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Vitamin D_3 modulates the innate immune response through regulation of the hCAP-18/LL-37 gene expression and cytokine production

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Abstract

The steroid hormone metabolite of vitamin D_3, 1α,25-dihydroxyvitamin D_3 (1,25D3), promotes osteogenic activity and regulates calcium and phosphate metabolism, which are actions regarded as classical vitamin D-regulated functions. Besides its role in these processes, 1,25D3 also seems implicated in the host defense against microbial/pro-inflammatory attacks. Low serum levels of vitamin D_3 (vitamin D deficiency) are associated with osteoporosis and increased risk of fractures but also inflammatory diseases and their disease progression, presumably via mechanisms associated with 1,25D3-evoked modulation of the innate immune system. 1,25D3 has been reported to modulate many inflammatory responses, suggesting that it regulates multiple transcriptional targets within the inflammatory system. Experimental studies in various experimental systems show that 1,25D3 differentially regulates the production of pro-inflammatory cytokines and chemokines depending on cell type. Importantly, many reports show that 1,25D3 up-regulates expression of the human antimicrobial peptide hCAP-18/LL-37 gene. The hCAP-18/LL-37 gene seems indeed to be an important transcriptional target for 1,25D3. However, only weak evidence is presented showing that 1,25D3 consistently increases the amount of biologically active LL-37 peptide. In the present review, we discuss 1,25D3-induced down-regulation of cytokine/chemokine production and stimulation of hCAP-18/LL-37 gene expression which represent two very important pathways for 1,25D3-evoked regulation of the innate immune response.

Key words: chemokine, cytokine, innate immune system, hCAP-18/LL-37, vitamin D_3

Introduction

The biologically active metabolite of vitamin D_3, 1α,25-dihydroxyvitamin D_3 (1,25D3), is a steroid hormone with high affinity for the intracellular/nuclear vitamin D receptor (VDR) [1]. The 1,25D3/VDR forms a complex with the retinoid X receptors (RXRs), and this complex then binds to vitamin D response elements of target genes causing up- or down-regulation of their transcriptional activity. The 1,25D3-regulated targets involve genes controlling cell proliferation and bone, calcium and phosphate metabolism, but also genes associated with regulation of the innate and adaptive immune systems [2-4]. 1,25D3 is reported to regulate the innate immune response via different pathways such as production of pro-inflammatory mediators, e.g. cyclooxygenases, differentiation of macrophages, production of cytokines and chemokines, and the expression of antimicrobial peptides, primarily via VDR-dependent transcriptional mechanisms. Importantly, many reports demonstrate that 1,25D3 is a powerful transcription factor for the human antimicrobial peptide hCAP-18/LL-37 gene in a wide variety of human cell types [1, 3, 5, 6].

In the present review, we focus on 1,25D3-evoked regulation of cytokine/chemokine production and hCAP-18/LL-37 expression, respectively, which may represent two important mechanisms by which 1,25D3 modulates the innate immune response. 1,25D3 seems to regulate pro-inflammatory cytokine and chemokine expression differentially depending on cell type. Although, much evidence shows that 1,25D3 enhances hCAP-18/LL-37 gene activity, less proof is available demonstrating that 1,25D3 also stimulates LL-37 protein production.

Metabolic transformation of the pro-hormone vitamin D_3 to 1,25D3

The steroid hormone precursor vitamin D_3 is produced in the skin keratinocytes through a UV light-dependent mechanism and/or supplied by the diet via intestinal uptake (Fig. 1). The pro-hormone vitamin D_3 is transported to the liver and hydroxylated at the C-25 position to 25-hydroxyvitamin D_3 (25D3). A second hydroxylation of 25D3 occurs primarily in the kidneys through an enzymatic reaction catalyzed by the cytochrome P-450 (CYP) enzyme CYP27B1, and the biologically active steroid hormone 1α,25-dihydroxyvitamin D_3 (1,25D3) is hereby formed (Fig. 1). The second hydroxylation of vitamin D_3 is regulated by parathyroid hormone, phosphate and calcium levels in serum. Transformation of 25D3 to 1,25D3 is not restricted only to the kidneys but may also occur in other tissues and cells [7, 8]. Interestingly, Toll-like receptor (TLR) activation has been demonstrated to enhance CYP27B1 mRNA expression in human monocytes, suggesting that transformation of 25D3 to 1,25D3 occurs in monocytes upon activation of TLRs [9]. 25D3 and 1,25D3 are circulating in the plasma mainly bound to vitamin D-binding protein but also albumin, while only a small fraction (less than 1%) occurs in the plasma as free 25D3 and 1,25D3 [10, 11]. The VDRs can only be activated by free 1,25D3, implying that this form of vitamin D_3 represents the biologically significant one.

