

Human neutrophil peptides induce interleukin-8 production through the P2Y₆ signaling pathway

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Antimicrobial human neutrophil peptides (HNPs) play a pivotal role in innate host defense against a broad spectrum of prokaryotic pathogens. In addition, HNPs modulate cellular immune responses by producing the chemokine interleukin-8 (IL-8) in myeloid and epithelial cells and by exerting chemotaxis to T cells, immature dendritic cells, and monocytes. However, the mechanisms by which HNPs

modulate the immune responses in the eukaryotic cells remain unclear. We demonstrated that, as with adenosine triphosphate (ATP) and uridine diphosphate (UDP), HNP stimulation of human lung epithelial cells selectively induced IL-8 production in 10 pro- and anti-inflammatory cytokines examined. HNP-induced IL-8 release was inhibited by treatment with the nucleotide receptor antagonists suramin and

reactive blue. Transfection of lung epithelial cells with antisense oligonucleotides targeting specific purinergic P2Y receptors revealed that the P2Y₆ (ligand of UDP) signaling pathway plays a predominant role in mediating HNP-induced IL-8 production. (Blood. 2006;107:2936-2942)

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Introduction

Among the antimicrobial compounds stored in the azurophilic granules, human neutrophil peptides (HNPs) are the most abundant, constituting up to 50% of the total protein content within human neutrophils.^{1,2} HNPs, also known as α -defensins, are small cationic peptides with 6 characteristic, highly conserved cysteine residues and 3 intramolecular disulfide bonds.³⁻⁵ HNPs exhibit antimicrobial activity against a variety of microorganisms, such as Gram-positive and Gram-negative bacteria, viruses, and fungi, through charge-dependent pore formation.

Normal plasma levels of HNPs range from undetectable levels to 50 to 100 ng/mL. In patients experiencing the onset of bacterial infection, those with nonbacterial infection, and those with pulmonary tuberculosis, mean HNP levels were 2- to 4-fold greater than they were in healthy volunteers.⁶ In patients with sepsis, HNP levels might be elevated to concentrations measured in milligrams per milliliter.³ In lung lavage fluid, HNP concentrations were 50-fold higher in patients with ARDS than in healthy controls,⁷ 5 orders of magnitude greater in patients with bacterial pneumonia than in healthy volunteers,⁶ and 31-fold higher in patients with diffuse panbronchiolitis than in controls.⁸ In sputum, we have shown an HNP concentration of $240 \pm 40 \mu\text{g/mL}$ in patients with cystic fibrosis compared with undetectable levels in healthy controls.⁹ These and other studies showing increased levels of HNP in the circulation and in the airways of patients with inflammatory lung diseases suggest that HNPs may contribute to the pathogenesis of inflammatory lung disorders.⁶⁻¹²

Indeed, in addition to their microbicidal role, extracellular HNPs can modulate immunologic responses. For example, HNPs are chemotactic to human CD4⁺/CD45RA⁺ naive T cells, CD8⁺

T cells, immature human dendritic cells,¹³ and monocytes.¹⁴ Stimulation of human lung epithelial cells and primary bronchial epithelial cells with HNPs induces the production of interleukin-8 (IL-8) through the transcriptional regulation of IL-8 mRNA expression.¹⁵ IL-8 is a 6- to 8-kDa protein produced by a variety of cell types, including monocytes, lymphocytes, granulocytes, fibroblasts, epithelial cells, and endothelial cells.¹⁶ IL-8 is an inflammatory chemokine that functions as a neutrophil chemoattractant and activating factor. It also attracts eosinophils, basophils, and a subpopulation of lymphocytes.¹⁷

HNPs and certain chemokines share characteristics such as size, cationic charge, and disulfide bonding and biologic activities such as chemotaxis and antimicrobial properties.^{18,19} Although they lack amino acid sequence homology, the 3-dimensional structures of human β -defensins 1 and 2 and the CC chemokine CCL20 are similar, and all 3 molecules specifically interact with the CCL20 receptor CCR6 and induce similar biologic responses.²⁰ However, HNP-dependent chemotaxis is not mediated by CCR6¹³ and the mechanisms by which HNPs induce IL-8 production remain unknown.

Purinergic P2 receptors, including the P2X and P2Y families, are functional ligands of extracellular nucleotides that mediate intracellular signal transduction. P2X receptors are a family of adenosine triphosphate (ATP)-activated cation channels (P2X₁ to P2X₇) that open in response to ATP binding.²¹ The P2Y family members are pertussis toxin-sensitive, G_i-protein-coupled receptors. At least 8 subtypes of P2Y receptors (P2Y₁, P2Y₂, P2Y₄, P2Y₆, P2Y₁₁, P2Y₁₂, P2Y₁₃, and P2Y₁₄) expressed by mammalian cells have been identified, and each has different nucleotide-binding affinity.²²⁻²⁴

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Four isoforms of P2Y receptors, including P2Y₂ (ATP = uridine triphosphate [UTP]), P2Y₄ (UTP >> ATP), P2Y₆ (uridine diphosphate [UDP]), and P2Y₁₄ (UDP glucose), are expressed in the lung.^{22,23} P2Y receptors mediate a wide range of cellular responses, including chemotaxis to CD4⁺ T cells and induction of IL-8 expression by a variety of human epithelial and immune cells.^{25,26} Recent studies have demonstrated UDP (ligand of P2Y₆)–dependent IL-8 production from THP-1 monocytes,²⁷ human eosinophils,²⁶ and human mature dendritic cells.²⁸ In addition, products of ATP hydrolysis, such as adenosine monophosphate (AMP)^{29,30} and adenosine³¹ by cell-surface–associated nucleotidases, can also mediate IL-8 induction (Figure 1).

