Regulation of Cathelicidin Antimicrobial Peptide Expression by an Endoplasmic Reticulum (ER) Stress Signaling, Vitamin D Receptor-independent Pathway

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Vitamin D receptor (VDR)-dependent mechanisms regulate human cathelicidin antimicrobial peptide (CAMP)/LL-37 in various cell types, but CAMP expression also increases after external perturbations (such as infection, injuries, UV irradiation, and permeability barrier disruption) in parallel with induction of endoplasmic reticulum (ER) stress. We demonstrate that CAMP mRNA and protein expression increase in epithelial cells (human primary keratinocytes, HaCaT keratinocytes, and HEK 293 cells), but not in myeloid (U937 and HL-60) cells, following ER stress generated by two mechanistically different, pharmacological stressors, thapsigargin or tunicamycin. The mechanism for increased CAMP following exposure to ER stress involves NF-κB activation leading to CCAAT/enhancer-binding protein α (C/EBPα) activation via MAP kinase-mediated phosphorylation. Furthermore, both increased CAMP secretion and its pro- and activation to LL-37 are required for antimicrobial activities occurring following ER stress. In addition, topical thapsigargin also increases production of the murine homologue of CAMP in mouse epidermis. Finally and paradoxically, ER stress instead suppresses the 1,25(OH)2 vitamin D3-induced activation of VDR, but blockade of VDR activity does not alter ER stress-induced CAMP up-regulation. Hence, ER stress increases CAMP expression via NF-κB-C/EBPα activation, independent of VDR, illuminating a novel VDR-independent role for ER stress in stimulating innate immunity.

Mammalian epithelial tissues, such as the respiratory system, gastrointestinal tract, and genitourinary tract, as well as mucosal epithelia and skin, are positioned at the interface with the environment, where they employ multiple protective strategies that protect underlying tissues from exogenous microbial pathogens (1–3). These protective mechanisms include antimicrobial peptides (AMP)2 that target a broad range of pathogens, including Gram-negative and Gram-positive bacteria, yeast, fungi, and viruses (3–6). An important AMP, the carboxy-terminal fragment of the cathelicidin antimicrobial protein (CAMP), comprises a 37-amino acid peptide (LL-37) that is not only an inducible but also a multifunctional modulator of cytokine secretion/production, angiogenesis, and adaptive immunity (5, 7). Decreased CAMP/LL-37 production parallels increased pathogen colonization/invasion in exposed epithelia in diseases such as cystic fibrosis (8), Crohn disease (9), streptococcus/viral invasion, and atopic dermatitis (10).

Although CAMP production potentially defends against pathogen-induced diseases in multiple epithelia (like many other AMPs, it is expressed only at low, constitutive levels in these epithelia), it is up-regulated in response to external perturbations, i.e. wounding, UV irradiation, and epidermal permeability barrier abrogation (6, 11, 12). Numerous transcriptional regulatory elements exist on the 5′-upstream promoter region of CAMP, including a vitamin D receptor (VDR)-responsive element, and VDR activation increases CAMP expression in a variety of epithelial and non-epithelial cell types, including immune cells (13). Toll-like receptor activation following bacterial challenges increases CYP27 expression, which generates a ligand for VDR, 1,25(OH)2 vitamin D3, thereby inducing CAMP production (14). However, whether this VDR-dependent mechanism is responsible for up-regulation CAMP in response to external perturbations remains unknown.

The ER is a central organelle, where both protein and lipid synthesis largely occur and where numerous secretory and membrane proteins are folded by ER-resident, molecular chaperones. The ER also contains a substantial pool of intracellular Ca2+, which, when released from the ER, modulates numerous metabolic pathways (15). Alterations in ER calcium levels in response to exposure to various pathological/pharmacological

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2 The abbreviations used are: AMP, antimicrobial peptide; BHQ®, Black Hole Quencher®, CRAMP, cathelin-related antimicrobial peptide; CAMP, human cathelicidin antimicrobial protein; ER, endoplasmic reticulum; FAM, 6-carboxyfluorescein acronym; KC, keratinocytes; qRT-PCR, quantitative real-time polymerase chain reaction; Tg, thapsigargin; Tm, tunicamycin; VDR, vitamin D receptor; C/EBP, CCAAT/enhancer-binding protein; Bis-Tris, 2-(bis-(2-hydroxyethyl)aminoo)-2-(hydroxymethyl)propane-1,3-diol; PARP, poly(ADP-ribose) polymerase; TUDCA, tauroursodeoxycholic acid; QNZ, 6-amino-4-quinazoline.
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stimuli lead to either accumulation of unfolded protein or over-accumulation of folded protein (16), conditions that together are referred to as ER stress (15). Although excessive ER stress can overwhelm these mechanisms, triggering apoptosis (16), two key intracellular signal pathways, termed the unfolded protein response and the ER-overload response, restore normal ER functions (17).