25D3 is the major circulating form of vitamin D_3 and it occurs normally in serum in the concentration range of 20-100 μg/L, corresponding to 50-250 nM [12, 13]. Measurement of serum 25D3 is regarded as the standard determinant of vitamin D status in humans [12]. In post-menopausal women, the serum 1,25D3 concentration, is reported to be as low as 130.5 pM [14]. In fact, this fits well with a mathematic modeling of the free serum concentrations of 25D3 and 1,25D3 performed by Chun et al. [15], showing that the predicted free 25D3 and

1,25D3 concentrations are 50 nM and 100 pM, respectively, in vivo. Thus, importantly, serum 1,25D3 concentrations are about 500 to 1000 times lower than those of 25D3.

Vitamin D signaling

The free pool of 1,25D3 readily diffuses over the plasma membrane and binds to the intracellular VDR. This complex forms a heterodimer with the RXRs [1, 16]. The 1,25D3/VDR/RXR complex binds to the vitamin D responsive elements of genes regulated by 1,25D3 and up- or down-regulates their gene activity. The 1,25D3-evoked regulation of transcriptional activity involves recruitment of co-activators and co-repressors which modulate the 1,25D3/VDR/RXR complex gene transcriptional activity. 1,25D3 regulates important physiological functions such as calcium, phosphate and bone metabolism as well as growth and differentiation of keratinocytes, osteoblasts and osteoclasts, mostly through transcriptional mechanisms but also rapid, non-genomic and VDR-independent signaling seems to be involved [2, 17]. Presumably, 1,25D3 also modulates the innate immune system through primarily transcriptional regulation, although possible involvement of non-transcriptional regulation cannot be ruled out. The VDR is an intracellular/nuclear receptor acting as a classical steroid hormone receptor. Similar to reports describing, rapid non-genomic estrogen, progesterone and testosterone signaling associated with activation of plasma membrane receptors for these steroids, there are reports showing that 1,25D3 may also have binding sites/receptors in the plasma membrane responsible for rapid, non-genomic 1,25D3–stimulated functional effects [17, 18]. The rapid 1,25D3-evoked responses involve for example opening of voltage-dependent Ca^{2+} channels in osteoblasts and migration of endothelial cells, and they are thought to involve interaction of 1,25D3 with proteins such as

protein disulfide isomerase family A member 3, phospholipase A2 and caveolin-1 in the plasma membrane caveolae [18].

Vitamin D_3 is associated with inflammatory diseases and the innate immune system

Altered 25D3 levels in serum are associated with common inflammatory diseases such as inflammatory bowel disease, atherosclerosis, asthma, periodontitis, rheumatoid arthritis and multiple sclerosis [3, 4, 19]. The importance and the possible dependence of serum 25D3 levels for the development of these pathogenic conditions are not completely understood. Importantly, experimental studies show that 1,25D3 has an impact on many aspects and levels of the innate immune system, some of these aspects are discussed below, providing several possible explanations for the mechanisms behind the connection between 1,25D3 and inflammatory diseases. 1,25D3 influences not only the innate immune system but also adaptive immune responses. In the present review, we primarily focus on some important aspects of 1,25D3-induced regulation of the innate immune response. As mentioned previously, altered 25D3 levels in serum are associated with many inflammatory diseases, and, here, we comment further on periodontitis which is a progressive inflammatory disease primarily initiated by bacteria residing in the dental and sub-gingival plaque. The initial gingival inflammation could either resolve or develop to a more profound inflammation, periodontitis, causing destruction of the alveolar bone, loss of attachment, and finally loss of teeth. Low levels of 25D3 in serum have been associated with periodontitis, suggesting that vitamin D_3 is a protective factor against tooth loss [19-21]. Interestingly, the beneficial effect of hormone replacement therapy (estrogen alone or estrogen + progesterone) on periodontal inflammation and tooth loss in post-menopausal women seems to be dependent on sufficient, high levels of 25D3, suggesting a positive synergistic effect between female sex hormones