In the present study, we tested the hypothesis that HNPs induce IL-8 production through purinergic receptor signaling in human lung epithelial cells. We demonstrate that HNPs selectively induce IL-8 production predominantly through a P2Y₆ signaling pathway (Figure 1).

Materials and methods

Source of HNPs

HNPs were purified from patients with cystic fibrosis, as previously described.^{32,33} To minimize variability from one patient to another, sputum was pooled from at least 20 patients with cystic fibrosis before purification. With respect to possible variability from one batch to another, at least 3 different batches of purified HNPs were used to stimulate cells in all experiments reported in the present study. HNPs were used as a mixture of HNP-1, HNP-2, and HNP-3 identified by 12.5% acid–urea polyacrylamide gel electrophoresis and by mass spectrometry (Mass Spectrometry Laboratory, Molecular Medicine Research Center, University of Toronto, Toronto, ON, Canada).³² Because the relative intensities of the HNP-1, HNP-2, and HNP-3 peaks are measured quantities, mass spectrometry allows an estimate of the content of the 3 components in the HNP mixture. Average percentage compositions calculated from 15 batches of the purified HNP mixture were 72.2% for HNP-1, 16.4% for HNP-2, and 11.4% for HNP-3. Purified HNPs were reconstituted in 0.01% acetic acid and were tested by bacterial killing and endotoxin detection assays before use.^{32,33} Synthetic HNP-1 (Sigma, St Louis, MO), followed by use of the purified HNPs, was used to conduct pilot experiments.

Cell cultures

Human small airway epithelial cells (SAECs; Cambrex, East Rutherford, NJ) were cultured in the SAEC medium (Cambrex) at 37°C in a 5% CO₂–humidified incubator. A549 human alveolar epithelial type 2–like cells (ATCC, Rockville, MD) were maintained as monolayers in DMEM with L-glutamine (Gibco, Grand Island, NY) supplemented with 50 μg/mL

gentamicin (Gibco) and 10% heat-inactivated fetal bovine serum (complete medium; Gibco).³⁴

Transfection of antisense oligonucleotides

Transfection of SAECs and A549 cells with the sense or antisense oligonucleotides (final concentration, 2.5 μM) corresponding to the translation initiation sites of human P2Y₂, P2Y₄, and P2Y₆ were performed with the use of Lipofectamine reagent (Gibco). Sequences included P2Y₂ sense, 5′-GCGATGGCAGCAGACCTGG-3′; P2Y₂ antisense, 5′-CCAGGTCT-GCTGCCAT-CGC-3′; P2Y₄ sense, 5′-GCCATGGCCAGTACAGAGT-3′; P2Y₄ antisense, 5′-ACTCTGT-ACTGGCCATGC-3′; P2Y₆ sense, 5′-GCCATGGAATGGGACAATG-3′; and P2Y₆ antisense, 5′-CATTGTC-CCATCCATGGC-3′. The transfection medium was replaced with complete DMEM 4 hours after transfection, and the cells were incubated overnight at 37°C. Cells were washed with phosphate-buffered saline (PBS) and serum-free medium before stimulation with HNPs.

Reverse transcriptase–polymerase chain reaction assay

Total RNA was extracted from A549 cells grown in a 6-well plate using Trizol reagent (Invitrogen, Life Technologies, Carlsbad, CA). First-strand cDNA was prepared by using the SuperScript first-strand synthesis system (Invitrogen), and reverse transcriptase–polymerase chain reaction (RT-PCR) was performed in a GeneAmp PCR apparatus (Amersham Pharmacia Biotech, Piscataway, NJ) with Platinum PCR supermix (Invitrogen). The following primers were used to amplify the conserved regions in the third and seventh transmembrane domains of P2Y receptors³⁵: P2Y₂ sense primer, 5′-CCAGCCCCCGTGC TCTACTTTG-3′; P2Y₂ antisense primer, 5′-CATGTTGATGGCGTTGAGGGTGTG-3′ (367 bp); P2Y₄ sense primer, 5′-CGTCTTCTCGCCTCCGCTCTCT-3′; P2Y₄ antisense primer, 5′-GCCCTGCACTCATCCCTTTTCT-3′ (433 bp); P2Y₆ sense primer, 5′-CCGCTGAACATCTGTGTC-3′; P2Y₆ antisense primer, 5′-AGAGCCAT-GCCATAGGGC-3′ (464 bp); housekeeping gene *GADPH* sense primer, 5′-CTACTGGCGCTGCCAAGGTGT-3′; and *GADPH* antisense primer, 5′-GCCATGAGGTCCACCACCCTGT-3′ (358 bp). PCR was performed by denaturation at 94°C for 5 minutes followed by 35 cycles of amplification (94°C for 30 seconds, 58°C for 30 seconds, 72°C for 50 seconds), and extension at 72°C for 7 minutes.³⁵ PCR products were resolved on a 1.5% agarose gel.

