Calcitermin, a novel antimicrobial peptide isolated from human airway secretions

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Abstract The human airways are protected from pathogenic colonization by a blanket of fluid impregnated with innate antimicrobial effector molecules. Among several previously uncharacterized components, we isolated a peptide that had activity primarily targeting Gram-negative bacteria. We named the peptide 'calcitermin' since its amino acid sequence and mass were equivalent to the 15 C-terminal residues of the S100 protein, calgranulin C. The antimicrobial activity of calcitermin was enhanced in acidic buffers (pH 5.4) and in the presence of micromolar concentrations of $ZnCl_2$. Analysis revealed a putative zinc-binding consensus sequence as well as an α-helical conformation in structure-promoting solvents.

Key words: Antimicrobial peptide; Airway; Secretion; Innate immunity; Calgranulin; S100

1. Introduction

Antimicrobial peptides are widely distributed molecular effectors of innate immunity. Several families of peptides exist that display similar mechanisms of action against microbes. Many are broad-spectrum microbicides that target Gram-positive and Gram-negative bacteria as well as fungi and some enveloped viruses [1–3]. Antimicrobial peptides share cationic charge and hydrophobicity at physiologic pH, features that facilitate peptide binding and insertion into microbial membranes. Some peptides then aggregate to form pores, and death ensues once a critical number of pores have formed [4,5].

Epithelia in the human airways, from the nasal aperture to the alveoli, are covered in a protective film of serous and mucous fluid containing a number of antimicrobial proteins, including lysozyme, lactoferrin and secretory leukoproteinase inhibitor (reviewed in [3]). Recently, two β-defensin antimicrobial peptides (HBD-1 and HBD-2) and one cathelicidin (LL-37) have been found in fluid from the lower and upper airways [6–8]. Additionally, neutrophils can be recruited into the airways during infection and inflammation and secrete molecules such as the abundant α-defensins (HNPs) [9–11]. The combined effects of antimicrobial proteins and peptides within the airways contribute to an effective antibiotic shield.

The S100 family of calcium-binding proteins currently comprises 19 members that are differentially expressed in a variety of tissues. The activities of S100 proteins vary widely and include calcium-dependent roles in inflammation and the regulation of cellular processes, including proteins phosphorylation, cell proliferation, structural membrane and cytoskeletal organization [12]. Calgranulin, which belong to the S100 family, have been shown to be antimicrobial. Calgranulin A and B from the heterodimer calprotectin, with demonstrated fungicidal and bactericidal activities [13,14]. Calgranulin C is a newly described member of the family produced by neutrophils and monocytes [15] and keratinocytes [16,17] and has only been reported to have antiparasitic activity [16]. The overexpression of calgranulin C in lesional psoriasis suggests a role in inflammatory host defense [18].

From our initial observations, human airway secretions contain a number of uncharacterized antimicrobial molecules. We therefore probed for (poly)peptides that contribute to the overall microbicidal activity of human nasal fluid. We report here the discovery and characterization of a novel 15-residue antimicrobial peptide, which is a putative C-terminal cleavage fragment of calgranulin C.

2. Materials and methods

2.1. Isolation and purification of antimicrobial peptides from human nasal secretions

Nasal secretions were collected from healthy volunteer donors according to a protocol approved by the UCLA Institutional Review Board. From most donors, nasal secretions were collected by vacuum-aided suction, without chemical stimulation (described in [11]), to avoid introducing foreign substances into the nasal fluids. Gentle manipulation of a narrow catheter tip inside the nasal passageways mildly stimulated nasal secretions. 40 ml of nasal fluid were solubilized by 1:5 dilution in 5% acetic acid for 18 h at 4°C. Fluid was cleared by centrifugation at $21\,000 \times g$ for 15 min and the resulting supernatant was applied to SepPak Vac 15-*g* C_{18} cartridges (12% carbon, 12.5-nm pore size, 80-μm particle size), and eluted with 40% acetonitrile/0.1% trifluoroacetic acid (TFA). The vacuum-concentrated eluate was resuspended in 0.01% acetic acid, and subjected to strong cation-exchange HPLC (Vydac, The Separations Group, Hesperia, CA, USA, 5-μm particle size, 9.8×100 mm; linear AB gradients where A is 5% acetonitrile/25 mM NaP, pH 6.0, and B is 5% acetonitrile/25 mM NaP, pH 5.0/1 M NaCl, with a 60-min gradient at

