemical analogue of PBA, α-methylhydrocinnamate (ST7), can also dramatically up-regulate human CAMP mRNA transcription [55]. These studies support a potential role for sodium butyrate and its derivatives in the treatment of human infections.

Defensins: There are few publications reporting the regulation of defensins by butyrate; however, it has been shown to induce HBD-2 mRNA expression in colonocytes [58] and pretreatment of gingival epithelial cells with sodium butyrate significantly induced HBD-2 expression in response to bacterial challenge [59].

Sulforaphane

Defensins: Sulforaphane (SFN) is a dietary HDACi found in cruciferous vegetables (e.g. broccoli and broccoli sprouts) that reactivates epigenetically-silenced genes [60]. Recently, it was demonstrated that SFN induces HBD-2 mRNA and protein expression in the Caco-2 human colon cancer cell line in a time- and dose-dependent manner [58]. Inhibition of VDR by an antagonist significantly blocked the SFN-induced HBD-2 mRNA expression in these cells, and

SFN treatment increased the expression of VDR in both Caco-2 and HT-29 cell lines, indicating that induction of HBD-2 by SFN was mediated by the vitamin D pathway [58]. Whether these observations were due to epigenetic changes was not examined. The MAPK/ERK and NF-κB signaling pathways were also involved [58]. It has not been determined if cathelicidin expression is regulated by SFN.

Retinoic acid

Retinoic acid (RA) is a metabolite of vitamin A which is important in several aspects of immune function [61]. The retinoic acid receptor (RAR) forms a heterodimer with the retinoid X receptor (RXR) and interacts with specific retinoic acid response elements (RAREs) in the promoters of target genes [62]. All-trans RA was shown to induce both porcine cathelicidin, PR-39 expression [63] and hCAP18 promoter activity [64]. In contrast, induction of HBD-2, -3 and -4 by Ca^{2+}, TNF-α, IL-1β, INF-γ, PMA or *P. aeruginosa* in human keratinocytes is inhibited by RA [65]. The promoter regions of all the inducible human β-defensin genes have several possible AP-1 binding sites [65] and RA may suppress expression of these genes by antagonizing AP-1 (c-Jun/c-Fos)-mediated gene expression pathways [66]. Therefore, RA may impair the innate immune response in human skin, thus increasing susceptibility to infections during RA therapeutic treatment [65]. On the other hand, α-defensin HNP-1 was induced by both all-trans RA and 9-*cis*-RA in a dose-dependent manner, suggesting that retinoic acid may be important for myeloid cell expression of HNP-1 [67].

Human Health

Increasing endogenous cathelicidin and defensin expression may be particularly useful in the treatment of infections. Clinical studies have shown that asthma patients have reduced hCAP18 levels [68]. High levels of circulating hCAP18 in hemodialysis patients at the beginning of their treatment was indicative of a significant decrease in 1-year mortality and there was a modest correlation with $1,25(OH)_2D_3$ levels, but not with $25(OH)D$ levels [19]. In sepsis patients, lower $25(OH)D$, vitamin D binding protein (DBP) and cathelicidin levels were associated with severe illness and a positive correlation between $25(OH)D$ and cathelicidin levels was seen in all subjects [69]. Additional studies are needed to substantiate these latest findings and determine if supplementation of vitamin D-deficient individuals with vitamin D or therapy with active analogs of $1,25(OH)_2D_3$ would boost plasma levels of cathelicidin and thus increase protection against infection and sepsis.

Supplementation may increase cathelicidin and defensin expression in tissues and immune cells, thus enhancing barrier function. Atopic dermatitis patients suffer from frequent skin infections; therefore, induction of CAMP expression in the skin may increase protection from infection [70]. Patients supplemented with 4,000 IU/d of oral vitamin D for 21 days showed increased cathelicidin expression in skin lesions and a mild increase in unaffected skin, but a decrease in skin infection was not determined [70]. *Ex vivo* infection of urinary bladder biopsies from post-menopausal women after vitamin D supplementation resulted in an increased induction of the CAMP gene and protein expression when compared to biopsies taken prior to supplementation [71]. The studies to date would argue that it is important for individuals to have sufficient serum levels of $25(OH)D$ to allow for the production of adequate levels of cathelicidin during infection.

