Nonenzymatic Conversion of ADP-Ribosylated Arginines to Ornithine Alters the Biological Activities of Human Neutrophil Peptide-1

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Nonenzymatic Conversion of ADP-Ribosylated Arginines to Ornithine Alters the Biological Activities of Human Neutrophil Peptide-1

Linda A. Stevens,* Joseph T. Barbieri,† Grzegorz Piszczek,‡ Amy N. Otuonye,* Rodney L. Levine,§ Gang Zheng,* and Joel Moss*

Activated neutrophils, recruited to the airway of diseased lung, release human neutrophil peptides (HNP1–4) that are cytotoxic to airway cells as well as microbes. Airway epithelial cells express arginine-specific ADP ribosyltransferase (ART)-1, a GPI-anchored ART that transfers ADP-ribose from NAD to arginines 14 and 24 of HNP-1. We previously reported that ADP-ribosyl-arginine is converted nonenzymatically to ornithine and that ADP-ribosylated HNP-1 and ADP-ribosyl-HNP-(ornithine) were isolated from bronchoalveolar lavage fluid of a patient with idiopathic pulmonary fibrosis, indicating that these reactions occur in vivo. To determine effects of HNP-ornithine on the airway, three analogs of HNP-1, HNP-(R14orn), HNP-(R24orn), and HNP-(R14,24orn), were tested for their activity against Pseudomonas aeruginosa, Escherichia coli, and Staphylococcus aureus; their cytotoxic effects on A549, NCI-H441, small airway epithelial-like cells, and normal human lung fibroblasts; and their ability to stimulate IL-8 and TGF-β1 release from A549 cells, and to serve as ART1 substrates. HNP and the three analogs had similar effects on IL-8 and TGF-β1 release from A549 cells and were all cytotoxic for small airway epithelial cells, NCI-H441, and normal human lung fibroblasts. HNP-(R14,24orn), when compared with HNP-1 and HNP-1 with a single ornithine substitution for arginine 14 or 24, exhibited reduced cytotoxicity, but it enhanced proliferation of A549 cells and had antibacterial activity. Thus, arginines 14 and 24, which can be ADP ribosylated by ART1, are critical to the regulation of the cytotoxic and antibacterial effects of HNP-1. The HNP analog, HNP-(R14,24orn), lacks the epithelial cell cytotoxicity of HNP-1, but partially retains its antibacterial activity and thus may have clinical applications in airway disease. The Journal of Immunology, 2014, 193: 000–000.

Polymorphonuclear leukocytes migrate into the lung to the site of infection, inflammation, or other disease processes in response to complex signaling events. After activation, neutrophils release antimicrobial α-defensins (human neutrophil peptides [HNP1–4]), cationic, amphipathic, arginine-rich peptides with diverse biological activities (1). In addition to killing microorganisms, defensins promote proliferation (2, 3) and stimulate the release of neutrophil chemotactic factor, IL-8, and TGF-β1 from lung epithelial cells and fibroblasts (4, 5). The in vitro effects on proliferation of airway epithelial cells suggested a role for defensins in wound repair. Indeed, wound closure and mucin gene expression were enhanced by HNP1–3 in vitro (6).

It has been proposed that defensins released at the site of inflammation or infections are also involved in the pathogenesis of disease (7–9). In the bronchoalveolar lavage fluid (BALF) of healthy volunteers, concentrations of HNP1–3 are 0.2 μg/mL, but may increase 6-fold during infection and 50-fold in inflammatory lung diseases (10). Elevated levels of defensins were found in the BALF of patients with α1-antitrypsin deficiency (11, 12), in the sputum of patients with cystic fibrosis (13) and chronic obstructive pulmonary disease (14), and in the serum of patients with interstitial lung disease [e.g., idiopathic pulmonary fibrosis (15), sarcoidosis (16, 17)]. Defensins at low concentrations increased proliferation and collagen synthesis in lung fibroblasts, possibly participating in the formation of the fibroproliferative lesions seen in inflammatory pulmonary diseases (3). Intratracheal instillation of defensins (HNP1–3) into the lungs of mice that do not express neutrophil-derived HNP caused acute lung inflammation and dysfunction (18). Transgenic mice that expressed α-defensins (HNP1, 2) in their neutrophils had more severe, acid-induced acute lung injury than did their wild-type counterparts (19).

