Human neutrophil peptides induce interleukin-8 in intestinal epithelial cells through the P2 receptor and ERK1/2 signaling pathways

KAZUNARI IBUSUKI, TOSHIO SAKIYAMA, SHUJI KANMURA, TAKURO MAEDA, YUJI IWASHITA, YUICHIRO NASU, FUMISATO SASAKI, HIROKI TAGUCHI, SHINICHI HASHIMOTO, MASATSUGU NUMATA, HIROFUMI UTO, HIROHITO TSUBOUCHI and AKIO IDO

Digestive and Lifestyle Diseases, Department of Human and Environmental Sciences, Kagoshima University Graduate School of Medical and Dental Sciences, Kagoshima 890-8544, Japan

Received December 8, 2014; Accepted March 6, 2015

DOI: 10.3892/ijmm_xxxxxxxx

Abstract. Human neutrophil peptides (HNPs) are antimicrobial peptides produced predominantly by neutrophils. We have previously reported that HNP 1-3 levels are increased in the sera and plasma of patients with active ulcerative colitis. The increased expression of interleukin-8 (IL-8) has also been demonstrated in the colonic mucosa of patients with active ulcerative colitis. HNPs induce IL-8 in lung epithelial cells and monocytes through the $P2Y_6$ signaling pathway. However, the association between HNPs and IL-8 in the intestinal mucosa has not yet been investigated. In the present study, we investigated the effects of HNP-1 on the production of IL-8 by human intestinal epithelial cells and the underlying signaling mechanisms. We observed a significant increase in IL-8 expression in the human colon carcinoma cell line, Caco-2, following treatment with HNP-1. The non-selective P2 receptor antagonists, suramin and pyridoxal phosphate-6-azo (benzene-2,4-disulfonic acid) tetrasodium salt hydrate (PPADS), significantly blocked the HNP-1-induced expression of IL-8 in the Caco-2 cells. The $P2Y_6$-specific antagonist, MRS2578, led to a significant but partial decrease in IL-8 expression, suggesting that P2 receptors in addition to $P2Y_6$ are involved in the HNP-1-induced production of IL-8 by Caco-2 cells. In agreement with this finding, HNP-1 also significantly increased IL-8 production in the $P2Y_6$-negative human colon cancer cell line, HT-29, and this increase was blocked by treatment with suramin and PPADS. HNP-1 significantly increased the phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2) and p38 mitogen-activated protein kinase (MAPK) in the HT-29 cells. However, the HNP-1-induced production of IL-8 was suppressed by the ERK1/2 inhibitor, U0126, but not by the p38 MAPK inhibitor, SB203580. In conclusion, our data demonstrate that HNP-1 induces IL-8 production not only through $P2Y_6$, but also through additional P2 receptors via an ERK1/2-dependent mechanism in intestinal epithelial cells.

Correspondence to: Dr Shuji Kanmura, Digestive and Lifestyle Diseases, Department of Human and Environmental Sciences, Kagoshima University Graduate School of Medical and Dental Sciences, 8-35-1 Sakuragaoka, Kagoshima 890-8544, Japan
E-mail: skanmura@m2.kufm.kagoshima-u.ac.jp

Key words: human neutrophil peptide, interleukin-8, intestinal epithelial cell, P2 receptor, extracellular signal-regulated kinase 1/2

Introduction

Inflammatory bowel disease (IBD), including ulcerative colitis (UC) and Crohn's disease (CD), is a group of chronic inflammatory disorders of the gastrointestinal tract. The incidence of IBD is more frequent in Western countries, but it is rapidly increasing in Asian populations (1). Although the pathogenesis of IBD remains unknown, genetic and environmental factors resulting in an aberrant immune response to commensal bacteria seem to play a pivotal role in the development of IBD (2). One of the key histological characteristics of IBD, particularly in UC, is the accumulation of neutrophils in crypt lumens. Neutrophils provide the first line of cellular immune defense against foreign microbes. However, uncontrolled neutrophil trafficking has been implicated in the pathogenesis of IBD (3).

