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Neutrophil-derived Alpha Defensins control
Inflammation by inhibiting Macrophage mRNA Translation.

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Neutrophils are the first and most numerous cells to arrive at the site of an inflammatory insult and amongst the first to die. We previously reported that alpha-defensins, released from apoptotic human neutrophils, augmented the antimicrobial capacity of macrophages whilst also inhibiting the biosynthesis of pro-inflammatory cytokines. In vivo, alpha defensin administration protected mice from inflammation, induced by thioglycollate induced peritonitis or following infection with S. enterica serovar Typhimurium. We have now dissected the anti-inflammatory mechanism of action of the most abundant neutrophil α-defensin, Human Neutrophil Peptide 1 (HNP1). Herein we show that HNP1 enters macrophages and inhibits protein translation without inducing the unfolded-protein response or affecting mRNA stability. In a cell-free in vitro translation system, HNP1 powerfully inhibited both cap-dependent and cap-independent mRNA translation, whilst maintaining mRNA polysomal association. This is the first demonstration of an eobiotic peptide released from one cell type (neutrophils), directly regulating mRNA translation in another (macrophages). By preventing protein translation, HNP1 functions as a ‘molecular brake’ on macrophage driven inflammation; ensuring both pathogen clearance and the resolution of inflammation with minimal bystander tissue damage.

Introduction

Neutrophils, via the release of key inflammatory mediators, convey signals to practically all other immune cells, orchestrating both the innate inflammatory and subsequent adaptive immune responses (1). Through the de novo generation of lipid mediators they are also key players in the resolution of inflammation [reviewed in (2)]. Following neutrophil apoptosis, their subsequent uptake by human monocyte derived macrophages (HMDMs) induces complex phenotypic changes, including the secretion of multiple cytokines from activated HMDMs, for antimicrobial peptides, (HMDMs) induce complex phenotypic changes, including the subsequent uptake by human monocyte derived macrophages whilst also inhibiting the biosynthesis of pro-inflammatory cytokines. In vivo, alpha defensin administration protected mice from inflammation, induced by thioglycollate induced peritonitis or following infection with S. enterica serovar Typhimurium. We have now dissected the anti-inflammatory mechanism of action of the most abundant neutrophil α-defensin, Human Neutrophil Peptide 1 (HNP1). Herein we show that HNP1 enters macrophages and inhibits protein translation without inducing the unfolded-protein response or affecting mRNA stability. In a cell-free in vitro translation system, HNP1 powerfully inhibited both cap-dependent and cap-independent mRNA translation, whilst maintaining mRNA polysomal association. This is the first demonstration of an eobiotic peptide released from one cell type (neutrophils), directly regulating mRNA translation in another (macrophages). By preventing protein translation, HNP1 functions as a ‘molecular brake’ on macrophage driven inflammation; ensuring both pathogen clearance and the resolution of inflammation with minimal bystander tissue damage.

macrophages | α-defensins | mRNA translation | inflammation

Significance

Neutrophils are the major effectors of acute inflammation responding to tissue injury or infection. The clearance of apoptotic neutrophils by inflammatory macrophages also provides a powerful pro-resolution signal. Apoptotic or necrotic neutrophils also release abundant amounts of the antimicrobial peptides, alpha defensins. In this report we show that the most abundant of these peptides, HNP1 profoundly inhibits protein translation. It achieves this without affecting mRNA stability or by preventing mRNA polysomal association. This is the first demonstration of a peptide released from one cell, a leukocyte, entering and directly modulating the translation of another cell. It alludes to a novel mechanism, driven by dying neutrophils, that ensures the timely resolution of macrophage driven inflammation, without compromising antimicrobial function.