Net charge, amphipathicity, hydrophobicity, and tertiary structure are critical for the antimicrobial activity of defensins (20). In addition to the hydrophobicity of tryptophan 26 (21), the positive charge of three arginine residues (22) is essential for the antimicrobial activity of HNP-1. ART1, an arginine-specific ADP ribosyltransferase (ART) expressed on the surface of airway epithelial cells and neutrophils (23, 24), catalyzes the transfer of the ADP-ribose moiety of NAD to arginine 14 of HNP-1, inhibiting its antibacterial and cytotoxic activities (25). The primary sequence of HNP1–3 contains arginines at positions 5, 14, 15, and 24. Arginine 5 forms a salt bridge with glutamic acid 13 that is required for folding stability (26). Arginines 14 and 24 in HNP-1 are ADP ribosylated by ART1, and ADP-ribosylated arginines can be converted nonenzymatically to ornithine by nucleophilic attack of water on the guanidino carbon with probable release of ADP-ribose-carbamate (27, 28). Mono- and

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Abbreviations used in this article: ART, ADP ribosyltransferase; BALF, bronchoalveolar lavage fluid; HNP, human neutrophil peptide; mART, mouse ART; NHLF, normal human lung fibroblast; SAEF, small airway epithelial cell.
were toxic for NHLF at concentrations analog, for HNP (analog concentrations differs significantly from each other (of three experiments performed in triplicate. The mean cell proliferation as percentage of control of the four peptides in plots (R24orn) is not significant (antibacterial activity would reduce damage to the airway epithelium from epithelial cells in response to HNP-1, amplifies the inflammatory resulting in injury to the airway (31, 32). Modifications of antimicrobial HNP-1 that decrease its eukaryotic cytotoxicity but preserve antibacterial activity would reduce damage to the airway epithelium while allowing the peptide to function in innate immunity.

ADP ribosylation of specific arginines in HNP-1 by ART1 was reported previously to have a role in modulating its activity, consistent with a critical role for these arginines (25). We hypothesized that replacement of ADP-ribo-arginine with ornithine in HNP-1 might also alter activity. To address this question, HNP-(R14orn), HNP-(R24orn), and HNP-(R14,24orn) were synthesized, and their antibacterial, cytotoxic, and other activities were compared with native HNP-1. The data demonstrate that replacement of specific arginines in HNP-1 with ornithine through an ADP-ribosylation–dependent reaction alters its biological activities and may protect airway cells from HNP-1–induced cytotoxicity while partially preserving antimicrobial activity.

Materials and Methods

Analogs

HNP, HNP-(R14orn), HNP-(R24orn), and HNP-(R14,24orn) were synthesized by Bachem (Torrance, CA) as acetate salts. HNP-1 (Bachem), synthesized by Bachem as trifluoroacetate salt, was included in indicated assays in addition to the acetate salt.

Cell culture

Human lung carcinoma epithelial cells (A549) and human lung adenocarcinoma epithelial cells (NCH-H441) were purchased from American Type Culture Collection (Manassas, VA) and grown in F-12K medium or RPMI 1640, respectively, with 10% FBS. Normal human lung fibroblasts (NHLF) and normal human small airway epithelial cells (SAEC) were purchased from Clonetics (Walkersville, MD) and grown, respectively, in fibroblast media or small airway epithelial cell complete medium supplied by the company. All cells were grown at 37°C in 5% CO2.

Preparation of mouse ADP-ribosyltransferase-1

Rat mammary adenocarcinoma cells (NMu) transfected with plasmids containing the mouse ART (mART1) gene were grown in Eagle’s MEM with 10% FBS and 0.5 mg/ml Genetec (G418). Proteins released from the cells by incubation with phosphatidylinositol-specific phospholipase C, which cleaves the GPI anchor, were collected in PBS (33), and ADP-ribosyltransferase activity was assayed, as described (34).