Human neutrophils generate four α-defensins, human neutrophil peptides (HNPs) 1 to 4. We have previously reported that the plasma concentrations of HNP 1-3 in patients with active UC are higher than in healthy subjects or in those with inactive UC, CD or infectious enterocolitis (4). Thus, HNP 1-3 are considered to be useful biomarkers that may be used to diagnose and predict treatment outcomes in patients with UC. Moreover, we demonstrated that high concentrations of HNP-1 aggravated dextran sodium sulfate (DSS)-induced colitis by elevating the levels of inflammatory cytokines, suggesting a potential pro-inflammatory role for HNP-1 in colitis (5). In addition to their direct antimicrobial abilities, HNPs have a broad range of immune activation functions. HNPs are chemotactic *in vitro* for human monocytes, T-cells and immature dendritic cells (6-8). HNPs induce the production of interleukin-8 (IL-8, also known as CXCL8) by epithelial cells of the lungs and bronchus (9-14),

monocytes (13), lung fibroblasts (14), conjunctival epithelial cells (15) and rheumatoid fibroblast-like synoviocytes (16). IL-8 primarily mediates the activation and migration of neutrophils into tissue from peripheral blood. In addition to this pro-inflammatory function, IL-8 is also known to be a potent promoter of angiogenesis (17). The increased expression of IL-8 in the colonic tissues of patients with UC has been demonstrated and may contribute to the pathogenesis of UC (18,19). The serum concentrations of IL-8 have also been shown to be related to the endoscopic and histological severity of UC (20). In lung epithelial cells and monocytes, the HNP-induced production of IL-8 is regulated by the $P2Y_6$ receptor (10,13). P2 receptors are activated by extracellular nucleotides. These receptors are divided into two subfamilies: ligand-gated ion channels (P2X) and G-protein-coupled receptors (P2Y). Both P2X and P2Y are expressed widely throughout the intestinal tract and participate in the regulation of a variety of physiological functions (21). However, the association among HNPs, IL-8 and P2 receptors in the intestinal mucosa has not yet been investigated. In the present study, we sought to determine whether HNP-1 induces IL-8 in intestinal epithelial cells, and if so, to elucidate the mechanisms that underlie this activity.

Materials and methods

Chemicals. The synthetic products of HNP-1 were purchased from Peptide Institute, Inc. (Osaka, Japan). MEM and McCoy's 5A medium, fetal bovine serum, penicillin-streptomycin, L-glutamine and the IL-8 ELISA kit were obtained from Life Technologies Corp. (Carlsbad, CA, USA). Suramin sodium (non-selective P2 receptor antagonist) was obtained from Wako Pure Chemical Industries (Osaka, Japan). Pyridoxal phosphate-6-azo (benzene-2,4-disulfonic acid) tetrasodium salt hydrate (PPADS, another non-selective P2 receptor antagonist) was obtained from Sigma-Aldrich Japan (Tokyo, Japan). U0126 [extracellular signal-regulated kinase 1/2 (ERK1/2) inhibitor] and SB203580 [p38 mitogen-activated protein kinase (MAPK) inhibitor] were obtained from Calbiochem (Darmstadt, Germany). MRS2578 ($P2Y_6$-specific antagonist) was obtained from Tocris Bioscience (Ellisville, MO, USA).