Reserved for Publication Footnotes
IL-6, IL-8 and IL-1 simultaneously inhibited the production of multiple cytokines (TNF-α, IL-6, IL-8 and IL-1β) (5). HNP1 also inhibited TNF-α, IL-1β and IL-8 production by HMDMs stimulated with the TLR7 agonist R848 (1μg/mL) (A) or with 3μg/mL CD40L + 5ng/mL IFNγ (B) for 18 hrs. TNFα (A), IL6 and IL1β (B) were assayed by ELISA. (C-D) HMDMs stimulated with R848 (1μg/mL) or the mutant peptides LHNP, W26A or Melle at the same (C) or variable concentrations (D). TNFs assayed by ELISA after 18 hours. Representative of five independent experiments. One way ANOVA with Dunnett’s multiple comparison tests. *P<0.01, **P<0.005. (E) Methionine starved HMDMs were then cultured with 10μg/mL 35S-methionine, or methionine +/- activation (with 3μg/mL CD40L and 5ng/mL IFNγ), and +/- addition of HNP1 (25μg/mL) for 4 or 18 hours. Secreted and intracellular proteins were resolved by SDS-PAGE. Phosphorimaging of radiolabelled cellular and secreted protein gels show de novo protein synthesis. (F) De novo protein synthesis of 35S-Methionine labeled proteins following 18 hours of culture, quantified by scintillation counting and normalised to untreated controls. N=3. Error bars represent the mean ±SEM; **P<0.01, *P<0.005 (Tukey’s post hoc test following a one-way ANOVA).

We wished to understand how α-defensins could simultaneously function as an effective antimicrobial antibiotic, whilst also inducing profound changes in HMDM gene expression. We report here that HNP1 enters HMDMs, where it profoundly inhibits protein translation in both resting and activated macrophages, without affecting mRNA stability or turnover. Instead it abrogates mRNA translation without affecting mRNA polysomal association.

Results

HNP1 inhibits the synthesis of proteins, which is dependent on HNP1 tertiary structure. We have previously shown that whilst alpha defensins augmented the macrophage’s ability to kill intracellular Pseudomonas aeruginosa, these peptides simultaneously inhibited the production of multiple cytokines (TNFα, IL-6, IL-8 and IL-1β) (5). HNP1 also inhibited TNFα biosynthesis from HMDMs stimulated with the toll-like receptor 7/8 (TLR7/8) agonist R848 [Fig 1A]. The biosynthesis of IL-6 and IL-1β induced via the T cell surrogate stimulus CD40L/IFNγ, was also reduced [Fig 1B], confirming that disparate stimuli and multiple secreted proteins were susceptible to HNP1-mediated inhibition. The structure of HNP1 was crucial for its cytokine inhibitory potential. When the intra-molecular disulphide bonds that stabilize the triple-stranded beta-sheet structure of HNP1 were disrupted (L-HNP), or when dimerization was prevented by replacing the tryptophan residue at position 26 with the non-polar amino acid alanine (W26A) (16), a complete loss of cytokine inhibitory potential was seen [Fig 1C and (5)]. In contrast N-Methylation of Ile20 (Melle), (which also prevents dimerization), had a minimal effect on the ability of HNP1 to inhibit R848-induced TNFα production by HMDMs [Fig 1C and 1D].

To test if HNP1 might inhibit protein synthesis per se, stimulated HMDMs were labelled with 35S-methionine in the presence of HNP1. 35S-methionine incorporation into proteins within cellular lysates (i.e. cellular proteins) and the culture media (i.e. secreted proteins) was visualised [Fig 1E] and quantified, following 18 hours of culture [Fig 1F]. Strikingly, HNP1 treatment significantly reduced the quantity of both 35S-labelled cellular and secreted proteins in un-stimulated HMDMs and robustly inhibited the labelling of secreted proteins in CD40L/IFNγ stimulated HMDMs, possibly reflecting the highly secretory phenotype of the stimulated macrophage. As expected, secreted TNFα was significantly reduced by HNP1 [Fig S1A]. However the overall cellular protein levels were unchanged during the time-course of the experiment [Fig S1B], consistent with a lack of increased global protein turnover and with maintenance of cell number and viability, as previously reported (5). Taken together neutrophil-derived HNP1 profoundly inhibits global protein synthesis within the resting or activated macrophage.