Preparation of ADP-ribosyl HNP analogs for identification of the site of modification

HNP analogs (10 or 30 μg), mART1 (9.14 nmol/h transferase activity), and 5 mM NAD in 50 mM potassium phosphate (pH 7.5) were incubated at 30°C overnight or as indicated. Products were separated by HPLC. ADP-ribosylation sites on the purified products were mapped by mass spectrometry (28).

Mass spectrometry and sequence analysis

HNP analogs were reduced, trypsinized, and analyzed by reverse-phase chromatography/mass spectrometry, as described (25), except that the reverse-phase column was a Zorbax 300SB-C18, 1.0 × 35 mm, 3.5 μm, and the mass spectrometer was an Agilent model 6520 QTOF capable of tandem mass-spectrometric sequencing of peptides. Spectra were deconvoluted with Agilent Masshunter software version 3, and spectra were matched to those predicted with GPMAW version 9 (Lighthouse Data, Odense, Denmark) (28).

Effects of HNP analogs and agmatine on nicotinamide release from [14C]NAD

HNP analogs or agmatine were incubated with mART1 (9.3 nmol/h) and 0.1 mM [nicotinamide-U-14C]NAD (0.05 μCi) in 50 mM potassium phosphate (pH 7.5) for 1.5 h at 30°C. Nicotinamide release resulting from ADP-ribose transfer to an acceptor was monitored, as described (35).

Effects of HNP analogs on proliferation, cytotoxicity, and release of IL-8 and TGF-β1

To measure the release of IL-8 (2 × 105 cells/ml) and TGF-β1 (1 × 104 cells/ml) from A549 cells, cells were grown in 96-well plates for 24 h in complete media prior to treatment, washed with serum-free media, and treated for 24 h with analogs dissolved in serum-free media. Levels of

FIGURE 1. Effect of HNP-1 analogs on the viability of A549 cells and NHLF. A549 cells (1 × 105/ml) (A–C) and NHLF (4 × 105/ml) (D–F) were plated 24 h before the cells were washed with serum-free media and addition of HNP (A and D), HNP-(R14orn), HNP-(R24orn), (B and E), and HNP-(R14,24orn), (C and F) solubilized in serum-free media at the indicated concentrations for 24 h, as described in Materials and Methods. The data are the means ± SEM of three experiments performed in triplicate. The mean cell proliferation as percentage of control of the four peptides in plots (A–C) over the range of HNP analog concentrations differs significantly from each other (p < 0.0001). The difference between the mean cell proliferation of HNP-(R14orn) and HNP-(R24orn) is not significant (p = 0.53). The mean proliferation of A549 cells increases significantly from 100% of control at concentrations <25 μg/ml analog, for HNP (p = 0.0009), HNP-(R14orn) (p < 0.0001), HNP-(R24orn) (p = 0.000s), and HNP-(R14,24orn) (p < 0.0001). HNP and the three analogs were toxic for NHLF at concentrations <10 μg/ml (p < 0.0001).
human IL-8 and TGF-β1 in the media were quantified by immunoassay (R&D Systems, Minneapolis, MN). To determine the number of viable A549 cells (1 × 10⁵ cells/ml), NCI-H441 (1 × 10⁵ cells/ml), SAEC (1 × 10⁵ cells/ml), or NHLF (4 × 10⁵/ml) cells were grown on 96-well plates in complete media prior to treatment and treated as above. Proliferation and cytotoxicity were determined with tetrazolium-based Cell Counting Kit-8 (Dojindo Molecular Technologies, Rockville, MD), according to the manufacturer’s instructions.