estrogen and progesterone and vitamin D_3 [22]. The common opinion is undoubtedly that high levels of 25D3 are beneficial, while low levels are deleterious for periodontal health. This opinion is, however, questioned by a recent study by Antonoglou et al. [23] reporting that serum levels of 25D3 are not related to periodontal pocket depths and gingival bleeding in a non-smoking cohort (n=1262) without diabetes, i.e. in a group of individuals at low risk for periodontitis.

1,25D3 enhances differentiation of monocytes into macrophages

Monocytes/macrophages represent the first-line of defense against invading microorganisms and harmful agents. 1,25D3 seems to promote maturation of monocytes into macrophages and, moreover, enhance their phagocytic capacity [24, 25]. Interestingly, 1,25D3 has been reported to inhibit the differentiation of human monocytes into dendritic cells, without any impact on cell survival, through stimulation of colony-stimulating factor 1 (CSF-1) production, suggesting that 1,25D3 maintains the monocytes as monocytes and/or macrophages and thus promotes the innate immune response through this mechanism [26].

1,25D3 differentially attenuates cytokine/chemokine production in a cell type-dependent manner

In human peripheral blood mononuclear cells, 1,25D3 (10-100 nM) attenuates dose-dependently *Mycobacterium tuberculosis*-evoked expression of pro-inflammatory cytokines IL-6, TNFα and IFNγ [27]. On the other hand, these authors show that 1,25D3 enhances the expression of anti-inflammatory IL-10 [27]. The mechanism behind the down-regulation of pro-inflammatory cytokine expression by 1,25D3 is suggested to involve a VDR-associated

reduction of pattern recognition receptor TLR2, TLR4, dectin-1 and mannose receptor expression [27]. Lipopolysaccharide-stimulated production of IL-1β, IL-6 and TNFα is suppressed by 1,25D3 in human and murine monocytes/macrophages [28-30]. Interestingly, the down-regulation of pro-inflammatory cytokines upon stimulation with 1,25D3 seems to vary with the stage of monocyte/macrophage maturation, and, moreover, this effect seems to be dependent on MAPK phosphatase-1 [29, 30]. Importantly, 1,25D3 enhances glucocorticoid-induced inhibition of lipolysaccharide-stimulated IL-6 in human monocytes, suggesting that 1,25D3 and glucocorticoids may act in synergy [31].

In human adipocytes and in 3T3-L1 adipocyte cell line, 1,25D3 attenuates the expression of IL-8, IL-6, IL-1β and CCL2 (MCP-1) [32, 33]. 1,25D3 has been shown to suppress IL-1α, IL-6 and IL-8 but activate TGFβ in keratinocytes [2]. On the other hand, 1,25D3 enhances mRNA expression of TNFα in the human keratinocyte cell line HaCaT [34]. Larsen et al. [35] demonstrate that 1,25D3, at an optimal concentration of 10 to 100 pM, inhibits IL-1α-evoked IL-8 mRNA expression and protein production in fibroblasts, keratinocytes and leukocytes but not in endothelial cells. Human periodontal ligament cells are fibroblast-like cells residing in the periodontal ligament which forms the interface between the root cement of the teeth and the alveolar jaw bone. The periodontal ligament cells show not only fibroblast-like functional properties but also immune cell-like properties [36]. These cells respond to stimulation with lipopolysaccharides with enhanced cytokine and chemokine production. 1,25D3 attenuates *Porphyromonas gingivalis* and lipopolysaccharide-induced IL-8 and CCL2 chemokine mRNA expression and protein production, while it has no effect on IL-6 in primary human periodontal ligament cells [37, 38]. On the other hand, Andrukhov et al. [38] report that 1,25D3 reduces the production of all three pro-inflammatory cytokines IL-6, IL-8 and CCL2 in commercially available human periodontal ligament fibroblasts. In periodontal ligament