Exogenous HNP1 accumulates in the macrophage. HNP1 gained entry to macrophages and was found within the membrane and cytoplasm. However there was no clear co-localisation of HNP1 (or the control peptide W26A) with the ER marker calreticulin [Figs 2A and Fig S2A and Fig S2C] or with ribosomes [stained with anti Rps20, Figs 2B, Fig S2B and Fig S2D]. Control experiments also showed no non-specific staining or cross-reactivity.
HNP1 binds non specifically to RNA but does not alter mRNA transcription or stability. As HNP1 enters the macrophage it may, by reason of its positive charge and amphipathic nature (10, 18), bind to mRNA, so altering its turnover and inhibiting protein synthesis. This was tested using electrophoretic mobility-shift assays (EMSA) with 25mer homopolymeric RNA oligonucleotides. In contrast to W26A, HNP1 showed concentration-dependent shifts of poly(C) [Fig 3A-B], poly(A) [Fig S4A-B] and poly(U) RNA [Fig S4C-D], which was observed both in the presence or absence of Mg$^{2+}$ [Fig S4E]; a cation often required for nucleic acid binding by proteins. An antibody supershift EMSA also confirmed that HNP1 could bind to mRNA (coding for the firefly luciferase (Lluc) or β-galactosidase (β-gal) reporters) [Fig S4F].

To ask if HNP1 affected mRNA transcription, we quantified the steady-state mRNA levels generated by CD40L/IFNγ stimulated HMDMs. The mRNA levels of TNFα, IL-10, cyclooxygenase (Cox2) and tristetraprolin (TTP) were unaffected by HNP1 treatment of HMDMs over a 24hr time course [Fig 3C-D], despite a clear reduction in TNFα protein production [Fig 3E]. To assess mRNA decay, HNP1 or W26A treated HMDMs were stimulated (with R848) for 1 hour resulting in maximal TNFα mRNA levels, prior to the addition of actinomycin D to arrest further transcription. The decay rate of TNFα mRNA was not significantly modulated in HNP1 versus W26A-treated HMDMs over a further 1 hour time-course [Fig 3C-D, L-HNP1(n) or 1µg/ml) stimulated (u)]. Hours represent time following stimulation N=3. Error bars= mean ± SD.
Eukaryotic mRNA has a 5' monomethylated cap structure (m'G) which is crucial for canonical translation initiation, the rate-limiting and primary node of translation regulation (reviewed in (20)). To interrogate the role of translation initiation in HNP1-mediated inhibition we employed reporter mRNAs that contained a viral internal ribosome entry site (IRES) in their 5' untranslated regions (5'UTR), bypassing some or all of the eukaryotic translation initiation factor (eIF) requirements and initiating translation cap-independently (reviewed in (21)). The Classical Swine Fever Virus (CSFV) IRES mRNA reporter initiates translation independently of the majority of eIFs but is dependent on the ternary complex (eIF2, GTP and tRNAi) whilst the Crimean-Congo Hemorrhagic Fever Virus (CCHFV) IRES mRNA reporter is translationally coupled to eIF4E (reviewed in (22)).

The classical swine fever virus (CSFV) IRES mRNA reporter was quantified in the presence of an increasing concentration of HNP1. The IC50 (shown by the dotted line) is 1.6±0.2μM. Mean ±SEM (n=3). For A and D: ***p<0.001, *p<0.05 (analysed by Tukey's multiple comparison post hoc test following one-way ANOVA). For A: 1 ng CSFV IRES-β-gal-Aβ reporter mRNA was translated as for (A). Values plotted relative to vehicle control. (n=3) Error bars represent mean ±SEM. For B and D: ***p<0.001, *p<0.05 (analysed by unpaired T test). Values are plotted relative to vehicle control. (f) RRL was pre-treated with 150μg/mL cycloheximide and either 25μg/mL HNP1 or vehicle control. 1 ng 5'M-labelled m'G-fLuc-Aβ reporter mRNA was then added for the indicated times (shown in minutes) prior to 15-30% sucrose density gradient fractionation. Graph depicts the relative amounts of mRNA sedimenting with initiating ribosomes, normalised to amount recruited at 5 min in vehicle control-treated RRL. Black bars are control and grey bars are HNP1 treated. Error bars represent mean ±SEM (n=3), *p<0.05 (unpaired T test).