SDS–polyacrylamide gel electrophoresis and Western blotting

A549 cells were detached with cell scrapers in cold cell-lysis buffer (50 mM HEPES pH 7.4, 150 mM NaCl, 5 mM EDTA, 1 mM DTT, 0.05% Triton X-100, 100 μg/mL PMSE, and 1:1000 protease inhibitor cocktail [Sigma, St Louis, MO]) and were lysed by passage through a 21-gauge needle 10 times. The cell lysate was incubated for 1 hour at 4°C and centrifuged at 10 000g for 10 minutes at 4°C. The protein concentration of the total cell lysate (supernatant) was measured using a Bio-Rad protein assay (Bio-Rad, Mississauga, ON, Canada). Cell lysates (5 μg each) were resolved on 12% SDS-PAGE using a Mini-Protein 3 Electrophoresis Cell (Bio-Rad) at 150 V, 400 mAmp for 1 hour and were transferred to nitrocellulose membrane using a semidry system (Bio-Rad). The membrane was washed twice in dH₂O and was blocked with PBS containing 0.1% Tween-20 and 0.5% nonfat dry milk before overnight incubation with 1 μg/mL polyclonal rabbit anti-human P2Y₆ primary antibody (Affinity BioReagents, Golden, CO) in blocking buffer at 4°C, followed by 1:5000 goat anti-rabbit IgG-HRP secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) in blocking buffer for 1 hour at room temperature. The membrane was washed before peroxidase substrate TMB (3,3′,5,5′-tetramethylbenzidine; Sigma) was added. Anti-human β-actin antibody 2 monoclonal antibody (Alpha Diagnostic International, San Antonio, TX) was used as a loading control for Western blots.

Lactate dehydrogenase cytotoxicity detection

LDH is a stable cytoplasmic enzyme present in most cells and is released into cell-culture supernatant when the cell membrane is damaged. To select the optimal dose of the blocking reagents used, cytotoxicity was evaluated. SAEC or A549 cells seeded in 24-well plates were treated for 30 minutes at

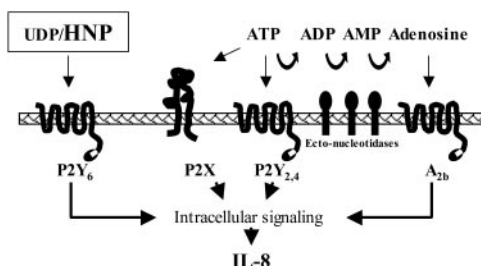


Figure 1. Alternative pathways of nucleotide/nucleoside receptor–mediated IL-8 induction. UDP/HNP induces IL-8 production through the 7-transmembrane, G-protein–coupled receptor P2Y₆, whereas ATP-dependent IL-8 production is mediated by P2Y₂ and P2Y₄ and by P2X ligand-gated ion channel. Extracellularly released ATP is hydrolyzed to ADP, AMP, and nucleoside adenosine by cell-surface–associated ecto-nucleotidases. Adenosine also induces IL-8 production by A_{2b} receptor.²¹

37°C with different concentrations of blocking reagents, including sense and antisense oligonucleotides and nonspecific inhibitors (suramin and reactive blue) of P2Y receptors, followed by incubation for 8 hours with HNP stimulation. Supernatants were then collected, and cells were washed with PBS. Serum-free DMEM (200 μ L) was added, and A549 cells were lysed by freeze-thaw cycles (frozen at -80°C for 30 minutes and thawed at 37°C). Supernatants were pooled and centrifuged at $250g$ for 5 minutes. LDH concentrations in cell-culture supernatants and cell lysates were measured using a Cytotoxicity Detection (LDH) Kit (Roche Applied Science, Penzberg, Germany).

LiquiChip multiple cytokine assay

Cell culture supernatants from indicated experiments were collected for simultaneous measurement of multiple cytokines (IL-1 β , IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, TNF- α , IFN- γ , and GM-CSF) using the LiquiChip Human 10-cytokine kit (Qiagen, Valencia, CA).

IL-8 enzyme-linked immunosorbent assay

To confirm the specificity of IL-8 production after HNP stimulation, IL-8 levels were also measured from cell-culture supernatants by using a human IL-8 ELISA kit (Biosource International, Camarillo, CA). Correlation was excellent ($r = 0.93$; $P < .05$) in IL-8 concentrations measured between the LiquiChip cytokine assay and the ELISA kit.

^{125}I -HNP binding to lung A549 cells

To demonstrate the binding of HNPs on the A549 cell surface, HNPs were labeled with sodium iodine I 125 (^{125}I) using IODO bead iodination reagent (Pierce Biotechnology, Rockford, IL) according to the manufacturer's instruction. ^{125}I -HNP, at a final concentration ranging from 0 to 0.8 μM , was added to A549 cells in DMEM for total binding. To determine specific ^{125}I -HNP binding, a parallel assay was carried out in A549 cells by 30-minute pretreatment with a 40-fold excess concentration of unlabeled HNPs before addition of the ^{125}I -HNP dilutions. Cells were then incubated on ice with ^{125}I -HNP for another 30 minutes, washed, and dissolved in NaOH before radioactive counting (Auto-Gamma 5650; Packard Instruments, Downers Grove, IL).