cells from young individuals, treatment with 1,25D3 is demonstrated to reduce LPS-stimulated mRNA expression of IL-6, CXCL1 (GRO1) but not IL-1β and CCL2 [39]. Information on the regulation of cytokine/chemokine production by 1,25D3 in some different human cell types is summarized in Table 1. Thus, it seems that 1,25D3 differentially regulates inflammation promoter-stimulated cytokine and chemokine expression depending on cell type and cell preparation. The differences in 1,25D3 response between cell types may reflect cell type-dependent VDR expression level and/or differences in 1,25D3 signaling pathways down-stream of VDR such as cell type specific expression of co-activators and co-repressors. Importantly, treatment with VDR siRNA abolishes the 1,25D3-evoked attenuation of pro-inflammatory cytokine/chemokine expression in human periodontal ligament fibroblasts stimulated with *Porphyromonas gingivalis* lipopolysaccharide, showing that 1,25D3 acts through VDR [38].

1,25D3 promotes expression of the human antimicrobial peptide hCAP-18/LL-37 gene

The antimicrobial peptide (AMP) LL-37 is produced in its pro-form (hCAP-18) by epithelial cells and neutrophils. The hCAP-18 is a member of the cathelicidin family, and, in fact, it is the only human member of the cathelicidin family [40]. The hCAP-18 peptide is secreted and then processed to LL-37 extracellularly in a reaction catalyzed by serine proteinase 3 [41]. The processing steps in the generation of active LL-37 is presented in Figure 2. LL-37 is thought to exert its antimicrobial properties through permeabilization of the bacterial cell wall thereby promoting osmotic lysis of the bacteria [42-44]. In fact, LL-37 perforates not only the bacterial cell wall but also host cell plasma membranes, as demonstrated by a LL-37-evoked rapid stimulation of Ca^{2+} influx in human osteoblasts [45]. LL-37 is supposed to act also via neutralizing lipopolysaccharides thereby inhibiting the production of pro-inflammatory

cytokines [46]. Additionally, LL-37 is reported to enhance chemokine production in keratinocytes, suggesting that LL-37 enhances neutrophil chemotaxis in the skin [47]. Interestingly, LL-37 may interact with immune cells via binding specific receptors in the plasma membrane such as P2X7 and formyl peptide receptor-like 1 [48, 49]. Thus, LL-37 seems to interact with the innate immune response through different mechanisms. LL-37 has been shown to attenuate *Porphyromonas gingivalis* and *E. coli* lipopolysaccharide-induced pro-inflammatory cytokine formation in human periodontal ligament cells and gingival fibroblasts, suggesting that LL-37 may antagonize periodontitis through this mechanism [50, 51]. LL-37 levels are increased locally in periodontitis; in fact they are well within the concentration range of LL-37 causing apoptosis of cultured osteoblasts, suggesting that LL-37 may have an impact on the destruction of the alveolar jaw bone observed in individuals suffering from periodontitis through this mechanism [45, 52]. Thus, LL-37 can antagonize loss of tooth attachment in periodontitis by inhibiting production of pro-inflammatory cytokines, but, on the other hand, LL-37 may enhance loss of attachment through stimulation of osteoblast apoptosis.

The human cathelicidin antimicrobial peptide (CAMP) gene hCAP-18/LL-37 is very convincingly demonstrated to be a 1,25D3/VDR-dependent target gene showing enhanced gene activity in response to stimulation with 1,25D3 in many human cell types [27, 53-59]. Normally, the concentrations of 1,25D3 used to stimulate hCAP-18/LL-37 gene activity are between 1 and 50 nM, but in some experimental systems higher concentrations (100-1000 nM) of 1,25D3 are needed to enhance hCAP-18/LL-37 gene activity [27, 53-56]. *In silico* screening analysis for VDR response elements demonstrates promoter-proximal elements in the hCAP-18/LL-37 gene, indicating that the hCAP-18/LL-37 gene expression is governed by the 1,25D3/VDR complex [57]. Monocytes cultured in vitamin D-binding protein-free