We recently demonstrated that epidermal permeability barrier perturbation generates ER stress (18), whereas other external stressors, e.g. UV irradiation (17, 19), oxidative stress, and wound injury (20), also initiate ER stress responses including downstream activation of the transcription factor, NF-κB (16). Because several stressors also up-regulate CAMP expression and because ER stress/activation ubiquitously occurs in mammalian cells, we hypothesized here that ER stress-mediated signals could stimulate CAMP expression in epithelial cells exposed to external perturbations, thereby restoring or enhancing antimicrobial defense barrier(s). We investigated here the role of ER stress in stimulating production of CAMP/LL-37 and its murine homologue, CRAMP, utilizing human and murine epidermis as our models of mammalian epithelial tissues. Our results demonstrate that ER stress stimulates synthesis of CAMP/LL-37 via a novel NF-κB/C/EBPα pathway, independent of the VDR, in epithelial cells such as KC and HeLa cells, but not in myeloid cells. As such, the insights from these studies could lead to the development of novel mechanism-based therapies for diseases with CAMP/LL-37 deficiency, as well as for improving antimicrobial defense.

EXPERIMENTAL PROCEDURES

Cell Culture—Normal human keratinocytes (KC) isolated from neonatal foreskins by a modification of the method of Pittelkow and Scott (21) from Holleran et al. (22) under an Institutional Review Board approval protocol (University of California, San Francisco) or immortalized, non-transformed (HaCaT) KC, derived from human epidermis (a gift from Dr. N. Fusieng (Heidelberg, Germany)), were grown as described previously (23). Culture medium was switched to serum-free KC growth medium containing 0.07 mM calcium chloride and growth supplements (Invitrogen) 1 day prior to thapsigargin (Tg) or tunicamycin (Tm) treatment. HeLa cells were cultured in 10% FCS-DMEM, whereas HL-60 cells and U937 were cultured in 10% FCS-DMEM, whereas HL-60 cells and U937 were cultured in RPMI 1640 containing 10% fetal bovine serum. Culture medium was then switched to the indicated medium containing 2.5% FCS 1 day prior to treatment. Cell toxicities, including apoptosis, were assessed by poly(ADP-ribose) polymerase (PARP) cleavage as well as a trypan blue dye exclusion assay (24).

Ex Vivo Murine Experiments—Full thickness pieces of murine skin harvested from mice (7 control of Vdr-null mice) were placed on filter paper, dermis side down, and maintained at the air-medium interface in KC growth medium (above). Tg (100 nM) or vehicle (DMSO) was epicitously applied (20 μl/cm²) followed by incubation for 24 h at 37 °C in 5% CO₂ in air. Skin was separated followed by isolation of the protein and mRNA fraction, as described previously (26).

Quantitative Real-time Polymerase Chain Reaction Analysis—Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using cDNA prepared from mRNA fractions of cell lysates, as we described previously (26). The following primer sets were used: CAMP (hCAP18), 5′-CACACGACGTCCACAGGATTG-3′ and 5′-GGCCTGTGTTGCCATCCTGAT-3′; intron CAMP, 5′-CCCTGTTGTCCATCCTGAT-3′ and 5′-GGCCTGTGACCTTGGACAGCAT-3′; CRAMP, 5′-GCTTTGGAACCATGCGTT-3′ and 5′-TTGGTGAAGTCACTCCACAGC-3′; human CCAAT/enhancer-binding protein α (C/EBPα), 5′-TCACGCGATATCAACACTTG-3′, 5′-AGTATCCGAGCAAAACCAAA-3′; human glyceraldehyde 3′-phosphate dehydrogenase (GAPDH), 5′-GGAGTCTAAGGATTTGGTGCTGA-3′ and 5′-GCAACAAATATCCACTTTACCAGGATTA-3′; and murine GAPDH, 5′-ACCTGCCATTGATGATGACATCA-3′ and 5′-GGTCTTACGTGTTGACCCCAAGAT-3′. For XBP1 analysis, both precursor and mature XBP1 mRNA expression were assessed using the TaqMan assay with primer pairs of XBP1 and probes for each form and Premix Ex Taq™ reagent (Takara, Madison, WI): 5′-CCAAGATCCCCAACAGGATA-3′ and 5′-AACGCCAGGGAATGAAT-3′; mature XBP1, 5′-dFAM 6-carboxyfluorescein acronym (FAM) GCTGAGTCCGCAGCAGGCT- TACGGACAGCT-BHQ-1-3 and 5′-dCAL® Fluor Gold 540-AACACTCGACATTGAGCATCAGC-TACGGACAGCT-BHQ-1-3. mRNA expression was normalized to levels of GAPDH.

