

Sphingolipids and Antimicrobial Peptides: Function and Roles in Atopic Dermatitis

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Abstract

Inflammatory skin diseases such as atopic dermatitis (AD) and rosacea were complicated by barrier abrogation and deficiency in innate immunity. The first defender of epidermal innate immune response is the antimicrobial peptides (AMPs) that exhibit a broad-spectrum antimicrobial activity against multiple pathogens, including Gram-positive and Gram-negative bacteria, viruses, and fungi. The deficiency of these AMPs in the skin of AD fails to protect our body against virulent pathogen infections. In contrast to AD where there is a suppression of AMPs, rosacea is characterized by overexpression of cathelicidin antimicrobial peptide (CAMP), the products of which result in chronic epidermal inflammation. In this regard, AMP generation that is controlled by a key ceramide metabolite S1P-dependent mechanism could be considered as alternate therapeutic approaches to treat these skin disorders, i.e., Increased S1P levels strongly stimulated the CAMP expression which elevated the antimicrobial activity against multiple pathogens resulting in the improved AD patient skin.

Key Words: Cathelicidin antimicrobial peptide, Atopic dermatitis, Endoplasmic reticulum stress, Sphingosine 1-phosphate, Innate immunity

INTRODUCTION

The skin of atopic dermatitis (AD) is highly susceptible to colonization by *Staphylococcus aureus*. The defense system of the skin against bacterial invasion is significantly disrupted in AD. The high incidence of *S. aureus* colonization was also observed in the decrease of IgA and the slight change toward an alkaline pH in AD skin. Recently, another important association between sphingolipid metabolites and the defense mechanism against *S. aureus* colonization has been ascribed to decreased levels of ceramide which reflects possible alteration of sphingolipid metabolites in the AD skin. Endoplasmic reticulum (ER) stress stimulates the production of cathelicidin antimicrobial peptide (CAMP), the proapoptotic sphingolipids, ceramide and sphingosine as well as an antiapoptotic sphingosine 1-phosphate (S1P). In previous studies, a variety of external perturbations including ER stress stimulated the production of ceramides (Uchida *et al.*, 2003; Lei *et al.*, 2008; Uchida *et al.*, 2010). Recently, we also suggested the CAMP production via a novel NF- κ B- and C/EBP α -mediated pathway

independent of vitamin D receptor mediated mechanism (Park *et al.*, 2011). Interestingly, sphingolipid metabolites especially ceramides were converted into sphingosine by ceramidase or dephosphorylated from S1P by S1P phosphohydrolase-1 in subtoxic levels of ER stress (Lepine *et al.*, 2011). Therefore, sphingolipid metabolites ceramides, sphingosine and S1P were possibly involved in triggering innate immunity in skin.

Here, we introduce the antimicrobial peptides released from skin epithelial cells in relation to the activation or upregulation of their defense mechanism mediated by the sphingolipid signaling molecules in AD.

FUNCTION AND IMPORTANCE OF KEY EPIDERMAL ANTIMICROBIAL PEPTIDES

Mammalian epithelial tissues, such as intestine, respiratory tract, reproductive tract, mucosal epithelia and skin, are positioned at the interface with the external environment, where they continuously encounter exogenous microbial pathogens.

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These tissues have developed protective mechanisms that include antimicrobial peptides (AMP) (also called host defense peptides) that exhibit a broad-spectrum antimicrobial activity against multiple pathogens, including Gram-positive and Gram-negative bacteria, fungi, enveloped viruses and even transformed or cancerous cells. (Liu *et al.*, 2013). In addition to antimicrobial activity, it has been highlighted that AMPs have the ability to enhance innate/adaptive immunity by functioning as immunomodulators (Beignon *et al.*, 2001). In human and other mammals, the two main AMP families are defensin and CAMP. Defensins are widely distributed in mammalian epithelial cells and phagocytes (Ali *et al.*, 2001). CAMP is a structurally and evolutionarily distinct antimicrobial peptide that is similar to defensins in abundance and distribution. In addition to CAMP and defensins, additional antimicrobial peptides also exist which including histatins (de Sousa-Pereira *et al.*, 2013), dermcidin (Schitteck *et al.*, 2001; Zeth, 2013) and anionic peptides. However, these antimicrobial peptides are restricted to a few animal species and tissues. In this review, the major characteristics of two major epidermal AMP families, β -defensins and CAMP will be present and discussed.