medium show stronger 1,25D3-evoked induction of mRNA for hCAP-18/LL-37 than cells cultured in medium containing vitamin D-binding protein, demonstrating that the vitamin D-binding protein regulates bio-available 1,25D3 to the monocytes [58]. Importantly, 1,25D3-induced stimulation of antimicrobial activity against *Mycobacterium tuberculosis* seems to be critically dependent on induction of the hCAP-18/LL-37 gene demonstrated by siRNA for hCAP-18/LL-37 [59]. Treatment with 1,25D3 is convincingly shown to enhance hCAP-18/LL-37 gene activity and pro-LL-37, hCAP-18 protein, but 1,25D3-induced extracellular cleavage of hCAP-18 into mature, active LL-37 has not been clearly demonstrated in experimental cell studies, although Liu et al. [9] show a peak at 4.5 kDa, corresponding to LL-37, by SELDI-TOF mass spectrometry in primary human monocytes stimulated with 1,25D3. Thus, it remains to be convincingly demonstrated in cell studies that 1,25D3 increases transformation of hCAP-18 into LL-37. Importantly, the 1,25D3-evoked up-regulation of hCAP-18/LL-37 transcript and hCAP-18 protein is blocked by treatment with VDR siRNA and VDR antagonists, showing that VDR, as expected, is involved in the 1,25D3-induced stimulation of hCAP-18/LL-37 gene expression and production of pro-LL-37 [9, 56]. The vast majority of studies show that 1,25D3 enhances hCAP-18/LL-37 gene activity, but also 1,25D3-induced attenuation of lipopolysaccharide- and ultraviolet B radiation-induced mRNA levels for hCAP-18/LL-37 is reported [60].

The LL-37 molecule shows both hydrophobic and hydrophilic properties and it carries a net positive charge of 6 at physiological pH, making it a highly polycationic agent that may interact with anionic binding sites within for example bacterial cell wall, host cell membranes but also extracellular and secreted proteins such as mucins [44, 61]. Furthermore, LL-37 probably may compete with other endogenous polycationic substances for negatively charged cellular and extracellular binding sites. Due to its polycationic structure and high affinity for

negatively charged residues within cellular and extracellular proteins, extracellularly cleaved, free LL-37 will rapidly bind to negatively charge moieties and therefore rapidly disappear from the extracellular fluid. This may explain why it is difficult to demonstrate 1,25D3-induced stimulation of LL-37 production on the peptide level. In order to determine the functional importance of 1,25D3-induced LL-37 in the immune system a successful approach can be to combine quantitative methods for peptide measurement, such as Western blotting, with functional studies using LL-37 siRNA in isolated cell systems. In fact, Yuk et al. [62] show, by using LL-37 siRNA, that 1,25D3 induces autophagy in human monocytes through LL-37, indicating that LL-37, besides its well known antimicrobial activity, also mediates 1,25D3-evoked autophagy.

Conclusions

Insufficient levels of 25D3 in serum are supposed to enhance the disease progression of various inflammatory diseases and increase the severity of the disease through mechanisms associated with the innate immune system. Experimental studies in various cellular systems show that 1,25D3 attenuates the production of pro-inflammatory cytokines and chemokines and up-regulate expression of the human antimicrobial peptide hCAP-18/LL-37 gene. 1,25D3 seems to evoke a differential down-regulation of some, but not all, inflammation promoter-induced cytokines/chemokines through a mechanism proposed to involve reduction of pattern recognition receptor expression. The hCAP-18/LL-37 gene is convincingly demonstrated to be a very strong and important transcriptional target for 1,25D3. The hCAP-18/LL-37 gene shows up-regulation and the cytokine/chemokine production is reduced in response to stimulation with physiological and high physiological concentrations of 1,25D3 (1-100 nM),

suggesting that 1,25D3 exerts its effect on these two branches of the innate immune response at relevant, physiological concentrations. A problem for the interpretation of the present data is, however, that only weak evidence is presented showing that 1,25D3 increases the amount of biologically active LL-37 peptide. Therefore, more experimental studies are needed to demonstrate that 1,25D3, in relevant physiological concentrations, triggers not only hCAP-18/LL-37 gene activity but also enhance the production of functional LL-37 peptide.

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Table 1. Effects of 1,25D3 on cytokine/chemokine production in some different human cell types. Stimulation and reduction of cytokine/chemokine production by 1,25D3 are indicated by + and -, respectively. PDL = periodontal ligament.