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16.7 mM NaCl min^{-1} and 1 ml min^{-1}). Peak fractions that exhibited antimicrobial activity by radial diffusion assay (RDA) were applied to a Vydac 218TP C18 (5-μm particle size, 4.6×250 mm) reverse-phase HPLC column (linear AB gradient where A is H_2O/0.1% TFA, and B is acetonitrile/0.1% TFA, with a 120-min gradient at 0.5% acetonitrile min^{-1} and 1 ml min^{-1}). Peak fractions were further purified on a microbore Vydac C18 column (5-μm particle size, 2.3×100 mm) using a linear three-ramp gradient of water–ACN (0.3 ml min^{-1}; see Fig. 1A). A single peak eluting at 30% ACN contained calcitermin. Matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF) and N-terminal Edman microsequencing of purified natural calcitermin were performed at the Emory University School of Medicine Microchemistry Facility (Jan Pohl, Ph.D., Director).

2.2. Calcitermin synthesis and purification

Peptides were synthesized at a 0.25-mmol scale with a Perkin-Elmer ABI 431 A Synthesizer, using pre-derivatized polyethylene glycol polystyrene glutamic acid resin (PerSeptive Biosystems, Framingham, MA, USA), FastMoc™ chemistry [19], and double coupling for all residues. The peptide was then purified by reverse-phase HPLC. Since calcitermin contains a tyrosine residue, its concentration was determined using an extinction coefficient of 1490 M^{-1} cm^{-1} [20].

2.3. CD spectroscopy

Circular dichroism (CD) measurements were performed with an AVIV 62DS spectropolarimeter (AVIV Associates, Lakewood, NJ, USA) that was calibrated with (+)-10-camphorsulfonic acid (1 mg ml^{-1}) in a 1-mm-path length cell [21]. The peptide–solvent solutions were measured in 0.1-mm light path demountable cells that were scanned from 260 nm to 190 nm at a rate of 10 nm min^{-1} with 0.2-nm intervals. The results were expressed as the mean residual ellipticity (MRE), $[\Theta]_{MRE}$ (degree cm^2 $dmol^{-1}$).

2.4. Atomic absorption

Nasal secretions were sonicated using a microtip dismembranator for 20 s, and 'wet-ashed' in concentrated HNO_3 (trace metal grade, Fisher Scientific): 100 μl of each sample received 900 μl of acid. These sat at ambient temperature overnight. For quality assurance, a reconstituted human serum standard (Utak Bi-Level Trace Elements, Utak Laboratories Inc., Valencia, CA, USA) and methods blanks were analyzed simultaneously. After acid treatment, 100 μl of each sample was diluted with $Mg(NO_3)_2$ matrix modifier to a volume of 1.0 ml and analyzed in a Perkin-Elmer 503 atomic absorption spectrophotometer (Foster City, CA, USA) fitted with a graphite furnace atomizer and a autosampler set for 25-μl sample injections. The minimum detection level (MDL), calculated as 3×standard deviation of method blanks, was 0.98 ng ml^{-1}. The human serum standard was 95±5 ng ml^{-1}, well within the acceptable range of 94±19 ng ml^{-1}.

2.5. Microbes and culture conditions

Escherichia coli ML-35p, *Listeria monocytogenes* EGD, *Staphylococcus aureus* (human clinical isolate, UCLA Clinical Microbiology Facility), *Candida albicans* (human clinical isolate), and *Staphylococcus epidermidis* (human clinical isolate, used for in vitro assays), were cultured 18 h at 37°C in 50 ml trypticase soy broth (TSB = 3% weight per water volume (w/v); all strains described in [22]). The *Pseudomonas aeruginosa* strain is a mucoid isolate [23] from a cystic fibrosis patient (isolate generously donated by Dr. M.J. Welsh, University of Iowa, IA, USA). Immediately prior to use each strain was subcultured in 50 ml TSB at either 1:100 (*L. monocytogenes*, *S. aureus*, *S. epidermidis*, *C. albicans* and *P. aeruginosa*) or 1:1000 (*E. coli*) dilution for 2.5 h at 37°C in an environmental shaking incubator (250 rpm) to obtain microbes in mid-logarithmic growth phase. Subcultures were then centrifuged at 1400×*g* for 10 min, washed once in 10 mM sodium phosphate (pH 7.4 or pH 5.4), and diluted to their desired concentrations in the same buffer. For bacteria, an $OD_{625} = 1.0$ was equivalent to 2.5×10^8 CFU ml^{-1}. For fungi, an $OD_{450} = 1.0$ was equivalent to 2.5×10^8 CFU ml^{-1}.