Defensins

The defensins, which have six conserved cysteine residues, are small and cationic peptides that are divided into three subfamilies, α -, β -, and θ -defensin based on the pairing of the cysteine residues to form three disulfide bridges (α -defensin [cys¹-cys⁶, cys²-cys⁴, cys³-cys⁵], β -defensin [cys¹-cys⁵, cys²-cys⁴, cys³-cys⁶], θ -defensin [cys¹-cys¹, cys²-cys³, cys²-cys³]) (Ali *et al.*, 2001; Huh *et al.*, 2002; Fazakerley *et al.*, 2010). Six α -defensins have been identified from five genes in human, human neutrophil peptide (HNP)-1, HNP-2, HNP-3, HNP-

4, and human defensin (HD)-5, and HD-6. The α -defensins are mainly produced by neutrophils and to our knowledge are not found in keratinocytes, which are human epidermal skin cells. Whereas, human β -defensins (hBDs) are encoded by more than 30 β -defensin genes (DEFBs) and the keratinocytes express hBD-1 through hBD-3 (Table 1). Unlike α - and β -defensins those are found in humans, the θ -defensin protein product has not been identified in humans.

hBD-1 is constitutively expressed in human skin, whereas both hBD-2 and hBD-3 are inducible AMP in epidermis dependent on a number of factors, including pathogenic bacteria, cytokines, growth factors or external stresses such as UVB irradiation. These factors have shown to differentially induce hBDs production in the skin. Toll-like receptor (TLR) 4 has been noted to recognize lipopolysaccharides (LPS) that induce hBD-2, not hBD-3, expression in keratinocytes (Luo *et al.*, 2012). Moreover, hBD-2 production was mainly regulated by IL-1, whereas hBD-3 induction is influenced primarily by IL-6 and epidermal growth factors, indicating that different stimulators and regulatory mechanisms are responsible for the generation of various hBDs (Harder *et al.*, 2000; Pioli *et al.*, 2006; Ahn *et al.*, 2013). hBD3 has potent antimicrobial activity against exogenous microbial pathogens, including Gram-negative and Gram-positive bacteria, while hBD2 has no noted effects against Gram-positive *Staphylococcus* (*S.*) *aureus*, which is the virulent bacteria that colonizes approximately 90% of AD patients (Fazakerley *et al.*, 2010; Ong, 2010).

Cathelicidins

The properties of CAMP were first discovered in porcine (Ritonja *et al.*, 1989), CAMP has been continuously investigated and found in multiple other mammalian species, e.g.,

Table 1. Antimicrobial peptides in the skin

Family	Representative peptide	Main cells or tissues	Main stimulators	Antimicrobial activity	Reference
Defensin	β -defensin1	Epidermis and other epithelia	Constitutive	Gram-positive bacteria Gram-negative bacteria Viruses, and Fungi	Ali <i>et al.</i> , 2001
	β -defensin2	Epidermis and other epithelia	IL-1	Gram-negative bacteria and Fungi	Huh <i>et al.</i> , 2002
	β -defensin3	Epidermis and other epithelia	IL-6, Growth factor	Gram-positive bacteria Gram-negative bacteria Viruses, and Fungi	Fazakerley <i>et al.</i> , 2010
Cathelicidin	LL-37	Epidermis and other epithelia	Vitamin D, ER stress	Gram-positive bacteria Gram-negative bacteria Viruses, and Fungi	Segaert, 2008
Psoriasin	N/A	Epidermis	Unknown	<i>Escherichia coli</i>	Glaser <i>et al.</i> , 2005
RNase	RNase7	Epidermis and other epithelia	Unknown	Gram-positive and Gram-negative bacteria	Wittersheim <i>et al.</i> , 2013
Catestatin	N/A	Epidermis	Injury	Gram-positive bacteria Gram-negative bacteria yeast, and Fungi	Radek <i>et al.</i> , 2008