Western Immunoblot Analysis—Western immunoblot analysis was performed as described previously (23), with modifications. Cell lysates, prepared in radioimmunoprecipitation assay buffer, were resolved by electrophoresis on 4–12% Bis-Tris Gel (Invitrogen). Nuclear and cytoplasmic fractions were prepared from cell lysates, as we described previously (26). The following primer sets were used: CAMP (hCAP18), 5′- ACAACGTGGAAGAATGAGAGGTG-3′ and 5′-ACCTGCCATTGATGATGACATCA-3′; intron CAMP, 5′-CCCTGTTGTCCATCCTGAT-3′ and 5′-GGCCTGTGACCTTGGACAGCAT-3′; mature XBP1, 5′-dFAM 6-carboxyfluorescein acronym (FAM) GCTGAGTCCGCAGCAGGCT-TACGGACAGCT-BHQ-1-3 and 5′-dCAL® Fluor Gold 540-AACACTCGACATTGAGCATCAGC-TACGGACAGCT-BHQ-1-3. mRNA expression was normalized to levels of GAPDH.

Dual-Luciferase Reporter Assay for NF-κB, C/EBP, and VDR Transcriptional Activity—Transcriptional activities of NF-κB, C/EBP, and VDR were assessed using their respective reporter luciferase constructs encoding the firefly luciferase reporter gene, as well as a constitutively expressing Renilla luciferase construct, using HilyMax (Dojindo, Rockville, MD), or anti-PARP (BD Biosciences), and detected using enhanced chemiluminescence (Thermo Fisher Scientific). The intensity of bands was measured with a LAS-3000 (Fuji Film, Tokyo, Japan).

Dual-Luciferase Reporter Assay for NF-κB, C/EBP, and VDR Transcriptional Activity—Transcriptional activities of NF-κB, C/EBP, and VDR were assessed using their respective reporter kits (SABiosciences, Frederick, MD). HaCaT KC were transfected with NF-κB-, C/EBP-, or VDR element-responsive luciferase constructs encoding the firefly luciferase reporter gene, as well as a constitutively expressing Renilla luciferase construct, using HilyMax (Dojindo, Rockville, MD). After transfection, cells were pretreated with or without 6-amino-4-quinozoline (QNZ, 100 nM) for 1 h and incubated with Tg and/or QNZ and/or 1,25(OH)₂ vitamin D₃ (100 nM) for 18 h. Promoter activ-
ity was assessed using the Dual-Luciferase assay system (Promega, Madison, WI).

siRNAs and Transfections—HaCaT KC were transfected with 20 nM siRNA for C/EBPα/H9251, C/EBPβ/H9280 (Santa Cruz Biotechnology), VDR, or non-targeted, control siRNA (Dharmacon, Lafayette, CO) using siLentFect (Bio-Rad), as described previously (27).

Statistical Analyses—Statistical comparisons were performed using an unpaired Student’s t test.

RESULTS
ER Stress Increases CAMP mRNA Expression in Human KC—We first ascertained whether activation of an ER stress response occurs in primary human and HaCaT KC after exposure to Tg, previously shown to produce ER stress by inhibiting the sarco/endoplasmic reticulum Ca2+/H1001-ATPase (28). ER stress in turn is indicated by the extent of activation of the transcriptional factor/ER stress marker, XBP1 (18). Accordingly, exogenous Tg (100 nM) accelerated conversion of the precursor to the mature (spliced) form of XBP1 mRNA in both primary human and HaCaT KC (Fig. 1A). Notably, concentrations (≤200 nM) of Tg neither decreased cell viability nor did they induce apoptosis, assessed both by trypan blue dye exclusion and by PARP cleavage, respectively (supplemental Fig. 1, A and B, respectively). Because higher Tg concentrations (500 nM) slightly decreased cell viability (supplemental Fig. 1A), we employed Tg concentrations of ≤200 nM as the ER stressor for the studies described below.