Cell culture. The human colon carcinoma cell line, Caco-2, was obtained from RIKEN BioResource Center (Ibaraki, Japan). The Caco-2 cells were grown in minimal essential medium (MEM) containing 20% heat-inactivated fetal bovine serum, 100 μg/ml streptomycin, 100 μg/ml penicillin and 2 mM L-glutamine. The Caco-2 cells were incubated with 50 μg/ml HNP-1 with or without 100 μM suramin, 100 μM PPADS or 10 μM MRS2578 for 24 h. The human colon cancer cell line, HT-29, was obtained from DS Pharma Biomedical Co., Ltd. (Osaka, Japan). The HT-29 cells were grown in McCoy's 5A medium containing 10% heat-inactivated fetal bovine serum, 100 μg/ml streptomycin, 100 μg/ml penicillin and 2 mM L-glutamine. The HT-29 cells were incubated with various concentrations of HNP-1 (0-50 μg/ml), or with 50 μg/ml HNP-1 with or without 100 μM suramin, 100 μM PPADS, 10 μM MRS2578, 1 or 5 μM U0126, or 1 or 5 μM SB203580 for 24 h. For western blot analysis, the HT-29 cells were incubated with 50 μg/ml HNP-1 for 30 min. Both cell lines were maintained in a humidified 5% CO_2 incubator at 37˚C.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from the cells using Isogen (Nippon Gene, Co., Ltd., Toyama, Japan) according to the manufacturer's instructions. The RNA was reverse transcribed using the PrimeScript RT reagent kit (Takara Bio, Otsu, Japan). The synthesized cDNA was amplified using SYBR Premix Ex Taq II (Takara Bio) and analyzed using the StepOnePlus Real-Time PCR system and StepOne Software version 2.0 (Applied Biosystems, Foster City, CA, USA). The primers for IL-8 (Primer set ID: HA032483), $P2Y_2$ (HA086668) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (HA067812) were purchased from Takara Bio. The cycling conditions were as follows: one cycle at 95˚C for 30 sec followed by 35 cycles each at 95˚C for 5 sec and 60˚C for 34 sec. To normalize the amount of total RNA present in each reaction, the GAPDH gene was used as an internal standard.

RNA silencing of the $P2Y_2$ receptor. Predesigned short interfering RNA (siRNA) specific for human $P2Y_2$ (Stealth RNAi, siRNA ID: HSS143207) and the negative control (Stealth RNAi siRNA Negative Control), Lipofectamine RNAiMAX transfection reagent and Opti-MEM were purchased from Life Technologies Corp. The siRNA was mixed with Lipofectamine RNAiMAX in Opti-MEM and allowed to form complexes for 20 min at room temperature. The complexes were then added to 50% confluent HT-29 cells.

Western blot analysis. Equal amounts of cell lysates from the HT-29 cells were run on 10% sodium dodecylsulfate polyacrylamide gels and electroblotted onto polyvinylidene fluoride membranes. After blocking overnight at 4˚C with 5% non-fat milk, the blots were probed with primary antibodies for 1 h at room temperature. Polyclonal rabbit antibodies against phosphorylated ERK1/2 (p-ERK1/2; 9101) and phosphorylated c-jun N-terminal kinase (p-JNK; 9251), as well as monoclonal rabbit antibody against phosphorylated p38 MAPK (p-p38 MAPK; 4511) were purchased from Cell Signaling Technology (Danvers, MA, USA). Monoclonal mouse antibody against β-actin (A5441) was purchased from Sigma-Aldrich (St. Louis, MO, USA). After incubating the membrane with the appropriate peroxidase-conjugated secondary antibodies (MP Biomedicals, Santa Ana, CA, USA) for 1 h at room temperature, the reactivity was visualized using an electrogenerated chemiluminescence detection kit (GE Healthcare Biosciences, Tokyo, Japan).

Statistical analysis. All experiments were repeated three times with cells at different passage numbers. Statistical analysis was performed using Tukey's honest significant difference method with SPSS 15.0J software (SPSS, Inc., Chicago, IL, USA) A value of $P<0.05$ was considered to indicate a statistically significant difference.