2.6. Gel overlay assay

The gel overlay assay was performed as described previously [24]. Briefly, acetic acid-extracted nasal fluids were separated by acid–urea-polyacrylamide gel electrophoresis (AU-PAGE). The gel was washed 20 min in 10 mM sodium phosphate, pH 7.4, then placed on a premade 1% agarose plate containing 10 mM sodium phosphate with 100 mM NaCl, pH 7.4, 1% TSB (0.03% w/v), and 4×10^6 *L. monocytogenes* or *E. coli*. The plate was then incubated at 37°C for 3 h to allow the proteins and peptides in the polyacrylamide gel to diffuse into the underlying bacterial layer. The polyacrylamide gel was then removed and the bacterial layer was overlaid with a nutrient layer that contained double-strength TSB (6% w/v) in 1% agarose. Clear zones without bacterial growth represented antibacterial activity. Duplicate AU-PAGE gels were electrophoresed and Coomassie-stained to correlate antibacterial activity with protein bands.

2.7. RDA

RDA were performed as described previously [24,25]. Briefly, the underlayer consisted of 1% agarose and 1% TSB (0.03% w/v) in 10 mM sodium phosphate buffer, pH 5.4. Overlay consisted of double-strength TSB and 1% agarose in 10 mM sodium phosphate buffer, pH 5.4. 4×10^6 bacteria or fungi were mixed with 10 ml of underlayer gel solutions kept molten at 48°C and poured into 100 cm^2 square petri dishes. A series of 3.2-mm diameter wells were punched after the agarose solidified, and 5 μl of calcitermin was added into designated wells. Plates were incubated at 37°C for 3 h to allow for peptide diffusion. The microbe-laden underlayer was then covered with 10 ml of molten overlay, and the plates were allowed to harden. Antimicrobial activity was identified as a clear zone around the well absent of microbial growth after 18 h incubation at 37°C. The activity was represented in radial diffusion units (RDU) defined as: (diameter of clear zone in mm – 3.2 mm)×10. Assays were performed in duplicate and repeated at least once.

2.8. Colony-forming unit (CFU) assay

Bacterial ($OD_{625} = 0.2$) or yeast ($OD_{450} = 1.0$) were diluted 100-fold in 10 mM sodium phosphate buffer (pH 5.4 or pH 7.4) for use in a microassay. The test sample consisted of 3 μl of either bacterial or yeast dilution plus 12 μl of calcitermin in 10 mM sodium phosphate buffer (pH 5.4 or pH 7.4)+1.3% TSB for each condition, which allowed triplicates of two time points (0- and 3-h incubation). Separate tubes with 3 μl bacteria or yeast and 12 μl of buffer/1.3% TSB were used as controls for microbial growth. Each well of a 72-well Terasaki microtiter plate (Nalge Nunc, International, Denmark) was loaded with 1.5 μl liquid wax (MJ Research, Watertown, MA, USA) to prevent evaporation. 2 μl of the test sample was loaded into each of six wells by pipetting directly underneath the liquid wax. The entire plate was incubated at 37°C. To recover the incubated fluid at the specified time points, wells were washed thoroughly with 45 μl of buffer and the wash was placed on ice in a microcentrifuge tube. The fluid was then plated on tryptic soy agar (TSA) plates to count CFUs the following day.

3. Results

3.1. Human nasal secretions contain unidentified antimicrobial peptides

In our analysis of the natural antimicrobial properties of minimally manipulated nasal secretions [11] we used a gel overlay technique that imprints proteins, electrophoresed by AU-PAGE, onto a lawn of *E. coli* or *L. monocytogenes*. Multiple microbicidal bands were detected that did not correspond to known antimicrobial (poly)peptides (data not shown). Reducing the ionic strength further increased the number of polypeptide bands that were measurably antimicrobial by gel overlay analysis (data not shown). The pattern of activity depended on the target microbe species. These initial studies served as the impetus to uncover novel antimicrobial polypeptides in human nasal secretions.