Cell type	**Cytokine/chemokine**	**Effect of 1,25D3**	**Reference**
Peripheral blood mononuclear cells	IL-6	-	[27]
	TNFα	-	
	IFNγ	-	
	IL-10	+	
Monocytes/macrophages	IL-6	-	[28-30]
	TNFα	-	
	IL-1β	-	
Adipocytes	IL-6	-	[32, 33]
	IL-8	-	
	IL-1β	-	
	CCL2 (MCP-1)	-	
Keratinocytes	IL-6	-	[2, 34, 35, 60]
	IL-8	-	
	IL-1α	-	
	TGFβ	+	
	TNFα	+	
Fibroblasts	IL-8	-	[35]
Leukocytes	IL-8	-	[35]
Endothelial cells	IL-8	no effect	[35]
PDL cells (primary)	IL-6	no effect	[37, 38]
	IL-8	-	
	CCL2 (MCP-1)	-	

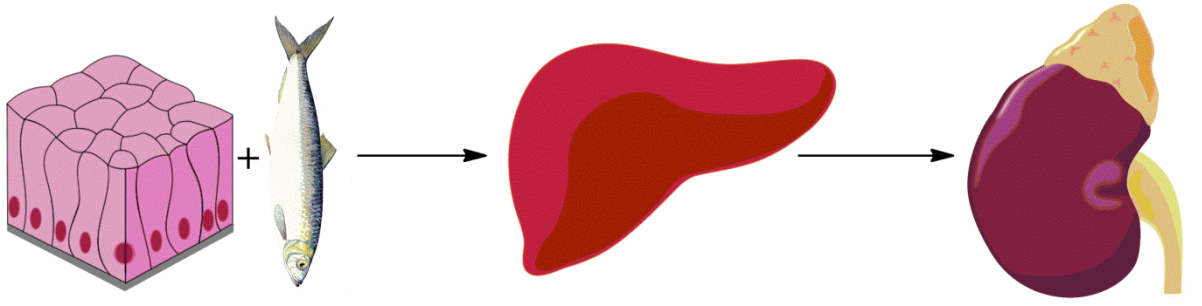
PDL cells (young individuals)	IL-6 IL-1β CXCL1 (GRO1) CCL2 (MCP-1)	- no effect - no effect	[39]
PDL fibroblasts (purchased)	IL-6 IL-8 CCL2 (MCP-1)	- - -	[38]

Figure legends

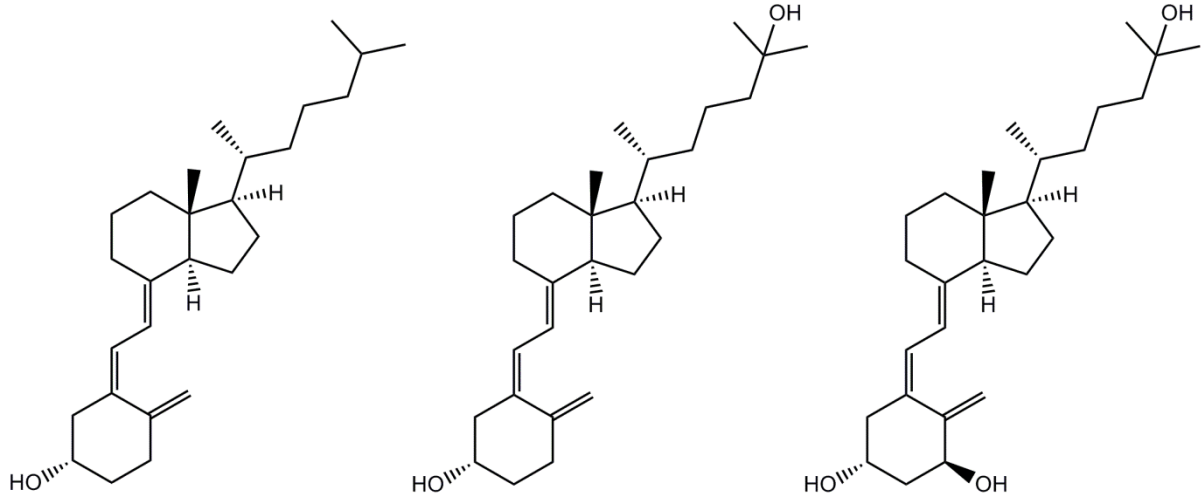
Fig. 1. The main sources of the pro-hormone vitamin D_3 are skin keratinocytes (formation of vitamin D_3 is catalyzed by UV-light) and the diet. The pro-hormone is metabolically transformed primarily in the liver and the kidneys to the biologically active 1α,25-dihydroxyvitamin D_3.