rabbit (Larrick *et al.*, 1991), humans (Larrick *et al.*, 1995), sheep (Bagella *et al.*, 1995), mice (Popsueva *et al.*, 1996), monkeys (Bals *et al.*, 2001), and recently in dogs (Sang *et al.*, 2007). These mammalian CAMPs are small, cationic, and amphipathic peptides, which range in size <100 amino acid residues. In humans, there is a single CAMP gene, which encodes the inactive precursor protein, known as CAP18. This inactive precursor consists of an N-terminal cathelin domain and a C-terminal peptide domain. Local proteases, *e.g.*, serine protease 3, kalikerin-5 or -7, cleave the C-terminal domain from the inactive precursor CAP18 to form active AMP such as LL-37, which is responsible for antimicrobial activity (Table 1) (Glaser *et al.*, 2005; Radek *et al.*, 2008; Segaert, 2008; Wittersheim *et al.*, 2013).

Epidermal CAMP is minimally detected in intact, unperturbed skin but significantly upregulated in response to exogenous infections, wounding, and permeability barrier perturbation. It has been well established that the Vitamin D₃-induced Vitamin D Receptor (VDR) activation is the primary transcriptional regulatory mechanism of CAMP production in the skin (Martineau *et al.*, 2007; Heilborn *et al.*, 2010; Kanda *et al.*, 2012). In general, while Vitamin D₃ is synthesized from 7-dehydrocholesterol in human skin upon exposure of UVB irradiation, with the two key enzymes being vitamin D 25-hydroxylase (CYP27A1) in liver and 25-hydroxyvitamin D₃ 1- α -hydroxylase (CYP27B1) in kidney. Both enzymes are required to form active Vitamin D₃, 1,25 dihydroxy vitamin D₃ (1,25D₃) (Lehmann *et al.*, 1999; Kragballe, 2002; Vantieghem *et al.*, 2006). In particular, CYP27B1 which can activate 1,25D₃ is also expressed in keratinocytes and is under the control of the signals that occur in bacterial infection or injury in the skin. Activated 1,25D₃ binds to the VDR and recruits transcriptional coactivator proteins such as steroid receptor coactivator (SRC) 3. This result is in the formation of a complex with activated VDR, leading to initiate CAMP expression and function (Fig. 1).

In addition to VDR-dependent mechanism, we have discovered that the subtoxic external perturbations such as UVB irradiation that induce endoplasmic reticulum (ER) stress and increase cellular ceramide production in parallel with stimulated metabolic conversion of sphingosine to sphingosine-1-

phosphate (S1P) lead to enhanced CAMP generation *via* an NF- κ B activation, independent of the VDR pathway (Fig. 1) (Uchida *et al.*, 2010). In addition to VDR, several consensus transcription factor-binding sequences, including C/EBP, are present on the promoter region of CAMP, but an NF- κ B binding element has not been identified in the region that includes the 693 bp upstream from the transcription initiating site. Since NF- κ B binding site(s) is not present within the CAMP promoter, the ER stress-induced activation of NF- κ B could not directly bind to the promoter region; instead, NF- κ B activation induces C/EBP α via p38 MAP kinase (Hayakawa *et al.*, 2010; Garcia-Garcia *et al.*, 2012). Although a detailed mechanism of how the ER stress-mediated increases in S1P activate NF- κ B still remains unresolved, our recent studies further demonstrated that CAMP generation likely occurs by an S1P receptor independent mechanism (Park *et al.*, 2013). Moreover, VDR- and S1P/NF- κ B-dependent mechanism are not operating simultaneously in response to ER stress, both pathways could alternatively regulate CAMP expression depending upon cellular environments, *i.e.* under basal (unstressed) vs. under stressed conditions in the skin (Fig. 1).