3.2. Isolation of an antimicrobial peptide from nasal secretions

Human nasal secretions were analyzed for antimicrobial peptides by methods successfully used for fish mucus secretions [26] and amphibians [27]. Acidified nasal secretions were subjected to batch reverse-phase separation (C18 SepPak), cation-exchange HPLC, and two rounds of reverse-phase

HPLC. The chromatogram from the final reverse-phase HPLC purification is shown in Fig. 1A, with the arrow indicating an antimicrobial peak fraction as tested by RDA against *E. coli* (10 mM sodium phosphate, pH 5.4). This fraction was subjected to mass spectrometry (Fig. 1B) yielding a mass of 1688.9 Da. Protein microsequencing revealed the full amino acid sequence shown in Fig. 1C. The measured mass (1688.9 Da) corresponded precisely to the theoretical mass of the amino acid sequence (1688.95 Da), and was 100% identical to the terminal 15 residues of calgranulin C (Fig. 1C) (calgranulin C SwissProt Accession #P80511). We thus named this peptide, 'calcitermin'.

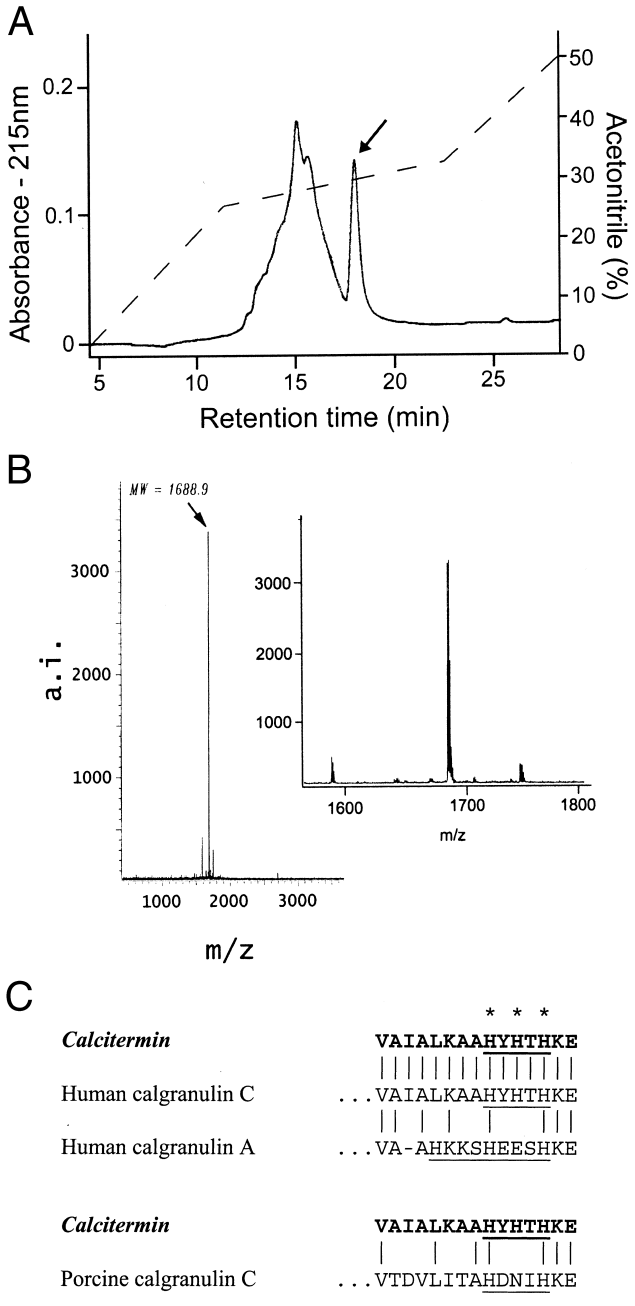

Fig. 1. Purification of an antimicrobial peptide from human nasal secretions. A: Absorbance at 215 nm for the final reverse-phase HPLC purification. Antimicrobial peptide is represented by an arrow. Acetonitrile gradient is given by a dashed line. B and inset: MALDI-TOF mass spectroscopy of the native peptide indicating the major product from purification was 1688.9 Da. The sequences of calcitermin, and the terminal 14 or 15 residues of human calgranulin A and C and porcine calgranulin C are shown in C. Identical residues are connected by vertical lines. Calcitermin was 100% identical to human calgranulin C, 53% identical to human calgranulin A, and 47% identical to porcine calgranulin C. Asterisks indicate histidine residues, and underlined residues indicate zinc-binding consensus sequences (HXXXH).