We next assessed whether Tg-mediated ER stress stimulates CAMP mRNA expression. qRT-PCR analysis revealed a significant increase in CAMP mRNA expression in both primary human KC and HaCaT KC following treatment with Tg (100 nM) (Fig. 1B).

In addition, basal CAMP expression is significantly increased in differentiated primary human KC (induced by raising Ca2+ concentration in medium) versus undifferentiated KC (Fig. 1B), whereas similar to undifferentiated KC, differentiated KC show increased CAMP mRNA expression following ER stress (Fig. 1B). Therefore, ER stress likely stimulates CAMP expression in each epidermal layer (basal through granular).

Although Tg is well known to provoke ER stress (29), we further ascertained whether increased CAMP expression is due specifically to an ER stress response. When HaCaT KC were co-treated with Tg and taurosodeoxycholic acid (TUDCA), an inhibitor of ER stress (30), the expected increases in XBP1 activation were attenuated significantly in comparison with cells treated with Tg alone; notably, TUDCA alone did not alter the extent of XBP1 activation (Fig. 1C). Finally, the Tg-induced
increase in CAMP mRNA expression was diminished in KC treated with TUDCA (Fig. 1D), suggesting that CAMP up-regulation is driven by an ER stress signal, rather than by unrelated, non-specific effects of Tg.

To further ascertain whether increased CAMP mRNA expression is initiated by ER stress, HaCaT cells were incubated with Tm, another chemically and mechanistically unrelated ER stressor, which stimulates accumulation of proteins in ER through inhibition of glycosylation (31). An increase in the mature (spliced) form of XBP1 mRNA again became evident in cells treated with Tm (Fig. 1A), whereas apoptosis did not occur at concentrations up to 960 nM (supplemental Fig. 1, A and B). Moreover, like Tg, Tm also significantly increased CAMP mRNA expression (Fig. 1B). Together, these results indicate that ER stress stimulates CAMP mRNA production in human KC.

**ER Stress Increases CAMP mRNA in Epithelial but Not in Myeloid Cells**—We next investigated whether increases in CAMP mRNA occur ubiquitously in epithelial cells (using HeLa cells and KC as models for epithelial cells), but not in non-epithelial cells, i.e. myeloid cells (using promyelocytic cells (HL-60) and monocyes (U937)). In response to Tg treatment, XBP1 activation occurred in all tested cells (supplemental Fig. 2) without evidence of cell toxicity (not shown), but CAMP mRNA expression increased significantly only in HeLa cells (Table 1). These results suggest that ER stress appears to generate signals that stimulate CAMP expression in epithelial cells, but not in myeloid cells.

**ER Stress Increases Extracellular LL-37 Levels**—CAMP must not only be synthesized but also secreted from cells and proteolytic hydrolysis must occur to generate the active antimicrobial peptide, LL-37. As shown by Western immunoblot analysis, increases in not only intracellular CAMP (cf. Fig. 3D, lanes 3 and 5 versus lane 1) but also extracellular LL-37 (~4.5 kDa) levels increase in conditioned cultured media from HaCaT KC exposed to ER stress (Fig. 2), revealing that ER stress increases not only formation but also secretion of fully functional CAMP in response to external stimuli.

**ER Stress-induced Stimulation of CAMP Expression Occurs at Transcriptional Level**—Because increased mRNA expression is either due to transcriptional up-regulation or due to increased mRNA stability (extending half-life), we next addressed which mechanism may be responsible for ER stress-induced stimulation of CAMP expression. To assess total RNA levels, which reflect bulk amounts of RNA transcript being formed, we used intron primers (not spliced RNA) (32, 33). qPCR analysis reveals that total RNA levels are increased in HaCaT KC following Tg treatment, and blockade of ER stress by TUDCA diminished increases in total RNA levels similar to that observed using the mRNA CAMP primers (Fig. 1E). These results suggest that the ER stress stimulates CAMP expression at the transcriptional level rather than via increased stability of CAMP mRNA.