A number of previous studies have revealed that CAMP is a major epidermal AMP with the most potent antimicrobial activity against virulent *S. aureus*, and a multifunctional peptide that also modulates cytokine production, cellular differentiation, and adaptive immunity (Table 1). Furthermore, recent studies revealed that CAMP combined with hBD-2 has synergistic antimicrobial activity by effectively killing *S. aureus* (Kim *et al.*, 2009; Goo *et al.*, 2010).

Role of antimicrobial peptides in inflammatory skin disorders

AD is the most common inflammatory skin disease and is characterized by T helper type 2 (Th2) cytokine-mediated inflammations and significant barrier disruption. Since Th2 cytokines such as IL-4 and IL-13, which are highly expressed in AD skin and suppress key epidermal AMPs, *e.g.*, hBD-2, hBD-3, and CAMP, expression levels of these AMPs decline in AD skin as compared to healthy skin (Reinholz *et al.*, 2012; Lancto *et al.*, 2013). Decreased expression of AMPs can explain the increased susceptibility of AD patients to bacterial infection, particularly methicillin-resistant *S. aureus* (MRSA), which poses a significant community health problem for atopic dermatitis patients since treatment is difficult. In contrast to AD, CAMP is strongly overexpressed in the skin of rosacea patient compared to the skin of healthy controls (Meyer-Hofert and Schroder, 2011). However, levels of hBDs in rosacea patient do not differ from healthy controls. As presented above, the active form LL-37, which is cleaved from the inactive precursor CAP18 by serine proteases, can be further processed to smaller peptide fragments and has pro-inflammatory response. In addition, increased expression/activity of serine proteases such as serine protease 3 and kalikerin-5 have been found in skin lesions of rosacea patients (Yamasaki *et al.*, 2007; Morizane *et al.*, 2010). Although further studies are needed to show how cutaneous serine proteases are activated in rosacea, increased CAMP expression due to enhanced serine protease activity can explain why patients suffering from rosacea show chronic inflammation.

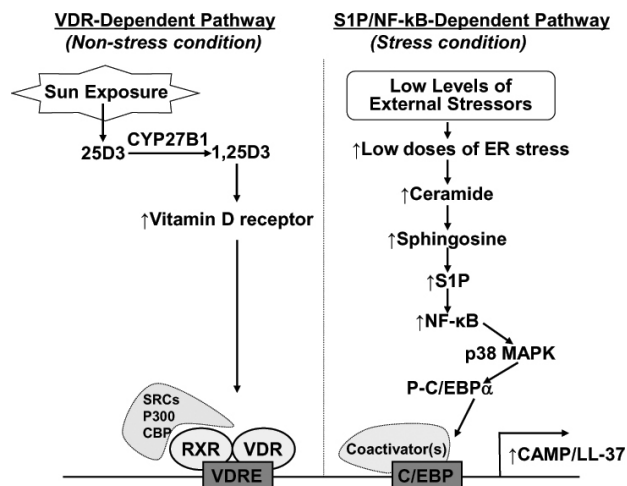


Fig. 1. Regulatory mechanisms of VDR- or S1P/NF- κ B-dependent induction of CAMP/LL-37 in human skin.

MAIN SPHINGOLIPID METABOLITES IN SKIN

Ceramide structure in skin

The stratum corneum (SC) is the outmost upper layer of the skin and provides the rate-limiting barrier function for penetration into and out of the skin. The SC of the skin contains relatively high amounts of ceramides as much as 50% of total skin lipids (Bouwstra *et al.*, 1999). Ceramides in skin exist both in the free form and bound to proteins. Interestingly, skin ceramides have unique structures which are not reported in other tissues. Typical skin ceramides structures contain the long-chain fatty acids linked to sphingosine or phytosphingosine (Stewart and Downing, 1999). Some of fatty acids with 2-hydroxyl group were also observed in skin ceramides structure (Huang and Chang, 2008). In addition, there are O-acyl ceramides structure consisted with a long-chain fatty acids, having a terminal hydroxyl group which esterified either with linoleate or a 2-hydroxy acid (Janusova *et al.*, 2011) (Fig. 2).