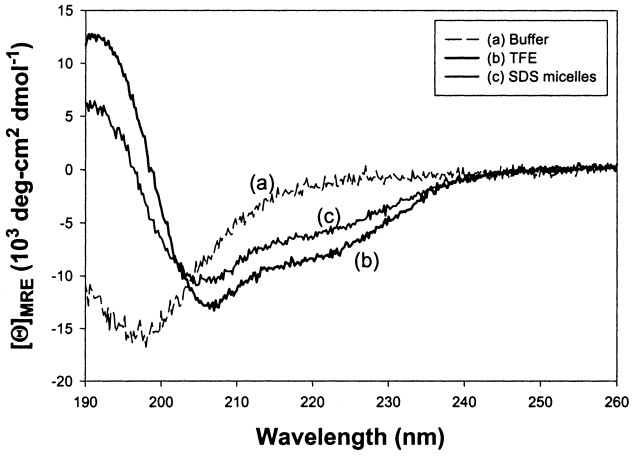

Fig. 2. CD spectrometry. CD was measured in 25 mM sodium phosphate buffer, pH 6.0 (curve a), 25 mM sodium phosphate, pH 6.0+50% trifluoroethanol (curve b), and 10% SDS+10 mM sodium phosphate, pH 6.0 (curve c). Note the transition from a random coil (curve a) to α-helical spectra (curves b and c).

3.3. Synthesis and conformational studies

We synthesized calcitermin using FastMoc™ chemistry and purified the peptide to >98% using C18 reverse-phase HPLC. After this step, the peptide appeared homogeneous and its mass, as confirmed by MALDI-TOF mass spectrometry measurement (1688.7 Da), agreed well with its theoretical mass (1688.95 Da). Synthetic and native calcitermin (5 μl of a 355-μM solution) were each tested using a RDA against *E. coli* (10 mM sodium phosphate, pH 5.4) and found to be equally antimicrobial (data not shown). Subsequent analyses were performed on synthetic calcitermin.

In CD spectroscopy measurements, α-helical secondary structures were revealed by double dichroic minima at 208 and 222 nm [28,29]. Our studies revealed a largely random coil structure in 25 mM sodium phosphate, pH 6.0 (Fig. 2). Once structure-promoting membrane mimetics were added, such as 50% trifluoroethanol (induces helical conformations) or 10% SDS micelles, calcitermin showed enhanced α-helical conformation (Fig. 2). The transition from random coil to α-helix has been shown by CD spectroscopy of other linear human antimicrobial peptides, including the cathelicidin LL-37 [30].

3.4. Antimicrobial activity of calcitermin is enhanced at acidic pH and in the presence of zinc

Calcitermin (1–50 μg ml^{-1}) was tested in a CFU assay against *E. coli*, *P. aeruginosa*, *C. albicans*, *S. epidermidis*, *S. aureus*, and *L. monocytogenes*. When assayed in 10 mM sodium phosphate buffer, pH 7.4, calcitermin could not kill *E. coli* (Fig. 3B) or any of the other strains tested (data not shown). However, when the acidity of the medium was increased (10 mM sodium phosphate, pH 5.4), 50 μg ml^{-1} cal-