Epidemiological studies link vitamin D deficiency and increased rates of respiratory infections and there is interest in using vitamin D supplementation to reduce influenza infections and treat tuberculosis [72]. In a small randomized trial of school age children, the vitamin D-supplemented group showed a nearly two-fold reduction in influenza A rates than the placebo group [73]. In another study with participants from different racial groups it was shown that the maintenance of serum 25-hydroxyvitamin D levels at 38 ng/ml or higher was correlated with a two-fold reduction in the incidence of acute viral respiratory tract infections [74]. In a double-blind randomized control trial of tuberculosis patients starting treatment, vitamin D supplementation did not significantly affect the time that patient sputum cultures converted from positive to negative for *M. tuberculosis* growth in the study population as a whole; however, a significantly shortened time of conversion was observed in a sub-group of participants with the tt genotype of the TaqI vitamin D receptor polymorphism [75]. The latter study demonstrates the importance that genetic differences among individuals may play in the outcomes of trials involving supplementation.

In contrast to the positive findings above, a number of negative studies with vitamin D have been reported. A randomized controlled trial of vitamin D3 supplementation showed that there was no obvious difference in the incidence and duration of severity of upper respiratory tract infections (URIs) between vitamin D (2000IU/day) and placebo groups although 25(OH)D level increased significantly after 12 weeks in the vitamin D group compared to the placebo group [76]. Another study found that there was no difference in serum 25(OH)D levels between groups of patients aged 1-25 months admitted to hospital with uncomplicated acute lower respiratory tract infection (ALRI) and healthy, similarly aged patients without a history of hospitalization for ALRI. This study suggested that vitamin D status was not a risk factor in hospitalization for

ALRI [77]. In a randomized, double-blind, placebo-controlled trial in TB clinics, the intervention and placebo groups were given 100,000 IU of cholecalciferol or vegetable oil at inclusion and again at five and eight months after the start of treatment, but no significant difference was observed between the groups on mortality in patients with TB [78].

Very recently, a randomized, double-blind, placebo controlled clinical trial to determine the efficacy of sodium butyrate as an adjunct therapy with antibiotics in the treatment of shigellosis in patients was performed. Reduced rectal luminal content of inflammatory cells and pro-inflammatory cytokines, increased expression of LL-37 in the rectal epithelia and improved rectal histopathology as compared to the placebo group was evident [79]. Nevertheless, efficacy of sodium butyrate treatment in clinical recovery was not observed as in studies with rabbits [54, 79]. Important differences between the animal and human studies included oral delivery in rabbits versus delivery by enema in the humans and the lack of antibiotics in rabbits [79]. It remains to be determined if oral delivery of butyrate compounds would be more efficacious as the treatment would not be eliminated as quickly as it is with an enema due to repeated bouts of diarrhea [79].

Conclusions

Accumulating evidence demonstrates that nutrients and microbial byproducts derived from the metabolism of dietary factors may play a critical role in modulating the innate immune response via regulation of AMP gene expression. The byproducts of gut microbe metabolism are potentially important for increasing AMP expression by epithelial cells of the luminal lining of the digestive tract, thus establishing a mucosal barrier preventing contact of microbes and pathogens with the intestinal epithelium [12, 50]. Increasing the consumption of dietary fiber,

thus increasing short chain fatty acid production, and/or food containing dietary HDAC inhibitors could increase the expression of AMPs in the digestive tract. This in turn would increase barrier protection in the gut lumen and reduce the rate or severity of intestinal infections. This is nicely demonstrated in the studies using a rabbit model of shigellosis [54, 56] and has shown some promise in a human trial [79].