**ER Stress-induced Stimulation of CAMP Expression Requires NF-κB**—Given the above results, we sought to further delineate the downstream mechanism(s) for the ER stress-dependent CAMP transcriptional up-regulation using HaCaT KC as the epithelial model. Because NF-κB-dependent gene expression is up-regulated in cells subjected to ER stress (34), we investigated the potential role of NF-κB in the regulation of CAMP expression in KC subjected to ER stress. First, Western immunoblot analysis demonstrated that NF-κB activation occurred in KC following ER stress, as assessed by phospho-NF-κB generation (Fig. 3A). Next, NF-κB transactivity, assessed by a reporter assay, was increased in HaCaT KC exposed to ER stress (Fig. 3B), in parallel with increased CAMP mRNA and protein expression (Fig. 3, C, D, and E, respectively).

However, co-incubation of HaCaT KC with Tg or Tm, along with two chemically unrelated but specific NF-κB inhibitors, QNZ or BAY11–7082 (35), diminished the expected ER stress-induced up-regulation of CAMP mRNA expression (Fig. 3C). Notably, neither QNZ nor BAY11–7082 alone altered basal CAMP mRNA expression. Western immunoblot analysis revealed that these inhibitors alone did not significantly alter CAMP protein levels (Fig. 3, D and E). Together, these results indicate that the NF-κB pathway likely plays a key role in the up-regulation of CAMP expression in KC subjected to ER stress.

**C/EBPα Is Required for ER Stress-induced Up-regulation of CAMP Expression**—The CCAAT/enhancer-binding protein (C/EBP), but not NF-κB, regulatory elements exist on the promoter region of CAMP (13), and C/EBPα transactivity
increases after either ER stress (36) or NF-κB activation (37). However, bone marrow cells from C/EBP mouse do not express CRAMP, the murine homologue of CAMP (38). Therefore, we next investigated whether C/EBP transactivation occurs following exposure of ER stress. Consistent with the prior studies noted above, total C/EBP transactivity significantly increased in HaCaT KC treated with Tg (Fig. 4A).

To further identify the responsible C/EBP transcription factor(s), we next assessed ER stress-stimulated CAMP expression, using siRNA against C/EBP in Tg-treated KC. C/EBP mRNA expression was suppressed by >60% versus scrambled siRNA (p < 0.01) in HaCaT KC exposed to ER stress. Moreover, ER stress-induced CAMP mRNA expression significantly declined in cells transfected with C/EBPα siRNA (Fig. 4B). Western immunoblot analysis further demonstrated reduced CAMP protein production in C/EBPα siRNA-transfected KC exposed to ER stress (Fig. 4C). In contrast, when C/EBPα mRNA expression was suppressed with siRNA (i.e. by ~50% versus scrambled siRNA) in HaCaT KC, ER stress-induced CAMP mRNA expression did not change (Fig. 4B).

C/EBPα Phosphorylation Is Required for ER Stress-induced Up-regulation of CAMP Expression—Because C/EBPα transactivation is also regulated by phosphorylation at both Ser-21 and Thr-222/226 (39, 40), we next assessed the levels of phosphorylated C/EBPα in HaCaT KC exposed to ER stress. Western immunoblot analysis revealed an increase in the levels of phosphorylated (both Ser-21 and Thr-222/226) C/EBPα in nuclear fractions from cells following Tg treatment (Fig. 5A). However, although phosphorylated Ser-21 returned to basal levels at 45–60 min, Thr-222/226 phosphorylated C/EBPα remained elevated in nuclear fractions at 60 min (Fig. 5A). Moreover, BAY11–7082, an inhibitor of NF-κB, significantly diminished the levels of ER stress-induced phosphorylated forms, whereas BAY11–7082 alone altered neither Ser-21 nor Thr-222/226-phospho-C/EBPα (Fig. 5B, upper panel). Additional studies were performed to ensure that the same pathway, i.e. ER stress-mediated NF-κB activation followed by increased C/EBPα phosphorylation, is also responsible for up-regulation of CAMP in primary human KC. As expected, we noted that significant increases in both Ser-21 and Thr-222/226-phospho-C/EBPα were evident following Tg treatment, whereas inhibition of NF-κB by Tg diminished these phosphorylation events (Fig. 5B, lower panel), suggesting that the same transcriptional regulation of CAMP is operative in both normal human KC and HaCaT KC.