Results

HNP-1 upregulates IL-8 expression partly through $P2Y_6$ receptors in Caco-2 cells. We first investigated whether HNP-1 increases IL-8 expression in intestinal epithelial cells by using Caco-2 cells that possess mRNA for several P2 receptor subtypes, including $P2Y_6$ (22,23). Incubation of the Caco-2 cells with

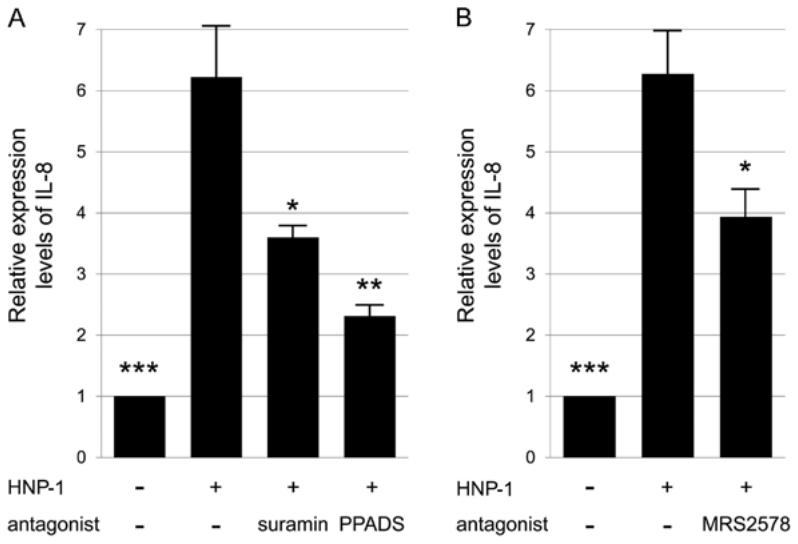

Figure 1. Involvement of P2 receptors in human neutrophil peptide-1 (HNP-1)-induced interleukin-8 (IL-8) expression in Caco-2 cells. (A) Caco-2 cells were incubated with 50 μg/ml HNP-1 with or without suramin (100 μM) or pyridoxal phosphate-6-azo(benzene-2,4-disulphonic acid) (PPADS) (100 μM) for 24 h. (B) Caco-2 cells were incubated with 50 μg/ml HNP-1 with or without 10 μM MRS2578 for 24 h. The mRNA expression of IL-8 was measured by RT-qPCR. The expression levels in the untreated cells were set to 1. Data are the means ± SE from three experiments. Significant differences relative to the HNP-1-treated cells with no antagonist are indicated as follows: *P<0.05; **P<0.01; ***P<0.001.

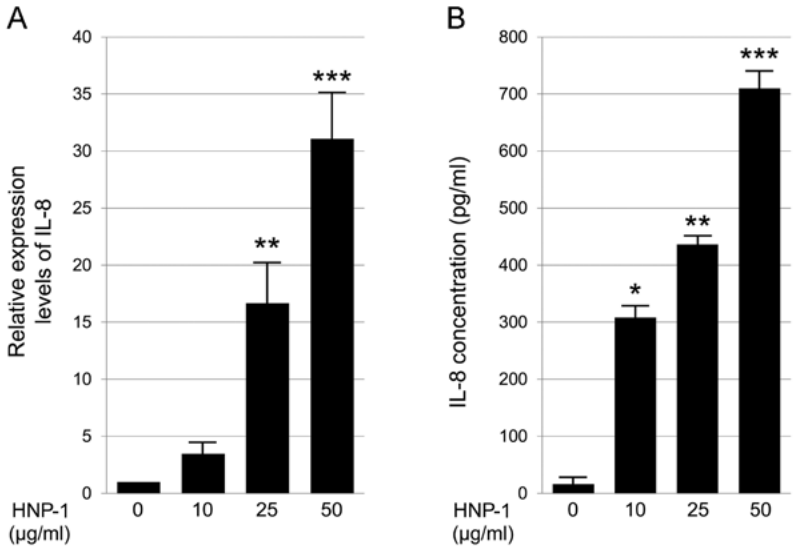

Figure 2. Human neutrophil peptide-1 (HNP-1) induces interleukin-8 (IL-8) production in a dose-dependent manner in $P2Y_6$-negative HT-29 cells. (A) HT-29 cells were incubated with varying concentrations of HNP-1 for 24 h and the mRNA expression of IL-8 was measured by RT-qPCR. The expression levels in the untreated cells were set to 1. (B) The IL-8 levels in culture supernatants from (A) were measured using ELISA. Data are the means ± SE from three experiments. Significant differences relative to untreated cells are indicated as follows: *P<0.05; **P<0.01; ***P<0.001.