Competitive binding of HNP with nucleotides on cell-surface P2Y receptors (cell ELISA)

A549 cells (5×10^4 /well) seeded overnight in 96-well plates were washed in PBS, and 50 μL serum-free DMEM was added. To perform binding assays at an equimolar ratio, molar concentrations of HNP and a 40-fold excess molar concentration of the competitors (ATP and UDP) were calculated (ie, 10 $\mu\text{g}/\text{mL}$ HNP is equal to 2.8 μM ; UDP or ATP concentration was calculated by $2.8 \times 40 \mu\text{M}$ multiplied by the molecular weight of UDP or ATP and then converted to a corresponding concentration [in $\mu\text{g}/\text{mL}$] for stock preparation, respectively). Because the composition of the HNP mixture was known after purification of the sputum from patients with cystic fibrosis and HNP-1 (MWt 3442) is a more abundant isoform than HNP-2 (MWt 3371) and HNP-3 (MWt 3486), we used the molecular mass of HNP-1, which is close to the mean value of the molecular masses of HNP-1, HNP-2, and HNP-3, to estimate the molar concentration of the HNP mixture. Cells were then incubated with serial dilutions of HNP (0–0.3 μM) at room temperature for 30 minutes, with or without pretreatment with serial dilutions of ATP or UDP (0–100 μM) (40 M excess of HNP concentrations) at room temperature for 30 minutes. After they were washed with PBS, the cells were fixed with 4% paraformaldehyde in PBS for 15 minutes, followed by blocking with 0.5% Tween-20 and 0.5% milk in PBS for 30 minutes. HNP binding to the cell surface was measured by using rabbit polyclonal anti-HNP antibody (10 ng/mL; Host Defense Research Center, Toronto, ON, Canada), HRP-conjugated goat anti-rabbit antibody (1:4000 dilution) (Jackson ImmunoResearch), and TMB substrate (Sigma) with thorough washing at every step. The reaction was stopped by the addition of 1 M sulfuric acid. The absorbance of each well was measured by a microtiter plate reader at 450 nm.

Statistical analysis

Data are expressed as mean \pm SEM. Two-way ANOVA, followed by the Turkey/Kramer test, was used for statistical analysis. Differences were considered statistically significant at P values less than .05.

Results

HNP selective induction of IL-8 in lung epithelial cells

Stimulation of A549 cells with various concentrations of HNP resulted in the induction of IL-8 in the 10 cytokines assayed (Figure 2A). HNP-induced IL-8 production was dose dependent, ranging from 33 ± 6 pg/mL in control to 1201 ± 79 pg/mL at 100 $\mu\text{g}/\text{mL}$ HNP, the highest concentration tested. Importantly, HNP-induced IL-8 release was apparent at doses as low as 3 $\mu\text{g}/\text{mL}$.

To examine whether the selective induction of IL-8 was caused by deficient production of other cytokines by A549 cells, recombinant human TNF- α at 0 to 50 ng/mL was used to challenge the cells because TNF- α is a known stimulus to induce multiple cytokines in A549 cells.³⁸ Stimulation of A549 cells with TNF- α induced the dose-dependent production of IL-1 β , IL-2, IL-6, and interferon- γ in addition to IL-8 (Figure 2B). Although the TNF- α level increased dramatically (data not shown), it was unclear whether the increase was associated with autocrine stimulation or with another mechanism.

ATP- and UDP-selective induction of IL-8 in lung epithelial cells

As discussed, we observed that HNPs selectively induced IL-8 production in 10 pro- and anti-inflammatory cytokines examined. Several nucleotides induced similar patterns of IL-8 response in a variety of cell types through different intracellular signaling pathways. To examine whether HNPs induced IL-8 production through nucleotide signaling pathways, A549 cells were incubated

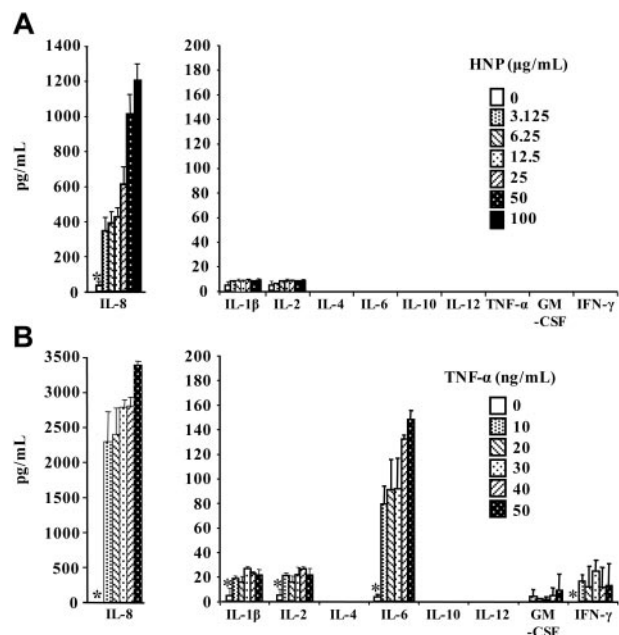
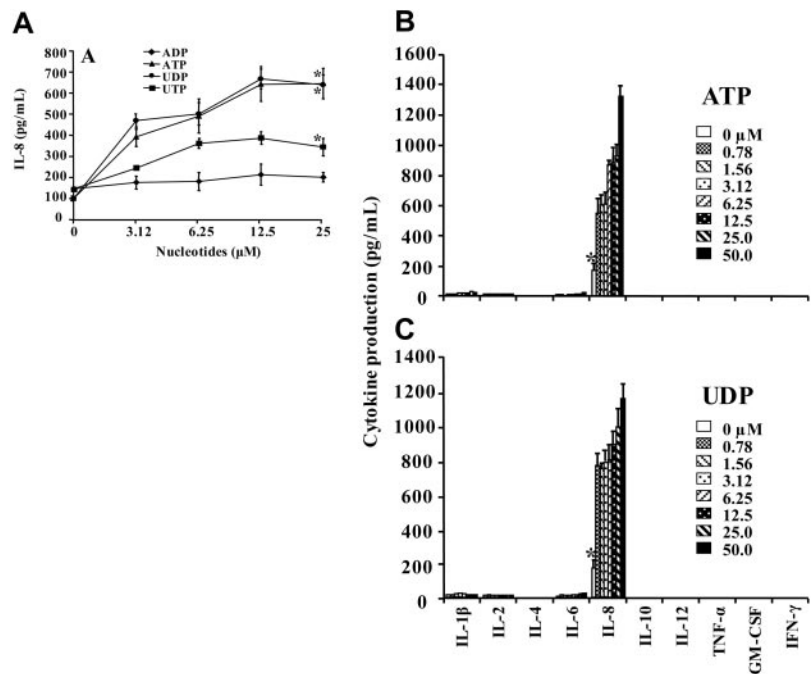