These ceramides have an essential function in the skin barrier properties, inhibiting water loss and protecting ingress of potentially harmful substances. This barrier functions have been impaired in skin diseases such as psoriasis and AD, presumably caused by an altered lipid composition and organization (Farwanah *et al.*, 2005). Indeed, the skin barrier disruption has been attributed to decreased levels of ceramides in SC of AD patients (Kita *et al.*, 2002).

Antimicrobial sphingosine in skin

Among various sphingoid bases, only sphingosine was clearly and profoundly effective against *Staphylococcus aureus* (4-log reduction at 20 μM ; 2-log reduction at 2.5 μM) (Arikawa *et al.*, 2002). The sphingosine was similarly active against *Streptococcus pyogenes*, *Micrococcus luteus*, *Propionibacterium acnes*, *Brevibacterium epidermidis*, and *Candida albicans*, moderately active against *Pseudomonas aeruginosa*, and ineffective against *Escherichia coli* and *Serratia marcescens* (Bibel *et al.*, 1992a). The positively charged structure

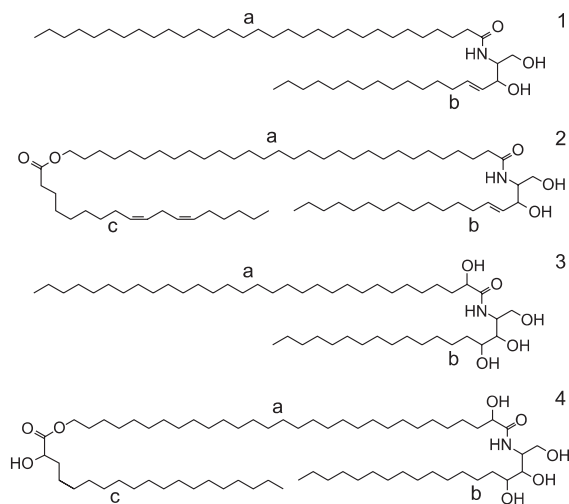


Fig. 2. Distinctive structures of skin ceramides. Sphingosine (1b,2b) or phytosphingosine (3b,4b) backbone were linked to a long-chain fatty acid (typically C_{30} ; 1a, 3a) or has a terminal hydroxy group (2a,4a) which esterified with linoleate (2c) or 2-hydroxy acid (4c).

of 2-amino position in sphingosine is responsible for enzyme inhibition such as a protein-kinase system because there was no activity at pH higher than 8.0 and N-acetyl sphingosine was ineffective, suggesting this chemical structure is important in antimicrobial activity (Bottega *et al.*, 1989; Igarashi *et al.*, 1989). In addition, both *erythro*- and *threo*-isomers were effective and essentially required Ca^{2+} ion for antimicrobial activity. Therefore, the existence of sphingosine in the SC suggests that sphingosine may have a major role in the resistance of skin to colocalization and infection by microorganisms (Bibel *et al.*, 1992b, Bibel *et al.*, 1993). The levels of sphingosine in the SC are regulated by two factors; the hydrolysis of ceramides to sphingosine by acidic ceramidase (CDase) and ceramides levels as a substrate pool. Actually, the levels of sphingosine are significantly down-regulated to 140-160 μM in SC of AD patients, compared with healthy controls (268 μM). This suggests that the decreased levels of ceramides and reduced activities of acidic CDase are in part associated with the deficiency of sphingosine in the skin of AD patients (Arikawa *et al.*, 2002).