50 μg/ml HNP-1 significantly increased the mRNA expression of IL-8 (Fig. 1A). To determine the involvement of P2 receptors in the HNP-1-induced expression of IL-8, the Caco-2 cells were treated with two non-selective P2 receptor antagonists, suramin and PPADS. Both antagonists significantly blocked the HNP-1-induced expression of IL-8 (Fig. 1A). In addition, treatment with the $P2Y_6$-specific antagonist, MRS2578, significantly decreased the expression of IL-8 (Fig. 1B). These data suggest that HNP-1 induces IL-8 expression through the $P2Y_6$ signaling pathway in intestinal epithelial cells. However, MRS2578 only caused a partial reduction (37%) in IL-8 expression (Fig. 1B), suggesting that P2 receptors other than $P2Y_6$ are involved in the HNP-1-induced IL-8 expression.

HNP-1 significantly increases IL-8 production through P2 receptors in $P2Y_6$-negative HT-29 cells. To determine the non-$P2Y_6$-mediated mechanisms underlying the HNP-1 induction of IL-8, we used HT-29 cells in the subsequent experiments, since HT-29 cells have no, or very low levels of $P2Y_6$ mRNA expression (24). Exposure of the HT-29 cells to HNP-1 significantly increased IL-8 mRNA expression in a dose-dependent manner (Fig. 2A). Consistent with the induction of IL-8 expression, the release of IL-8 protein by the HT-29 cells was significantly enhanced by HNP-1 (Fig. 2B). This increase was effectively blocked by suramin and slightly, although significantly by PPADS (Fig. 3A), indicating the involvement of P2 receptors in the HNP-1-induced production

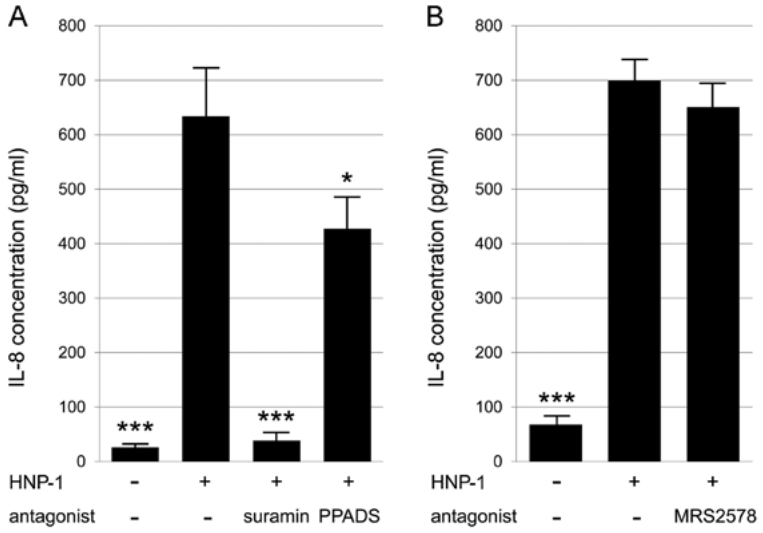

Figure 3. Involvement of P2 receptors in the human neutrophil peptide-1 (HNP-1)-induced production of interleukin-8 (IL-8) in HT-29 cells. (A) HT-29 cells were incubated with 50 μg/ml HNP-1 with or without suramin (100 μM) or pyridoxal phosphate-6-azo(benzene-2,4-disulphonic acid) (PPADS) (100 μM) for 24 h. (B) HT-29 cells were incubated with 50 μg/ml HNP-1 with or without 10 μM MRS2578 for 24 h. The IL-8 levels in culture supernatants were measured using ELISA. Data are the means ± SE from three experiments. Significant differences relative to the HNP-1-treated cells without an antagonist are indicated as follows: *P<0.05; ***P<0.001.