Because mitogen-activated protein (MAP) kinases phosphorylate C/EBPα (41), we assessed the roles of these kinases in ER stress-mediated signal stimulation of CAMP expression. A p38 MAP kinase-specific inhibitor, SB203580, significantly diminished the increases in CAMP mRNA expression in HaCaT KC treated with either Tg or Tm (Fig. 5C), whereas an inhibitor of
extracellular-signal-regulated kinase (ERK) (U0126) did not attenuate the ER stress-induced increase (actually, a modest increase was noted). Together, these results suggest that C/EBPα, but not C/EBPε, accompanied by phosphorylation, serves as a key downstream, transcriptional signal for ER stress-stimulated CAMP expression (see Fig. 8) and a requirement of NF-κB for C/EBPα phosphorylation.

**VDR Activation Is Not Required for ER Stress-mediated Up-regulation of CAMP Expression**—Prior studies have demonstrated that CAMP expression is up-regulated by VDR signaling in multiple cell types, including epithelial cells (13). Therefore, we next addressed whether VDR activation occurs as a consequence of ER stress and the potential role of VDR in modulating the downstream increase in CAMP expression. As expected, exogenous 1,25(OH)₂ vitamin D₃ (100 nM) significantly increased VDR transcriptional activity in HaCaT cells, assessed by a VDR reporter assay (Fig. 6A). However, co-incubation of 1,25(OH)₂ vitamin D₃ with Tg reduced the expected, 1,25(OH)₂ vitamin D₃-induced increases in VDR transcriptional activity (Fig. 6A). Moreover, Tg-induced ER stress alone modestly decreased, rather than increasing, VDR transcriptional activity (Fig. 6A) and revealed that Tg treatment significantly decreased VDR mRNA expression in HaCaT cells (Tg, 0.63 ± 0.04; vehicle control, 1.00 ± 0.07; p < 0.01, qRT-PCR analysis).

We next assessed the potential involvement of VDR activation in the ER stress-mediated increase in CAMP mRNA expression. Expression of VDR was first reduced with a VDR-specific siRNA (decreased by ~70% versus scrambled siRNA; p < 0.01) followed by exposure of HaCaT cells to ER stress. As expected, the 1,25(OH)₂ vitamin D₃-induced CAMP expression was attenuated significantly in cells transfected with siRNA against VDR (by 38% versus scrambled siRNA-treated cells incubated with 1,25(OH)₂ vitamin D₃, Fig. 6B). However, Tg still up-regulated CAMP expression, even in VDR-silenced cells (Fig. 6B). Finally, we assessed whether inhibition of NF-κB itself alters VDR transactivation. As shown in Fig. 6A, QNZ co-treatment did not reverse the ER stress-induced suppression of VDR transactivation. Together, these results suggest that: (i) ER stress suppresses VDR transcription activity, but not via NF-κB signaling; (ii) VDR activation is not required for ER stress-mediated up-regulation of CAMP expression in KC; and (iii) ER stress-mediated increases in CAMP mRNA expression likely occur independent of the VDR-mediated regulation.

**ER Stress Increases CAMP Expression in Murine Epidermis Independent from VDR Pathway**—Prior studies have shown that a variety of external perturbants increase murine CAMP (CRAMP) production in vivo (11, 12, 42). Hence, we next ascertained whether ER stress also increases CRAMP production not only in cultured cells but also in epidermis after epicutaneous applications of Tg to intact mouse skin. As shown in Fig. 7A, topical Tg activated XBP1, an indicator of ER stress (18). In parallel, CRAMP mRNA and protein expression also increased in murine epidermis after topical Tg treatment (Fig. 7, B and C, respectively). Together, these results demonstrate that ER stress stimulates CRAMP production in whole epidermis, validating the above described in vitro studies in stressed epithelial cells, i.e. epidermal KC and HeLa cells.

Finally, although VDR-responsive element(s) have not been identified in the 5′-upstream CRAMP promoter region, it is unlikely, but still possible, that (an) unidentified VDR-responsive element(s) is (are) present and regulates (regulate) CRAMP expression via the VDR pathway. Thus, to exclude a VDR-dependent pathway stimulating CRAMP expression following ER...
stress, we employed Vdr-null mice. Again topically applied Tg (but not vehicle alone) increased CRAMP mRNA expression in WT mouse skin, whereas Vdr-null mice also showed similar up-regulation of CRAMP mRNA expression (Fig. 7D). These results suggest that a VDR-independent pathway accounts for ER stress-mediated CRAMP expression.