Sphingosine 1-phosphate (S1P) in skin

In skin, sphingosine can be phosphorylated by sphingosine kinases into S1P, which acting both the maintenance of the epidermal permeability and as a critical signaling molecule, which binds to a family of G-protein coupled receptors, termed S1PR_1 - S1PR_5 (Maceyka *et al.*, 2009). This S1P signaling via S1PR receptors (S1PR s) significantly regulate the immunosuppressive action by causing lymphopenia as demonstrated by a S1PR_1 agonist, FTY720 (Fingolimod) treatment *in vivo* (Bohler *et al.*, 2003). The immunosuppressive ligand S1P is irreversibly metabolized into hexadecenal and ethanolamine phosphate by S1P lyase (Schwab *et al.*, 2005) (Fig. 3). Therefore, the regulation of S1P lyase activity may mainly control the intracellular S1P concentrations (Berdyshev *et al.*, 2011). Therefore, S1P lyase activity may also exhibit a central role for the regulation of immunological disorders, suggesting that an imbalance in the S1P-S1P lyase axis may directly related to the incidence of skin diseases. Interestingly, the S1P lyase mRNA and S1P lyase activity were greatly enhanced in atopic skin lesions in humans, supposing that S1P concentration in

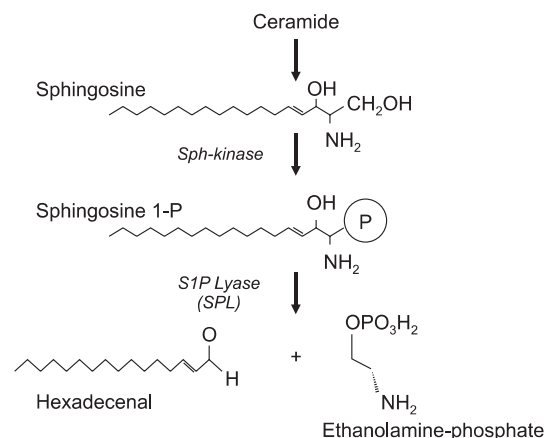


Fig. 3. S1P lyase (SPL) play a central role for regulating endogenous S1P concentrations.

AD patients skin may be much less compared to healthy skin (Baumer *et al.*, 2011). In this report, the S1P concentration in lesional skin of dogs suffering from AD was significantly lower, 0.18 nmol/mg protein while healthy skin was 0.63 nmol/mg protein.

Sphingolipid metabolism in ER stress

In a recent paper, we demonstrated that ER stresses by cell-permeable ceramide (C2Cer) in human keratinocytes upregulated the mRNA expression of CAMP as well as the endogenous sphingosine in 10-fold concentrations and S1P in 1.3-fold increase (Park *et al.*, 2013). In this conditions, the inhibition of S1P lyase activity by siRNA treatment profoundly increased S1P level, indicating the possible involvement of sphingolipid metabolites, ceramides, sphingosine and S1P for the induction of CAMP mRNA and protein expression. Especially the enzyme of sphingosine kinase type 1 (SK-1) which produce S1P was significantly activated in CAMP expression in human keratinocytes which were treated with C2Cer. In addition, a pharmaceutical inhibitor (SKI) or siRNA treatment of SK-1 attenuated CAMP expression in cells. From *in vivo* studies using S1P lyase-deficient mouse skin, C2Cer (7.5 μ M) treatment induced ER stress and subsequently increased the protein and mRNA levels of CAMP compared to vehicle. Exogenously added S1P or synthetic S1PR1 ligands (SEW 2871) did not enhanced CAMP mRNA expression. These results suggested that S1P regulation of enhanced CAMP expression may not be related to S1P receptor-mediated S1P signaling pathway.

CONCLUSIONS

Taken together, the antimicrobial peptide, CAMP is an indispensable factor of the skin's innate immune defense system against microorganisms' infection. Previously, the CAMP protein in keratinocytes is regulated by vitamin D3 through the vitamin D receptor (Gombart *et al.*, 2005). We newly identified S1P as an ER-stress signaling molecule which increases production of CAMP when the epidermis is under stress; e.g., UV exposure (D'Orazio *et al.*, 2013; Wallingford *et al.*, 2013), wound healing (Kawanabe *et al.*, 2007), or attack by microorganisms (Arikawa *et al.*, 2002). Identification of this new signaling mechanism will allow the development of new topical compounds, both synthetic and natural, that could be used to enhance epidermal innate immunity and antimicrobial defense, when the skin is under microbial attack from the outside.

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