**Antibacterial assays**

*Escherichia coli* (TG1 or ATCC 25922), *Pseudomonas aeruginosa* (PA103), and *Staphylococcus aureus* (ATCC 29213) were grown overnight in LB Broth, Miller (Luria-Bertani) (Difco: tryptone, 10 g/L; yeast extract, 5 g/L; and NaCl, 10 g/L) at 37°C. Bacteria were diluted 1/50, cultured at 37°C, and harvested after 2 h. The bacterial OD was measured (5 × 10⁷/ml = 1, A = 600 nm), and cells were diluted in 10 mM NaPO₄ (pH 7.4) to 5 × 10⁵/ml. Bacteria (20 μl) were added to 50 μl HNP or analogs in 10 mM NaPO₄ (pH 7.4). After 1 h at 37°C, without agitation, an aliquot was plated to determine CFUs. Extending the incubation time to 2 h did not change the amount of cell killing. HNP or analogs and amounts used in the assay were HNP, 1.0, 0.1, and 0.01 μg; HNP-(R14orn), 3.0, 1.0, and 0.1 μg; HNP-(R24orn), 3.0, 1.0, and 0.1 μg; and HNP-(R14,24orn), 3.0, 1.0, and 0.1 μg. Toxicity was similar in one or two incubations with HNP.

**Analytical ultracentrifugation**

Sedimentation velocity. Analytical ultracentrifugation experiments were performed using the Beckman Optima XL-A analytical ultracentrifuge and a four-place AN-Ti rotor. All HNP samples were dialyzed in PBS buffer (pH 7.4) and accepted fits had a root mean square of deviation to 250,000 rpm. Interference and absorbance scans at 280 nm were started immediately after thermal equilibrium was reached at rest at 20°C. Double-sector centrifuge cells were filled with 0.4 ml sample or reference buffer. Centrifuge rotor was accelerated to 60,000 rpm (60,000 rpm) from 1 h at 37°C, without agitation, an aliquot was plated to determine CFUs. Extending the incubation time to 2 h did not change the amount of cell killing. HNP or analogs and amounts used in the assay were HNP, 1.0, 0.1, and 0.01 μg; HNP-(R14orn), 3.0, 1.0, and 0.1 μg; HNP-(R24orn), 3.0, 1.0, and 0.1 μg; and HNP-(R14,24orn), 3.0, 1.0, and 0.1 μg. Toxicity was similar in one or two incubations with HNP.

**Statistical methods**

The ANOVA and the test for trends were used to analyze the data presented in the figures. The effects on the mean outcome (e.g., cell proliferation) by the change in concentration levels or analogs were tested by a two-way ANOVA. The test for trends, based on a linear regression model with concentration as the independent variable, was used to test whether the mean outcome changes with concentration levels; analysis was done using SAS v9.3 software.

**Results**

**HNP-(R14,24orn) is cytotoxic for normal human fibroblasts, but not for A549 cells**

To determine the effect of the HNP analogs on eukaryotic cells, lung epithelial carcinoma cells (A549) or NHLF were incubated with increasing amounts of HNP or the three HNP-ornithine analogs for 24 h in serum-free media (Fig. 1A–F). HNP and HNP-(R14,24orn) at 10 μg/ml and HNP-(R14orn) and HNP-(R24orn) at 25 μg/ml increased the number of A549 cells (as measured by a tetrazolium reduction assay). HNP and the three analogs were toxic for NHLF at concentrations <10 μg/ml (Fig. 1D–F). HNP-(R14orn) and HNP-(R24orn) were toxic for A549 cells at concentrations >40 μg/ml. In contrast, replacement of arginines with ornithines at aa 14 and 24 abolished the cytotoxic effect of HNP on A549 cells at concentrations to 100 μg/ml (Fig. 1C). The toxicity of HNP and ornithine analogs (50 μg/ml) was also tested on SAEC and NCI-H441 cells and compared with that seen with A549 and NHLF cells. The HNP analogs showed similar or less toxicity than HNP (Fig. 2).