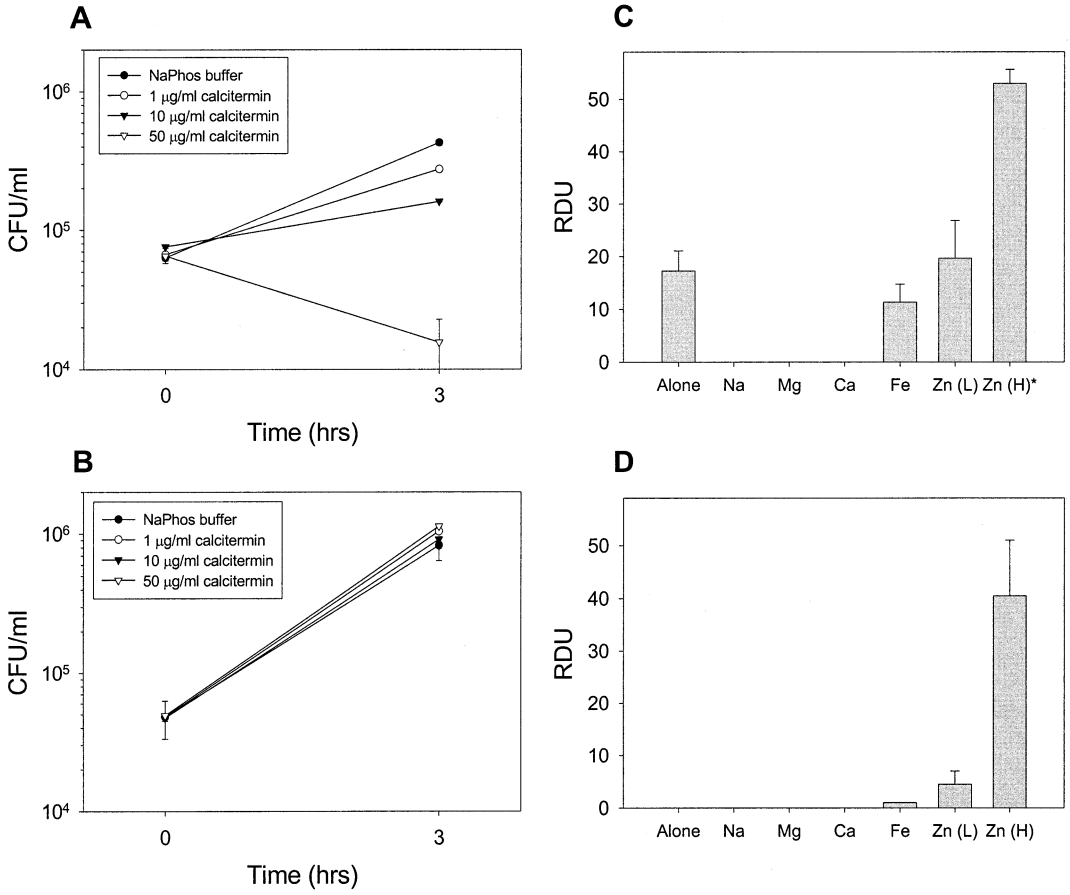

Fig. 3. Antimicrobial activity of calcitermin is dependent on pH and cations. CFU assays were performed with 0, 1, 10 or 50 μg ml^{-1} calcitermin against *E. coli* in 10 mM sodium phosphate at (A) pH 5.4 and (B) pH 7.4. Calcitermin was microbicidal only in mildly acidic conditions. Error bars represent S.E.M. ($n=3$). The effect of cations on the antimicrobial activity of calcitermin was also tested. Radical diffusion assays were performed on 5 μg of calcitermin against (C) *E. coli* and (D) *L. monocytogenes* either alone (in 10 mM sodium phosphate, pH 5.4) or in the presence of 100 mM NaCl (Na), 1 mM $MgCl_2$ (Mg), 1 mM $CaCl_2$ (Ca), 1 mM $FeC_6H_5O_7$ (Fe), 10 μM $ZnCl_2$ (Zn (L)), 100 μM $ZnCl_2$ (Zn (H)). Lower (L) and higher (H) concentrations of $ZnCl_2$ dramatically increased the activity of calcitermin. Asterisk indicates that 100 μM $ZnCl_2$ alone was weakly antimicrobial (<10 RDU). Error bars represent S.E.M. ($n=3$).

citermin was active against *E. coli* (Fig. 3A) and *P. aeruginosa* and *C. albicans*, but not *S. aureus* and *L. monocytogenes* (data not shown). As reported previously with clavanins [22], reducing the pH protonates calcitermin's three histidine residues. Since the pH of inflammatory fluid often becomes acidic [31], the concomitant increase in cationic charge likely enhances the peptide's interaction with negatively charged groups on bacterial surfaces.