DISCUSSION

LL-37, produced from its precursor, CAMP, is a major, inducible AMP that plays a crucial role in defense against pathogen invasion in a variety of epithelia, including epidermis (2). Prior studies have demonstrated that external perturbations, i.e. epidermal permeability barrier defects (12), UV irradiation (11), and pathogen colonization (43), increase CAMP/LL-37 production in epidermis, perturbants that are also known to trigger ER stress (17, 19, 20). Here we employed epidermal keratinocytes as a model of epithelial cells that are continuously exposed to external perturbants in vivo and demonstrated that ER stress, induced by two chemically and mechanistically unrelated, established ER stressors, Tg and Tm (28, 31), up-regulates both CAMP expression in vitro and its murine homologue, CRAMP, in mouse epidermis. Pertinently, our present study suggests further that the ER stress-mediated stimulation of CAMP expression is due to an NF-κB-dependent mechanism. Importantly, we demonstrated that ER stress suppresses VDR transactivity in KC. Hence, the NF-κB-C/EBPα-dependent mechanism stimulates CAMP expression via its VDR-independent mechanism. Our present studies likely are consistent for other epithelial cells because ER stress/activation ubiquitously occurs in mammalian cells. Indeed, CAMP expression was significantly increased in HeLa cells in response to ER stressors. However, myeloid cells (as shown here), and probably other non-epithelial cells, are unlikely to have the same regulatory mechanism of CAMP expression (i.e. NF-κB-C/EBPα-dependent) in response to external perturbant-medi-
ated ER stress. Hence, epithelial cells, which are continuously exposed to external stress, appear to utilize ER stress as a signal to maintain the innate immune system. However, excess levels of external perturbation (24) or ER stress cause apoptosis. Accordingly, excessive ER stress, as occurs following lengthy exposure to Tg (or high concentrations of Tg) leads to cell death and inflammation, as well as contributing to disease pathogenesis (44). However, lower levels of ER stress are essential for normal cellular functions in professional secretory cells, i.e. B cells, pancreatic β cells, and hepatocytes (45). ER stress-dependent signals also regulate KC differentiation (46). The present study demonstrates further that relatively low levels of ER stress stimulate production of CAMP, suggesting that ER stress stimulates the restoration of epithelial innate immune function after external perturbation.

Our studies demonstrated that increases not only in CAMP mRNA and protein expression, but also in CAMP secretion from cells and its proteolytic processing to LL-37, were required for antimicrobial activities in cells exposed to ER stress. Although the regulatory mechanisms for CAMP secretion and proteolysis to generate active LL-37 have not been elucidated, the present studies reveal that ER stress stimulates the restoration of epithelial innate immune function after external perturbation.

In contrast to human CAMP, the promoter region of murine CRAMP gene, a homologue of CAMP, contains both NF-κB and C/EBP (but not VDR) binding consensus sequences (13). A recent study also shows the requirement of NF-κB for lipopolysaccharide (LPS)-induced stimulation of CRAMP in murine mast cells (47). Although we demonstrated that ER stress increases CRAMP expression in murine epidermis ex vivo, it remains to be resolved whether the C/EBPα signal is also
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**FIGURE 8. Proposed mechanism of ER stress-mediated induction of CAMP in human KC.** A VDR-dependent pathway has been shown previously to regulate CAMP expression in human cells including KC (13). In addition to the VDR-dependent pathway, ER stress regulates CAMP via a novel NF-κB-C/EBPα signal in human KC. Both pathways are likely to alternatively regulate CAMP expression depending upon cellular environments: e.g. non-stress versus low levels of stress. VDRE, VDR element; RXR, retinoid X receptor.

required for the regulation of CRAMP under non-stressed conditions. Nevertheless, our studies suggest that ER stress regulates CAMP via an NF-κB-C/EBPα signal in human KC (and likely for its murine homologue, CRAMP).