**The three HNP ornithine analogs stimulate release of IL-8 from A549 cells**

IL-8, a chemoattractant, modulates the inflammatory response by recruiting neutrophils to the lung (37). Higher levels of IL-8 in patients compared with healthy volunteers (15, 38). HNP-1 has been reported to induce release of IL-8 and other chemokines and cytokines from epithelial cells and fibroblasts (4, 5, 39, 40). To determine the effects of HNP and the ornithine analogs on IL-8 release by A549 cells, cells were incubated with the ornithine analogs, and IL-8 in the medium was measured by immunoassay. HNP with a single ornithine substitution at concentrations up to 20 μg/ml increased IL-8 release (Fig. 3A) as did HNP-(R14,24orn), up to 100 μg/ml (Fig. 3B), a range of concentrations not cytotoxic for A549 cells.

**Two HNP ornithine analogs release more TGF-β1 from A549 cells than does HNP**

HNP-1 increased TGF-β1 release by fibroblasts, but reduced TGF-β1 production by A549 cells (4). A549 cells were incubated with increasing amounts of HNP analogs, and TGF-β1 release was determined by immunoassay. HNP-(R24orn) and HNP-(R14,24orn)

![Image](http://www.jimmunol.org/)
To evaluate how well the ornithine analogs serve as substrates for ART1, nicotinamide release concurrent with ADP-ribose transfer from NAD to the acceptor was measured after incubation of the peptides with ART1 and NAD: HNP was compared with agmatine, a decarboxylated form of arginine, which is a known ADP-ribose acceptor used by ART1 (Fig. 6A). HNP and HNP-(R14orn) were better acceptors of ADP-ribose than agmatine. A small amount of nicotinamide release was observed in the presence of ART1, NAD, and HNP-(R14,24orn), indicating that, under these assay conditions, arginine 5 and arginine 15 are poor ADP-ribose acceptors in the ART1-catalyzed reaction.

To identify the products of the reaction of the ornithine analogs, ART1 and NAD, the products were separated by HPLC, purified, and analyzed by mass spectroscopy (Fig. 6B). The early peaks in the HPLC separations were identified as ADP-ribosylated HNP-(R14orn), (Fig. 6Bb) or ADP-ribosylated-HNP-(R24orn), (Fig. 6Bc) whereas the later peaks were identified as the unmodified forms. In contrast, three products were separated from the reaction of HNP-1 with NAD and ART1 (Fig. 6Ba) (28). Compared with HNP-1, (Fig. 6Bd) the unmodified analogs were found at an earlier HPLC elution time, suggesting that they are less hydrophobic than HNP. ADP-ribosylated HNP-(R24orn) (Fig. 6Bc) eluted at a significantly earlier time, suggesting the product is as hydrophobic as the dimodified HNP-1. ADP-ribosylarginine 5 or 15 HNP was not identified in the reaction products or in HNP-(R14,24orn) (Fig. 6Be). Increased ADP ribosylation of HNP-1 and HNP-(R14,24orn) was observed after reduction of the disulfide bridges with DTT (Fig. 6Bf). These data suggest that the quaternary structure of HNP and the analogs limit the accessibility of arginines for ADP ribosylation by ART1.

The three HNP ornithine analogs weakly dimerize

Previous studies showed that dimerization was important for the antimicrobial activity of HNP-1 (41). We questioned whether the difference in antimicrobial activity of HNP-(R14,24orn) and HNP with a single ornithine substitution could be due to differences in dimerization. The degree of ornithine analog oligomerization in solution was investigated by analytical ultracentrifugation. The distribution of sedimentation coefficients of all HNP analogs was investigated by analytical ultracentrifugation. The three HNP ornithine analogs weakly dimerize