Figure 2. Cytokine profile of A549 cells in response to HNP and TNF- α . A549 cells (2.5×10^5 cells/well in 24-well plate) were incubated in serum-free DMEM containing indicated concentrations of HNP (A) and recombinant human TNF- α (B) for 8 hours. Multiple cytokines in culture supernatants were simultaneously measured ($n = 3$). Concentrations of HNP and TNF- α were progressively increased, as indicated in the bar graphs. * $P < .05$; control (0) compared with HNP or TNF- α at all concentrations, respectively.

Figure 3. Selective induction of IL-8 by ATP and UDP in A549 cells. (A) A549 cells were incubated for 8 hours in serum-free DMEM containing indicated concentrations of ATP, ADP, UTP, and UDP, and IL-8 levels in cell-culture supernatants were measured with the use of enzyme-linked immunosorbent assay (ELISA). (B) Because only ATP and UDP induce significant IL-8 production, multiple cytokines were measured in the cells stimulated with indicated concentrations of ATP and UDP for 8 hours (n = 3). ATP and UDP concentrations were progressively increased, as indicated in the bar graphs. **P* < .05; control (0) compared with ATP or UDP at all concentrations, respectively.



for 8 hours in the presence and absence of the nucleotides ATP, adenosine diphosphate (ADP), UTP, and UDP (3.12-25 µM). IL-8 concentrations in the cell-culture supernatants were then determined. Figure 3A shows that ATP and UDP induced the dose-dependent production of IL-8 from a concentration as low as 3.12 µM. UTP tended to increase, but ADP had no effect on IL-8 production. UTP is degraded into UDP, which may contribute to IL-8 production.

We further focused on cytokine profiles of the cells in response to a broad range of ATP and UDP concentrations. As with HNP stimulation, ATP and UDP selectively induced IL-8 production in 10 cytokines tested (Figure 3B-C). These observations suggest that HNP and nucleotides may share common cellular signaling pathways in mediating IL-8 production.

Nucleotides mediate HNP-induced IL-8 production from lung epithelial cells

Given that nucleotides are ligands for P2Y receptors, we examined whether blocking P2Y receptors would result in the attenuation of HNP-induced IL-8 production. When A549 cells were pretreated with the nonspecific P2Y receptor antagonists suramin and reactive blue at 100 µM, HNP-induced IL-8 production was blunted (Figure 4A). This inhibitory effect was not caused by cytotoxicity, as assessed by cell viability and LDH release (data not shown).

We theorized 2 mechanisms by which HNPs induce IL-8 production: (1) HNPs act directly on cell-surface nucleotide receptors; (2) HNPs act on cells through unknown mechanisms, resulting in the release of nucleotides that induce IL-8 production through nucleotide receptors.

To examine whether HNPs can directly bind to surface nucleotide receptors, competitive HNP binding on the A549 cell surface was assessed by the addition of ¹²⁵I-HNP. Figure 4B illustrates that HNPs bound to the cell surface, but this binding was not blocked by ATP or UDP (Figure 4C), suggesting that HNPs do not bind directly to surface P2Y receptors or, at least, that they do not share binding sites with the nucleotides.

We measured extracellularly released ATP and found no significant increase after HNP stimulation (data not shown). Other nucleotides were not measured because of the lack of reliable

assays. Nucleotides can be readily degraded or can undergo interconversion by cell-surface-associated enzymes. Thus, we focused on examining the role of nucleotide signaling by inhibiting the expression of nucleotide receptors rather than indirectly measuring extracellularly released nucleotides.

ATP-dependent signaling plays a minor role in HNP-induced IL-8 production

Although HNPs did not induce ATP release, it remained unknown whether HNP-induced IL-8 release is mediated by ATP signaling