[Fig 5A] Hence the profound inhibition of protein synthesis by HNP1 was not the result of an induced UPR.

**HNP1 does not block translation initiation.** To ask if HNP1 affected translation directly, and to avoid the confounding effects of mRNA transcription, processing or nuclear export, we utilised the cell-free rabbit reticulocyte lysate (RRL) in vitro translation system. Translation of the canonical fLuc reporter mRNA, was profoundly inhibited in the presence of HNP1, but not by the mutant control peptides, L-HNP nor W26A [Fig 5A]. As with TNFα mRNA, HNP1 did not destabilise the reporter mRNA because input mRNA levels were maintained [Fig 5B]. The IC50 value for this effect was approximately 1.6μM (or 5.5pg/ml) [Fig 5C], a concentration that significantly reduces the production of pro-inflammatory cytokines from stimulated HMDMs in vitro [Fig 1].
that whilst HNP1 profoundly alters protein translation at a point after translation initiation, it does not prevent mRNA polysomal association.

**Discussion**

Cells of the immune system have developed tightly regulated systems to ensure the timely resolution of inflammation. The control of mRNA translation is emerging as a major mechanism that regulates the levels of proteins within leukocytes [reviewed in (23, 24)]. We have now identified a novel mechanism in which the most abundant neutrophil α-defensin, HNP1, [which is readily released as these cells die (5)], inhibits bulk protein translation within macrophages. Whilst the characteristic hydrophobic, amphipathic nature of α-defensins allows them to partition into the membrane lipid layer (25), it also ensures ready access to the cell’s interior. Confocal imaging showed that HNP1 entered macrophages [Fig 2], without inducing an unfolded protein response [Fig 4] or affecting mRNA stability [Fig 3]. To our knowledge, this is the first description of an eobiotic peptide released as these cells die (5), inhibiting bulk protein translation within macrophages. Therefore, the characteristic hydrophobic, amphipathic nature of α-defensins allows them to partition into the membrane lipid layer (25), it also ensures ready access to the cell’s interior. Confocal imaging showed that HNP1 entered macrophages [Fig 2], without inducing an unfolded protein response [Fig 4] or affecting mRNA stability [Fig 3]. To our knowledge, this is the first description of an eobiotic peptide released as these cells die (5), inhibiting bulk protein translation within macrophages. Therefore, the characteristic hydrophobic, amphipathic nature of α-defensins allows them to partition into the membrane lipid layer (25), it also ensures ready access to the cell’s interior. Confocal imaging showed that HNP1 entered macrophages [Fig 2], without inducing an unfolded protein response [Fig 4] or affecting mRNA stability [Fig 3]. To our knowledge, this is the first description of an eobiotic peptide released as these cells die (5), inhibiting bulk protein translation within macrophages.

**Conclusion**

Since HNP1 binds non-specifically to RNA, the mechanism by which it inhibits translation could not be modulated by ribosome engagement with mRNA. However, both reporter and cellular mRNAs remained polysome-associated [Figs 5 and 6] and the polysomal distribution of these mRNAs was similar in control and HNP1-treated RRL and HMDMs. Translational repression could be occurring via either elongation and/or termination (27) and we would speculate that HNP1 prevents translation elongation (22), which has recently been established as a major control point for protein synthesis (30).

Previous studies also allude to the greater importance of protein synthesis rate over degradation rate in determining overall protein levels (28, 29). However, the lack of a significant change in overall HMDM cellular protein level [Fig S1B] argues against HNP1-mediated inhibition in non-specific cellular protein degradation. Further, HNP1 profoundly inhibits reporter protein synthesis in cell-free assays in which protein turnover pathways are fundamentally compromised and HNP1 itself has no known protease activity. Taken altogether, we believe these data indicate that HNP1 affects de novo protein synthesis.