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FIGURE 6. (A) Effect of HNP analogs or agmatine on nicotinamide release from NAD catalyzed by ART1. HNP analogs or agmatine in the amounts indicated were incubated with ART1 (9.3 nmol/h) and 0.1 mM [nicotinamide-U-14C]NAD (0.05 μCi/reaction) in 50 mM potassium phosphate (pH 7.5) for 1.5 h at 30˚C. ADP ribosylation of HNP analogs or agmatine was measured by nicotinamide release in the standard assay described in Materials and Methods, HNP-(R14orn) (▴), HNP (♦), agmatine (▪), HNP-(R24orn) (▼), HNP-(R14, 24orn) (__). The data are the mean ± SEM of two experiments with assays run in duplicate. There is no significant difference between the mean nicotinamide release levels of HNP and HNP-(R14orn), p > 0.0001, but both were significantly different from the nicotinamide release by agmatine (p < 0.0001). Nicotinamide release in the presence of HNP-(R14,24orn) increased with analog amounts (p < 0.0001). However, HNP-(R14,24orn) was a poor ADP-ribose acceptor in the presence of ART1, compared with HNP and the mono-ornithine analogs. (B) HPLC separation of reaction products from the incubation of ornithine analogs with ART1. HNP-1 analogs (10 μg) were incubated with ART1 (4.7 nmol/h) and 5 mM NAD in 50 mM potassium phosphate (pH 7.5) for 8 h at 30˚C. HNP-1 or products from the reaction mix were separated by HPLC and monitored at 210 nm, as in Materials and Methods. The data are representative of three experiments. Peaks were identified by mass spectroscopy as (Ba), HNP-1, 48.3 min (●); ADP-ribosyl-HNP, 47.7 min; (+) ADP-ribosyl-HNP-ornithine, 46.8 min; (♦), di-ADP-ribosyl-HNP, 46.1 min, (○). (Bb) HNP-(R14orn), 48.3 min (▲); ADP-ribosyl-arginine 24-HNP-(R14orn), 47.6 min (▲). (Bc) HNP-(R24orn), 48.1 min (◆); ADP-ribosyl-arginine 14-HNP-(R24orn), 46.4 min (●). (Bd) HNP-1, 48.5 min. (Be) HNP-(R14,24orn), 47.9 min. (Bf) HNP-1 (3 μg) or HNP-(R14,24orn) (3 μg) was incubated with ART1 (9.3 nmol/h) for 1 h in 50 mM potassium phosphate (pH 7.5), 10 μM (adenylate-[32P])NAD (1 μCi/assay), followed by 5 mM unlabeled NAD overnight, with or without 20 mM dithiodreitol at 30˚C. Proteins were trichloroacetic acid precipitated, separated on 16% tricine gels (Invitrogen), which were stained with Coomassie Blue, dried, and exposed to x-ray film (BioMax; Kodak). Data represent one of three experiments.
oligomerization of HNP isoforms in solution and no significant difference among the analogs. In summary, ADP ribosylation of arginines at residues 14 and 24 by ART1 regulates HNP-1 biological activities (Fig. 8). Site-specific ADP ribosylation by ART1 abolishes HNP-1 antimicrobial and cytotoxic activities. HNP with ADP-ribosylated arginines 14 and 24 nonenzymatically converted to ornithines is not cytotoxic for epithelial cells, but the antibacterial activity is preserved.

Discussion

The airway epithelium plays a critical role in host defense against infection (42). The repair process of the injured epithelium involves cell migration and proliferation, processes that increase neutrophil defensins. As reported previously (31, 32, 43, 44), the proliferative and cytotoxic effects of HNP-1 on human lung fibroblasts and epithelial and endothelial cells are dose dependent. Low concentrations of HNP-1 have a proliferative effect on NHLF (43) and A549 carcinoma epithelial cells (45). However, at concentrations >20 μg/ml, HNP is cytotoxic for NHLF and A549 cells (31, 43). The di-ornithine form of HNP retained proliferative effects, was nontoxic for A549 cells up to 100 μg/ml, but as toxic as HNP-1 on NHLF, demonstrating that the biological effects were dependent on cell type. For the four cell lines tested (Fig. 2), the ornithine analogs showed similar toxicity (i.e., for the NHLF and SAEC) or were less toxic than HNP.

As observed by confocal laser microscopy, HNP-1 at high concentrations (10–20 μg/ml) rapidly entered A549 cells, colocalized with the endoplasmic reticulum, and stimulated apoptotic cell death (44). The reduced cytotoxicity of HNP after di-ornithine substitution could result from changes to HNP folding that may affect its capacity to enter the cell or localize to the endoplasmic reticulum. Substitution of ornithines for two arginines in oncocin, a 19-aa peptide sequence derived from the insect Oncopeltus peptid 4, resulted in a peptide that was active against E. coli, but nontoxic to HeLa cells and stable in serum (46). Conversion of HNP-1 to the di-ornithine form resulted in reduction of injury to the epithelial cells in the airway and preservation of the proliferative response believed to be involved in wound repair.