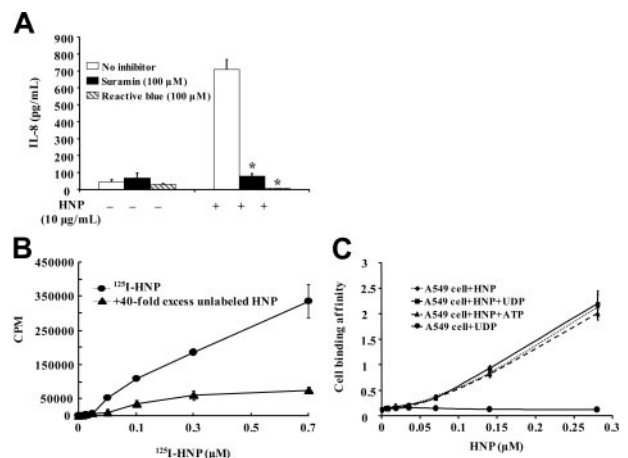


Figure 4. Effects of P2Y receptor antagonists suramin and reactive blue on HNP-induced IL-8 production, and HNP binding on A549 cells. (A) A549 cells (2.5×10^5 cells/well in 6-well plate) were incubated in serum-free DMEM containing 100 µM suramin or reactive blue for 30 minutes, followed by the addition of 10 µg/mL HNP for 8 hours. IL-8 levels in supernatants were measured. **P* < .05 suramin or reactive blue compared with no inhibitor, respectively. (B) A549 cells (5×10^4 cells/well in 96-well plate) were incubated in serum-free DMEM containing 0 to 0.7 µM ¹²⁵I-labeled HNP for 30 minutes on ice, with or without pretreatment with 40-fold molar excess concentration of unlabeled HNP for 30 minutes. Cell-associated HNP binding was measured by radioactivity counting. CPM indicates counts per minute. (C) HNPs do not compete with ATP or UDP in engaging cell-surface binding sites. A549 cells were incubated in serum-free DMEM containing 0 to 2.8 µM HNP for 30 minutes, with or without pretreatment with 0 to 100 µM ATP or UDP for 30 minutes. A control group of cells incubated with UDP in the absence of HNP was included. Cell-surface HNP binding was measured by HNP-cell ELISA (n = 3).

pathways including P2Y₂ (ATP = UTP) and P2Y₄ (UTP >> ATP) receptors, P2X ligand-gated ion channels,²⁶ and adenosine resulting from ATP degradation by nucleotidases through A_{2b} receptor^{30,31} (Figure 1). Involvement of these pathways was investigated with the use of specific blockers.

Given that no inhibitors or antibodies are available against specific P2Y receptor phenotypes, antisense oligonucleotides targeting translation initiation sites of P2Y mRNA were used to examine the roles of P2Y₂ and P2Y₄ receptors in mediating HNP-induced IL-8 production. Figure 5A shows that resting A549 cells constitutively expressed P2Y₄ mRNA but not P2Y₂ mRNA. Treatment of the cells with P2Y₂ and P2Y₄ antisense oligonucleotides did not attenuate HNP-induced IL-8 production (Figure 5B).

We next examined the roles of P2X receptor and adenosine (A_{2b}) receptor in HNP-induced IL-8 induction by using specific antagonists. Although PPADS, a P2X receptor inhibitor, and alloxazine, an inhibitor of adenosine A_{2b} receptor, decreased HNP-induced IL-8 production compared with nontreated cells, the differences did not reach statistical significance, indicating a minor role of P2X and adenosine receptors in mediating HNP-induced IL-8 production (Figure 5C).

P2Y₆ signaling regulates HNP-induced IL-8 production

No reliable, sensitive assay is available to measure the extracellular concentration of UDP (the ligand of P2Y₆). The role of the UDP-P2Y₆ signaling pathway in mediating HNP-induced IL-8 production was examined using P2Y₆ antisense oligonucleotides. A549 cells constitutively express high levels of P2Y₆ mRNA (Figure 6A). The use of P2Y₆ antisense oligonucleotides blunted the expression of P2Y₆ at mRNA (Figure 6A) and protein (Figure 6B) levels. Treatment of cells with P2Y₆ antisense oligonucleotides resulted in approximately 60% reduction in HNP-induced IL-8 production compared with control ($P < .05$) (Figure 6C). Similarly, when the experiments were repeated in primary human small airway epithelial cells, the use of P2Y₆ antisense oligonucleotide attenuated HNP-induced IL-8 release by approximately 70% compared with control ($P < .05$; Figure 6D).

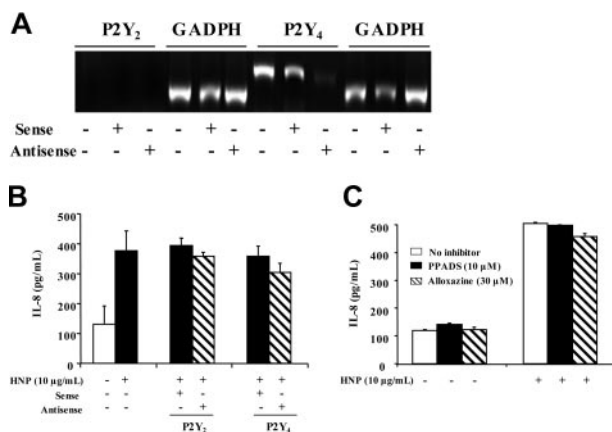


Figure 5. Role of P2X, P2Y₂, P2Y₄, and A_{2b} receptors in HNP-induced IL-8 production. (A) A549 cells were incubated in serum-free DMEM and lipofectamine reagent preparation containing 2.5 µM P2Y₂ and P2Y₄ sense or antisense oligonucleotides, respectively, for 4 hours. RT-PCR of P2Y₂ mRNA and P2Y₄ mRNA analysis was performed. (B) Cells were incubated in serum-free DMEM and transfected with P2Y₂ and P2Y₄ sense or antisense oligonucleotides for 4 hours, washed with complete DMEM, and incubated overnight. After washing with PBS and incubation in serum-free DMEM containing 10 µg/mL HNP for 8 hours and IL-8 concentration was measured in supernatants ($n = 3$). (C) Cells were incubated in serum-free DMEM containing a final concentration of 10 µM PPADS or 30 µM alloxazine for 30 minutes, followed by the addition of 10 µg/mL HNP for 8 hours. IL-8 levels were measured in cell-culture supernatants ($n = 3$).