We then tested the activity of calcitermin in a sensitive RDA. Similar to CFU assays, calcitermin was not active against any tested microorganisms in 10 mM sodium phosphate, pH 7.4 (data not shown), and only moderately active against *E. coli* (Fig. 3C) and inactive against *L. monocytogenes* (Fig. 3D) in 10 mM sodium phosphate, pH 5.4. The addition of 100 mM NaCl, 1 mM $MgCl_2$ 1 mM $CaCl_2$, or 1 mM $FeC_6H_5O_7$ reduced or totally inhibited the active of calcitermin against *E. coli* and *L. monocytogenes* (Fig. 3C,D). However, since calcitermin contains a putative zinc-binding domain (Fig. 1C), we admixed calcitermin and either 10 μM or 100 μM $ZnCl_2$ prior to analysis by RDA. $ZnCl_2$ increased the antimicrobial activity of calcitermin against *E. coli* (Fig. 3C) and most notably against *L. monocytogenes* (Fig. 3D). However, $ZnCl_2$ did not affect the activity of calcitermin against *C. albicans* (data not shown).

Utilizing atomic absorption, we next measured the concentration of zinc in human nasal fluids to determine if zinc levels in vivo were high enough to synergize with calcitermin. Three types of donors were tested: healthy donors ($n=5$), cystic fibrosis patients ($n=4$), and nasal carriers of *S. aureus* ($n=3$). Zinc concentrations ranged from below the detection limit (<0.98 ng ml^{-1}) to 83 ng ml^{-1}, with the cystic fibrosis samples at the high end. However, we did not detect significant differences among the three groups of donors (Student's *t*-test with Bonferroni adjustment; individual datapoints not shown). It is notable that 83 ng ml^{-1} (12.8 μM) zinc is sufficient to synergize with calcitermin against *L. monocytogenes*, which required only 10 μM $ZnCl_2$ for synergy in our in vitro assays (Fig. 3D).

4. Discussion

Human airways are exposed to colonization by inhaled pathogens, and thus the innate defense mechanisms in the airways must be highly effective. Many antimicrobial polypeptides are present in the airways, including larger and more abundant molecules, such as lysozyme and lactoferrin, and smaller and less abundant moieties, such as defensins. However, our studies indicated that additional as yet uncharacter-

ized antimicrobial substances contributed to the activity of nasal fluid [11].

Although calcitermin is the first antimicrobial fragment of a larger parent protein discovered in upper airway secretions, several studies have reported that segments of proteins have potent antimicrobial activity. Lactoferricin, a pepsin-digested fragment of lactoferrin, is broadly antimicrobial and has been shown to synergize with other polypeptides such as lysozyme [32]. Fragments of lysozyme have been reported to contain direct antibacterial activity independent of its muramidase action [33]. In fact, the calgranulin family of proteins may actually be pro-proteins similar to inactive pro-defensins and pro-cathelicidins that, when enzymatically cleaved, liberate active defensin and cathelicidin molecules, respectively [3]. Interestingly, histone fragments have been known for decades as potent cationic antimicrobials [34–36]. We also detected histone fragments in nasal secretions (A.M.C. and T.G., unpublished data), lending credence to the role of processed proteins in innate host defense.

The increase in activity of calcitermin may result from a zinc-induced conformational change that stabilizes peptide structure. Indeed, the structure and moderate microbicidal activity of calcitermin are reminiscent of the cationic, histidine-rich human salivary histatins. Most histatins contain at least one C-terminal zinc-binding consensus sequence, and through NMR studies zinc has been demonstrated to bind the zinc-binding consensus sequence of histatin-5 [37,38]. Two-dimensional NMR and Fourier-transformed infrared and CD spectroscopy indicate that histatins can form membrane-induced α-helical conformations [39]. As with calcitermin, the antimicrobial activities of several histatins are low at neutral pH and increase at acidic pH [40]. However, histatins have not been identified in the airways. Calcitermin may serve as a comparable sentinel molecule of the airways with a role similar to that of histatins in saliva.

Like calgranulin C, calgranulins A and B also contain zinc-binding domains in their C-terminal sequence. However, zinc chelation, rather than zinc binding, has been reported to increase the activity of calprotectin (heterodimer of calgranulins A and B) [41–43].

Up to 5% of neutrophil cytosolic proteins are calgranulins, and these proteins are released upon death and lysis of neutrophils at inflammatory sites [44,45]. The concentration of calgranulins in abscess fluids can approach 20 mg ml^{-1} [42]. Not only does the inflammatory fluid provide abundant precursors for the generation of calcitermin, but the pH often becomes acidic favoring the activity of calcitermin. Under these or similar conditions, calcitermin may contribute to the antimicrobial activity of body fluids.

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