A better understanding of the essential properties of the antimicrobial defensins may lead to new analogs with activity against bacteria resistant to current antibiotics. Cationic arginine residues, quaternary structure, and hydrophobicity are critical to HNP-1 antimicrobial activity. Bacterial lysis is thought to result from the positively charged peptide interacting with the prokaryotic negatively charged membrane, resulting in pore formation (47). Surprisingly, arginines or ornithines at positions 14 and 24 in the HNP primary sequence are critical for antibacterial activity. Although lysine, arginine, and ornithine have equivalent charge, the replacement of arginine by lysine affected the antimicrobial activity of α-defensins (48). The virtual lethal dose for 90% of bacteria increased when ornithine replaced arginine or lysine at residues 14, 15, and 24 (22). HNP-(R14,24orn) with arginine at residue 15, however, retained significant antibacterial activity against P. aeruginosa and E. coli, suggesting that ornithine 15 had an effect other than charge (22). Moreover, in contrast to the toxicity of replacing arginine 14 and 24 as in HNP-(R14,24orn), the replacement of arginine 24 with ornithine abolished the antibacterial activity against P. aeruginosa and E. coli, demonstrating that the activity of HNP-1 is affected by the number and position of arginine residues replaced by ornithine.

Previous studies have reported that, in addition to charge, hydrophobicity and dimerization contribute to the antimicrobial activity of HNP-1 (21, 41). Analytical ultracentrifugation analysis showed that HNP and the ornithine analogs sediment as a monomer, indicating that there was no difference in oligomerization between the analogs and HNP. All ornithine analogs eluted earlier than HNP-1 in the HPLC separations, suggesting that they are less hydrophobic than HNP-1 and that the decrease in hydrophobicity was unrelated to the antibacterial activity of the HNP analogs.

In contrast to the lack of cytotoxicity, the properties of HNP-1 that included the ability to form oligomers in solution, proliferation, antibacterial activity, and the release of TGF-β1 and IL-8 from A549 cells were not significantly affected by substitution of ornithines in place of arginines 14 and 24. ART1 was unable to ADP-ribosylate arginines 5 and 15 in HNP or di-ornithine HNP. Increased ADP ribosylation required disruption of its tertiary structure with reduction of the disulfide bonds in HNP-1 and HNP-(R14,24orn). Ornithine in the primary sequence can change the stability by making the peptide bond resistant to proteolytic cleavage (49). After incubation with trypsin, liquid chromatography–mass spectrometry analysis of the ornithine analogs did not find evidence of cleavage sites C-terminal to the ornithines (data not shown). Taken together, the data suggest that arginines 14 and 24, targeted by ART1 for ADP ribosylation, in addition to effects on properties such as charge, structure, and hydrophobicity, contribute to the biological activities of HNP-1.
Antimicrobial peptides are strong candidates in the search for alternative antibiotics. Mouse neutrophils lack defensins and thus cannot be used as a model for these studies (50). Our data show that HNP analogs generated in disease exhibit altered activities. Studies of the diseased human airway are necessary, to understand both the posttranslational processing of HNP-1 and the effects of the analogs of HNP-1 that can be important for healing the damaged lung. These results may help identify potential therapeutic agents. However, the use of the analogs in a clinical setting is complicated due to the fact that they are structurally complex (e.g., disulfide bridges) and increase release of IL-8 and TGF-β from cells, as observed with the A549 cells, and thus may be proinflammatory.

HNP-(R14,24 orn) is a promising novel candidate: a stable peptide antibiotic that shows diminished toxicity for the epithelial cells that line the airway.

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Disclosures

L.S., R.L., and J.M have patents and patents pending related to the modification of defensins, in particular HNP-1.

References