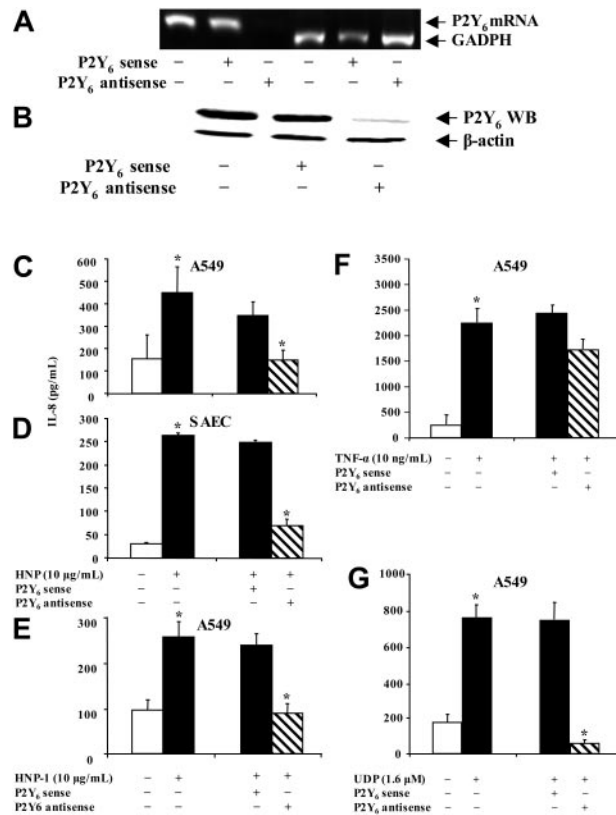


Figure 6. P2Y₆ mediates HNP-induced IL-8 production in human lung epithelial cells. A549 cells grown in complete DMEM were transfected with 2.5 µM P2Y₆ sense or antisense oligonucleotides for 4 hours. After washing, cells were incubated in complete medium overnight. (A) Total RNA was extracted, and RT-PCR of P2Y₆ mRNA was performed. (B) Total P2Y₆ protein level was determined from cell lysates by Western blotting (WB). After transfection with the oligonucleotides overnight, A549 cells (C) or SAEC cells (D) were incubated in serum-free DMEM containing the purified HNP for 8 hours. Three control groups were included to test the specificity of the antisense oligonucleotides on IL-8 release after stimulation with commercial synthetic HNP-1 (E), TNF-α (F), or UDP (G) in A549 cells. IL-8 levels in cell-culture supernatants were measured ($n = 3$). * $P < .05$; sense compared with antisense.

To confirm the results obtained by using the HNP mixture purified from patients with cystic fibrosis, experiments were repeated using commercially available synthetic HNP-1 and A549 cells because HNP-1 is the most abundant isoform among HNPs that include HNP-1, HNP-2, and HNP-3 in granule content in neutrophils^{36,37,39,40} and in the purified HNP mixture used. A similar inhibition of the HNP-1-induced IL-8 production was reproduced by the use of P2Y₆ antisense oligonucleotide, but the cells showed much less IL-8 release in response to the commercially synthesized HNP-1 than to the purified HNP mixture (Figure 6E). These results indicate that HNP-1 stimulated the cells to produce IL-8, but the potency of the commercial HNP-1 was low. This may be explained by several factors: (1) the synthesized form contained molecular components that were not HNP-1; (2) proper folding was not achieved; and (3) the stimulating activity of individual peptide (ie, HNP-1) was low compared with the results using combined HNPs.

To further test the specificity of the blocking effect of P2Y₆ antisense nucleotides on HNP-stimulated IL-8 release, A549 cells were stimulated with TNF-α and UDP, respectively, in the presence and absence of the P2Y₆ antisense nucleotides for 8 hours. TNF-α-induced IL-8 release was not affected, but UDP-induced IL-8 release was completely blunted by the antisense nucleotide (Figure 6F-G), suggesting that HNP-induced IL-8 release may occur through the UDP pathway, distinct from the TNF-α signaling pathway.

Discussion

The main findings of the present study are that HNPs selectively induced IL-8 production in 10 common pro- and anti-inflammatory cytokines examined. NP-induced IL-8 production is dominantly regulated through the P2Y₆ signaling pathway in the otherwise quiescent, nonprimed lung epithelial cells.

Neutrophils release large amounts of protein into the extracellular milieu as a consequence of degranulation, leakage during phagosome formation, and cell death and lysis. High concentrations of HNP associated with increased levels of IL-8 have been reported in the lung lavage fluid, sputum, and plasma of patients with a variety of inflammatory lung diseases.^{6,7,41} We have demonstrated that HNPs at clinically relevant concentrations seen in the lung diseases can initiate inflammation by producing IL-8 and neutrophil migration, resulting in acute lung injury in vivo in mice.³³ Because HNPs are not direct chemoattractants for neutrophils, neutrophil infiltration is largely dependent on the release of IL-8 by lung epithelial cells in response to HNP stimulation.^{42,43} Thus, an understanding of the mechanisms by which HNPs induce IL-8 production would provide potential therapeutic approaches to control inflammatory courses.

We demonstrated selective induction of IL-8 production in 10 cytokines tested by HNPs in resting epithelial cells. By comparison, at a given time, the same cells stimulated with TNF- α or LPS were able to release multiple cytokines, including IL-1 β , IL-6, and TNF- α . A recent study reported that though HNPs induced the up-regulation of IL-1 β and IL-8 at gene levels, only IL-8 production was increased at the protein level in primary human bronchial epithelial cells.⁴³ Our findings are in agreement with other studies reporting that HNPs can induce multiple cytokines in the presence of a costimulus. When human monocytes were concurrently activated with *Staphylococcus aureus* or PMA, HNPs induced TNF- α and IL-1 β expression.⁴⁴ In subcultures in which A549 cells were preincubated overnight with dexamethasone and subsequently were stimulated for 6 hours with HNPs, van Wetering et al¹⁵ reported increased levels of IL-8 and epithelial neutrophil activating peptide 78 (ENA-78). When CD3e-activated splenic and Peyer patch T cells isolated from mice were incubated with HNPs, an enhanced secretion of T_H1 and T_H2 cytokines was observed.³⁷ Taken together, these data suggest that HNPs selectively induce IL-8 production through specific mechanisms in resting eukaryotic cells.