Fig. 2. Schematic figure showing the processing steps in the generation of active LL-37.

Figure 1

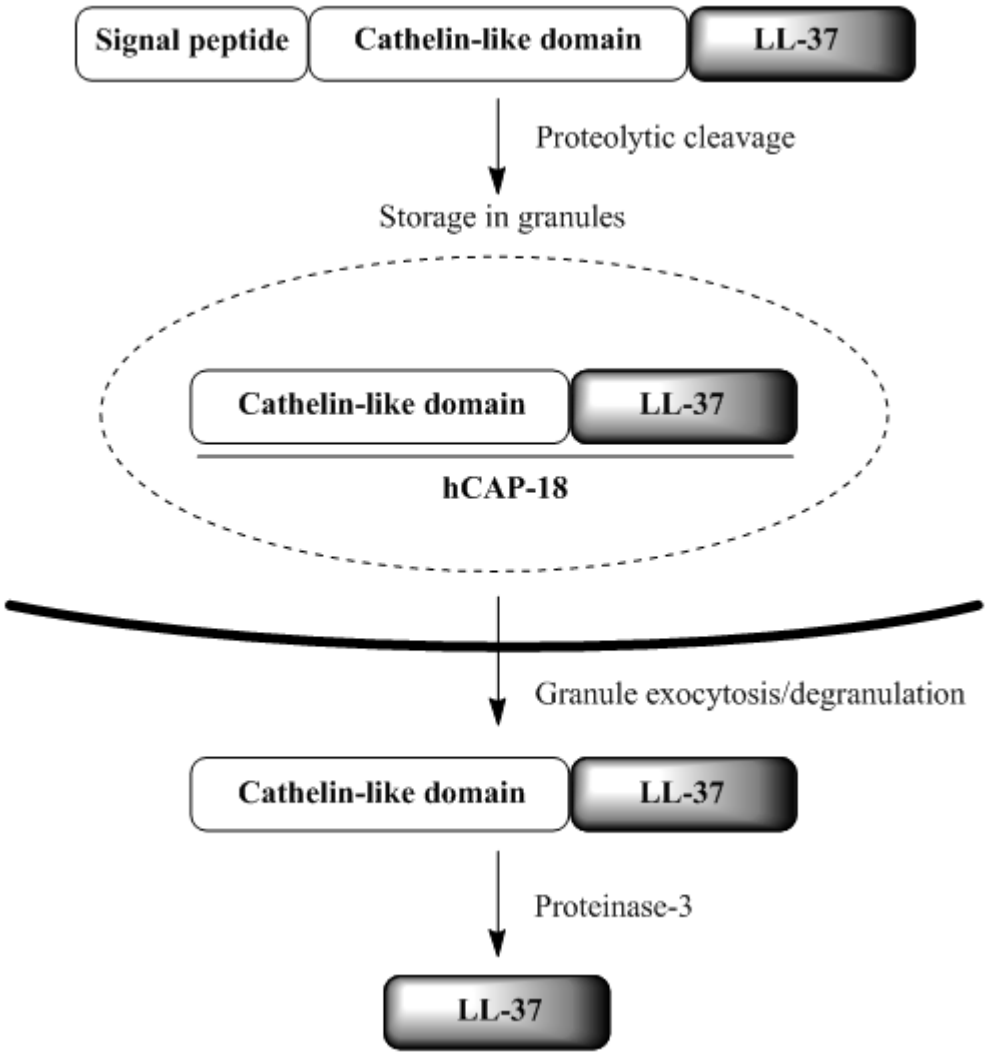


Skin + diet Liver Kidney



Vitamin D_3 25-hydroxyvitamin D_3 1α,25-dihydroxyvitamin D_3

Figure 2


Signal peptide
Cathelin-like domain
LL-37
Proteolytic cleavage
Storage in granules
Cathelin-like domain
LL-37
hCAP-18
Granule exocytosis/degranulation
Cathelin-like domain
LL-37
Proteinase-3
LL-37