The tertiary structure of monomeric HNP1 is also clearly important for translational inhibition, as highlighted by the loss of efficac of observed for linearized HNP1 (L-HNP1) or W26A [Fig 1C]. However, the N-methylation of HNP1 Ile-20 (Melle), which prevents dimerization, does not alter the ability of Melle to inhibit TNF-α production, confirming that HNP1 dimerization is
not required to inhibit macrophage protein translation [Fig 1D].

The concentration of HNP1-3 in the synovial fluid of patients with rheumatoid arthritis is between 3 and 25 μg/mL, with an average of 12.4 μg/mL, suggesting that the concentration reached in tissues is similar to that used in our assays (5). Our previous studies have shown that HMDMs fully recover their pro-inflammatory potential within 72 hours following exposure to α-defensins; so whilst they clearly disable the macrophage protein translation machinery, they do not induce macrophage apoptosis (5). A previous study reported that α-defensins reduced the release of IL1β from activated monocytes, whilst not affecting the transcription of IL1β mRNA (30). Based on our findings, these observations can likely be explained by the translation of pro-IL1β being impaired.

In summary we have uncovered that neutrophil α-defensins abrogate the bulk mRNA translation of proteins within HMDMs, without affecting mRNA transcription or stability. In this way they prevent an excessive pro-inflammatory response that would create its own collateral damage, whilst still acting as powerful antimicrobial peptides. This is the first demonstration of an anti-microbial peptide that also has a translation-based anti-inflammatory role, acting as a ‘molecular brake’. It opens the way forward to developing similar peptide-based therapeutics that would act as effective combined anti-inflammatory and antimicrobial agents.

Materials and Methods

All materials and the following protocols are and fully described in the SI Appendix. Briefly, synthetic HNP1 and mutant derivatives were prepared by solid-phase synthesis as previously described (31). Template plasmids pCSFV-lacZ (32), p77-Luc (33) for reporter mRNA transcription were previously described and pGL2-CFP-RLuc, Renilla luciferase downstream of a Cricket Paralysis Virus (CrPV) IRES, was a kind gift from Matthias Hentze (EMBL, Heidelberg). Healthy donor peripheral blood mononuclear cells (PBMCs) were purified from whole blood as previously described (5). Stimuli included 1μg/mL R848 (InvivoGen), 3μg/mL CD40L (Peprotech) and IFNγ (Peprotech). Cytokines were quantified by sandwich ELISA (R&D Systems). For assessment of protein synthesis HMDMs were incubated in L-(-)-Methionine-free DMEM (MP Biomedicals) for 2 hours at 37°C, 5% CO₂, followed by 10μCi/mL[35S]-Methionine (Perkin Elmer), stimulation with CD40L and IFNγ and defence peptides. In vitro transcription was assayed by mG- or ApG-capped, nonadenylated, [32P]-UTP-labelled or non-labelled reporter mRNAs were synthesized as previously described (35). In vitro translation was assessed using the nuclear-treated rabbit reticulocyte lysate (RRL) in vitro translation kit (Promega) according to manufacturers’ recommendations. For electrophoretic mobility shift assays 10 pmol 5'-Cy5 labelled 25mer oligonucleotide (poly-Adenine, poly-Cytosine or poly-Uracil) (Eurogentec) was incubated with HNP1 or W26A peptide in 10μL binding and then resolved by electrophoresis. For immunocytochemistry HMDMs were grown on glass coverslips and stained with mouse monoclonal anti-human HNP1-3 antibody (Millipore) together with polyclonal rabbit anti-human ribosomal protein p110a (Abcam, dilution 1:250) or polyclonal rabbit anti-human calreticulin (Abcam, dilution 1:250). ER stress and the UPR, the mRNA stability assay along with RNA quantification and polysome analysis are fully explained in the supplemental Materials and Methods section.