The potential for VDR and FXR to bind to the VDRE in the human CAMP gene offers an abundance of new compounds that can be synthesized or obtained from the diet to produce potent ligands for these steroid hormone receptors and used to enhance AMP expression. For example, the therapeutic use of active vitamin D is hampered by the toxic side-effects of hypercalcemia [12] and although synthetic analogs reduce these side-effects they still exist. On the other hand, analogs of LCA have been shown to lack this side-effect, but activate VDR-target genes like CAMP [51].

The VDR may act as a receptor for additional nutritional ligands including curcumin and polyunsaturated fats including α-linolenic acid, docosahexaenoic acid, eicosapentaenoic acid and arachidonic acid [80]; however, we recently demonstrated that in tissue culture only curcumin modestly induced CAMP gene expression through a yet uncharacterized VDR-independent mechanism (Guo et al., in press). Identification of additional nutritional compounds and their synthetic analogs would provide further options for increasing AMP gene expression for therapeutic uses.

AMPs are critical to the barrier defense provided by the innate immune system and deficits in AMP production can increase susceptibility to infections. As discussed, numerous nutritional compounds and microbial byproducts from metabolism of dietary factors have been shown to regulate the expression of AMPs. The possibility of improving the innate immune response or

boosting barrier defenses toward infection by increasing the consumption of food items rich in these nutrients is an exciting prospect, but well-designed clinical and animal model studies need to be performed to demonstrate that compounds obtained from the diet can improve immune function by increasing AMP levels.

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Authors' Contributions

YC performed the initial literature search, wrote the preliminary document, assisted with and completed the final version. MLF modified the preliminary document. AFG directed and augmented the literature search as well as modified and revised the preliminary document.

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Figure Legends

Figure 1. Regulation of AMP gene expression through the VDR or FXR pathways. (A) Vitamin D-pathway dependent TLR activation of CAMP gene expression. TLR-signaling activates NF-κB binding and induces VDR and CYP27B1 expression, the enzyme that catalyzes the conversion of 25(OH)D to 1,25(OH)$_2$D. The expression of the vitamin D receptor (VDR) is increased. In the presence of locally high levels of 1,25(OH)$_2$D, ligand-bound VDR:RXR heterodimers translocate into the nucleus and bind to the VDRE in the promoter of the human CAMP gene inducing its expression.   (B) Vitamin D-mediated regulation of the human DEFB4 gene. (1) Direct induction: TLR stimulation activates the vitamin D-pathway as described in Fig. 1A. Also it up-regulates expression of IL-1β and IL-1R1 and down-regulates expression of IL-1R antagonist (IL-1RA). IL-1R1 activates the NF-κB transcription factor which binds to the promoter proximal NF-κB binding site in the DEFB4 gene and induces HBD-2 expression together with the VDR:RXR heterodimer that binds to the VDRE in the promoter of DEFB4 gene. Indirect induction: In the presence of 1,25(OH)$_2$D$_3$ the VDR:RXR heterodimer that binds

the VDRE in the NOD2 gene promoter and induces expression of the NOD2 protein. Activation of NOD2 by its agonist muramyl dipeptide stimulates the NF-κB transcription factor that binds to the promoter proximal NF-κB binding site in the DEFB4 gene to induce its expression. (C) Regulation of human CAMP gene expression by vitamin D and bile salts in biliary cells. 1) $1,25(OH)_2D_3$ activates the VDR:RXR heterodimer that then binds to the VDRE in the CAMP gene promoter; 2) UDCA activates the ERK1/2 signaling pathway that, in turn, induces VDR protein expression and induction of CAMP gene expression in the presence of $1,25(OH)_2D_3$ and 3) CDCA binds to the FXR:RXR heterodimer that binds to the VDRE in the CAMP gene promoter.