As with HNPs, lung epithelial-cell stimulation with ATP and UDP selectively induced IL-8 production, suggesting a linkage between HNPs and nucleotides in the sharing of common signaling pathways. UDP alone has been shown to induce IL-8 release in the THP-1 monocytic cell line and human mature dendritic cells,^{27,28} whereas ATP and UDP stimulate human eosinophils to produce IL-8.²⁶ In light of the direct stimulatory role of nucleotides to selectively induce IL-8 in different cell types^{26,27} and the similar effects of HNPs and nucleotides, we demonstrated that the use of the P2 receptor antagonists suramin and reactive blue almost completely blocked HNP-induced IL-8 production. However, there appeared to be no direct interaction between HNP and P2 receptors because ATP and UDP did not compete with HNPs on cell-surface binding, or at least HNPs did not appear to share the same binding sites with ATP and UDP.

To further investigate the specific pathways involved in HNP-mediated IL-8 production, we focused on identifying the specific types of nucleotide receptors rather than the specific nucleotides

involved, and we did so for several reasons. First, nucleotides are rapidly released in response to stimuli, and their autocrine functions are rapidly regulated by cell-surface-associated extracellular nucleotidases.^{29,45} Second, cell-surface-associated nucleoside diphosphokinase enzymes can rapidly convert the adenine nucleotides into uracil nucleotides, or vice versa. Third, nucleotides and their hydrolyzed products, nucleosides, exert biologic activities through a large family of receptors that display overlapping sensitivity to different agonists.²² Measurement of the nucleotide concentrations thus may not provide an accurate explanation to identify the specific pathways.

Several nucleotide receptor signaling pathways can be involved in IL-8 production in response to HNPs. ATP induces IL-8 production partially through ATP-gated ion channel P2X²⁶ and adenosine receptor as a result of ATP degradation.^{30,31,45} Through the use of specific inhibitors, our results indicated that neither the P2X receptor nor the adenosine A_{2b} receptor played a significant role in mediating IL-8 production in response to HNP stimulation. Interestingly, a recent study demonstrated that the human cathelicidin-derived cationic peptide LL37, found in neutrophils, in bone marrow-derived cells, and in epithelial cells may promote IL-1 β production in LPS-primed monocytes through the activation of P2X₇ receptors.⁴⁶

We demonstrate that the mRNAs of P2Y receptors P2Y₄ and P2Y₆, but not P2Y₂, express in lung epithelial cells. These P2Y receptors are ligands for UTP, UDP, and ATP, respectively, though P2Y₄ also recognizes ATP. Antisense oligonucleotides were used to block expression of the P2Y receptors because of the lack of available specific inhibitors and antibodies. We found that the inhibition of P2Y₆ receptor expression dramatically reduced HNP-induced IL-8 production by approximately 60% and by more than 70% in A549 cells and in primary human small airway epithelial cells, respectively. These results, obtained by using P2Y₆ receptor antisense oligonucleotides, are consistent with the fact that an almost complete attenuation of IL-8 was achieved by using the P2Y inhibitor reactive blue, which is more specific to P2Y₆ than to P2Y₄ receptor.^{22,28}

The exact mechanisms by which HNPs signal through the P2Y₆ receptor remain unknown. Several possibilities are under investigation. One is that HNPs interact with the P2Y₆ receptor through binding sites that are distinct from those for UDP binding; HNPs act at the cell surface through interaction with one or more specific ligands, which in turn activate P2Y₆. Another is that an anchor or a coligand(s) associated with the P2Y₆ receptor is required for HNP binding and action. Yet another is that HNP induces the up-regulation of UDP after an unknown mechanism that activates the P2Y₆ receptor, resulting in specific IL-8 induction. Selective induction of IL-8 by UDP has been well documented under different in vitro conditions.^{27,28,47,48} Additionally, HNPs modulate the affinity of P2Y₆ for UDP. Finally, HNPs may synergize with UDP.

The P2Y₁₄ receptor has been recently cloned, and P2Y₁₄ mRNA is expressed in A549 cells, the bronchial epithelial cells (BEAS-2B), and primary alveolar epithelial type 2 cells.²⁴ It has been demonstrated that the P2Y₁₄ receptor specifically responds to UDP glucose but not to ATP, ADP, UTP, or UDP.^{23,49} Furthermore, the present study shows that the use of P2Y₆ antisense nucleotides resulted in complete inhibition of IL-8 release induced by stimulation with HNP. Thus, we do not anticipate that P2Y₁₄ is significantly involved in the HNP-P2Y₆ signaling pathway.

In conclusion, we demonstrate that HNP-induced IL-8 production in epithelial cells is predominantly regulated by the 7-transmembrane, G-coupled-protein P2Y₆ receptor. However, the binding of HNPs to the cell surface is not competitively blocked by excessive

amounts of UDP, a specific ligand of P2Y₆ receptor, suggesting that mechanisms other than direct interaction between HNPs and P2Y₆ are involved. The current study provides potential therapeutic

implications to modulate HNP-P2Y₆-induced excessive inflammatory responses without interrupting charge-dependent antimicrobial activity of HNPs in inflammatory conditions.

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