Pertinently, recent studies suggest that Th2 cytokines, i.e. IL-4 or IL-13, down-regulate CAMP expression via the activation of NF-κB signal, whereas blockade of the NF-κB pathway enhances 1,25(OH)2 vitamin D3-induced up-regulation of CAMP expression in KC (48). Thus, NF-κB plays a pivotal role in regulating CAMP expression. In addition, although it is likely that NF-κB and VDR pathways are not operating simultaneously in response to ER stress, both pathways could alternatively regulate CAMP expression depending upon cellular environments (Fig. 8), i.e. under stressed versus under basal (unperturbed/un-stressed) conditions in epithelial cells. Pertinently, the increased level of CAMP expression following ER stress is nearly equivalent to the magnitude in response to 1,25(OH)2 vitamin D3, suggesting divergent physiological versus perturbant-induced regulation of CAMP expression. Thus, the NF-κB-C/EBPα pathway could be important to restore and/or to enhance the antimicrobial defense barrier in epithelial cells exposed to external perturbations, which likely result in ER stress in cells. However, it is important to note that blockade of NF-κB activation using a specific pharmacological inhibitor (QNIZ) does not alter the down-regulation of VDR transactivation by ER stress (Fig. 6). Hence, the ER stress-mediated inhibition of VDR activity is likely due to other ER stress signals rather than an NF-κB-dependent signal. Together, these results reveal that ER stress-mediated up-regulation of CAMP represents a previously unidentified VDR-independent and NF-κB-dependent pathway.

The C/EBPs are a family of basic leucine zipper transcription factors that serve to regulate cell growth and differentiation, including that in KC (46). C/EBPα transactivity is regulated by phosphorylation (Ser/Thr), whereas phosphorylation of these sites has been shown to be catalyzed by MAP kinases (40, 49), including signal-regulated kinase (ERK)1/2 and p38 MAP kinase. We demonstrate here that C/EBPα phosphorylation by these kinases is activated by NF-κB activation and is required for CAMP up-regulation in response to ER stress. Importantly, prior studies demonstrate that Ser-21 phosphorylation down-regulates transcriptional activities due to conformational changes of C/EBPα structure (40). Our results revealed that Thr-222/226 phosphorylation remains elevated following ER stress (versus Ser-21 phosphorylation, which is increased acutely but normalizes), suggesting that the decreases in Ser-21 phosphorylation could contribute to the up-regulation of CAMP expression. In addition, because ERK kinase inhibition does not suppress the ER stress-induced stimulation of CAMP expression (it even increases it, as shown in Fig. 5), ERK kinase may catalyze Ser-21 phosphorylation and serve as a negative regulator of CAMP expression. Further details of roles/requirements for specific phosphorylation sites on C/EBPα as well as the specific kinase(s) responsible for the increased CAMP expression remain unresolved.

Interestingly, the expression of C/EBPα, which is required for granulocyte and monocyte development, is itself regulated by C/EBPα (50), whereas C/EBPα-knock-out mice do not express CRAMP in bone marrow cells (expression in other cells and tissues was not examined) (13, 38). However, our present studies demonstrate that C/EBPα does not directly regulate ER stress-mediated induction of CAMP in human KC. Moreover, we showed that myeloid cells do not increase CAMP expression following ER stress. Hence, a role of EBPα or C/EBPα in regulation of CAMP expression appears to be cell/tissue-specific and/or different expression profiles of coactivators/corepressors required for transcriptional regulation.

Finally, ER stress is known to generate unique cellular signaling pathways, i.e. 1) the inositol-requiring enzyme 1 (IRE1)-tumor necrosis factor receptor-associated factor 2 (TRAF2) pathway, 2) the PKR (protein kinase R)-like ER kinase (PERK)-eukaryotic initiation factor 2α (eIF2α) pathway, and/or 3) the activating transcription factor 6 (ATF 6)-AKT (or protein kinase B) pathway. All these, in turn, activate the NF-κB signaling pathway (51). Our present studies now show further that NF-κB activation accounts for transcriptional up-regulation of CAMP in KC subjected to ER stress. Although it is possible that this ER stress-induced NF-κB activation is due to the IRE1, PKR-like ER kinase, and/or ATF 6 pathway(s), our recent studies reveal that ER stress-induced production of a certain class of sphingolipid species stimulates the NF-κB-C/EBPα pathway, resulting in increased CAMP expression in KC. Therefore, ER stress-mediated CAMP expression may involve a unique lipid signaling pathway in epithelial cells.

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3 K. Park and Y. Uchida, unpublished data.