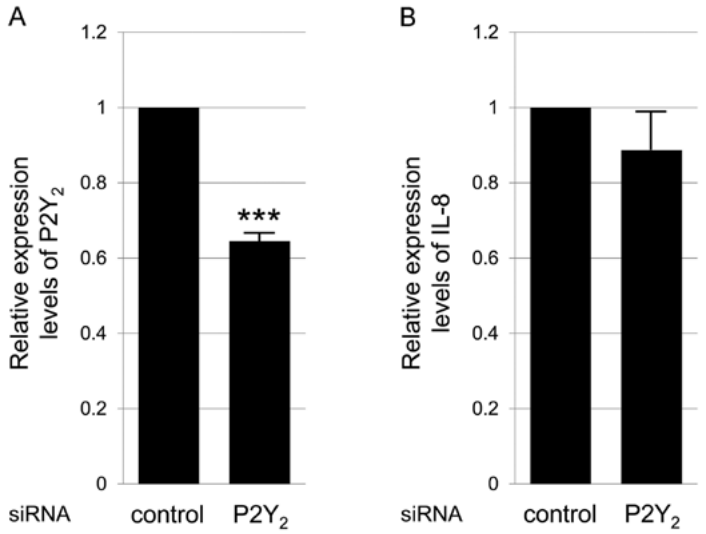

Figure 4. The $P2Y_2$ signaling pathway is not involved in human neutrophil peptide-1 (HNP-1)-induced interleukin-8 (IL-8) expression in HT-29 cells. Silencing oligonucleotides ($P2Y_2$) or non-silencing siRNA (control) was introduced into the HT-29 cells. Twenty-four hours following transfection, the cells were incubated with 50 μg/ml HNP-1 for 24 h. The mRNA expression of (A) $P2Y_2$ and (B) IL-8 was measured by RT-qPCR. Data are the means ± SE from three experiments. ***P<0.001 compared with cells transfected with non-silencing siRNA.

of IL-8 by HT-29 cells, despite the absence of $P2Y_6$. Treatment of the HT-29 cells with MRS2578 had no effect on the expression of IL-8, as was expected (Fig. 3B).

P2 receptors, other than $P2Y_2$ and $P2Y_6$ subtypes are involved in the HNP-1-induced production of IL-8 by HT-29 cells. In addition to $P2Y_6$ receptors, the $P2Y_2$ and $P2X_7$ receptors are involved in the production of IL-8 by epithelial cells. The activation of $P2Y_2$ and $P2X_7$ induces the release of IL-8 in renal epithelial cells and bronchial epithelial cells, respectively (25,26). HT-29 cells express the receptor for $P2Y_2$ (24,27) but not the one for $P2X_7$ (28). Although $P2Y_2$ was not antagonized by PPADS, the involvement of $P2Y_2$ in the HNP-1-induced production of IL-8 could not be excluded, as the inhibitory effects of PPADS on IL-8 production were much weaker than those of suramin. Therefore, we investigated the possibility that $P2Y_2$ is the receptor primarily responsible for the HNP-1-induced production of IL-8 in HT-29 cells. As definitive antagonists of $P2Y_2$ are not currently available, we applied $P2Y_2$ siRNA and analyzed IL-8 expression following treatment with HNP-1. The silencing of $P2Y_2$ decreased the mRNA expression level of $P2Y_2$ by 40%, which was a significant decrease (Fig. 4A); however, the expression of IL-8 following treatment with HNP-1 was not altered (Fig. 4B). These results indicate that P2 receptors, other than the $P2Y_2$ and $P2Y_6$ subtypes are involved in the HNP-1-induced production of IL-8 by HT-29 cells.

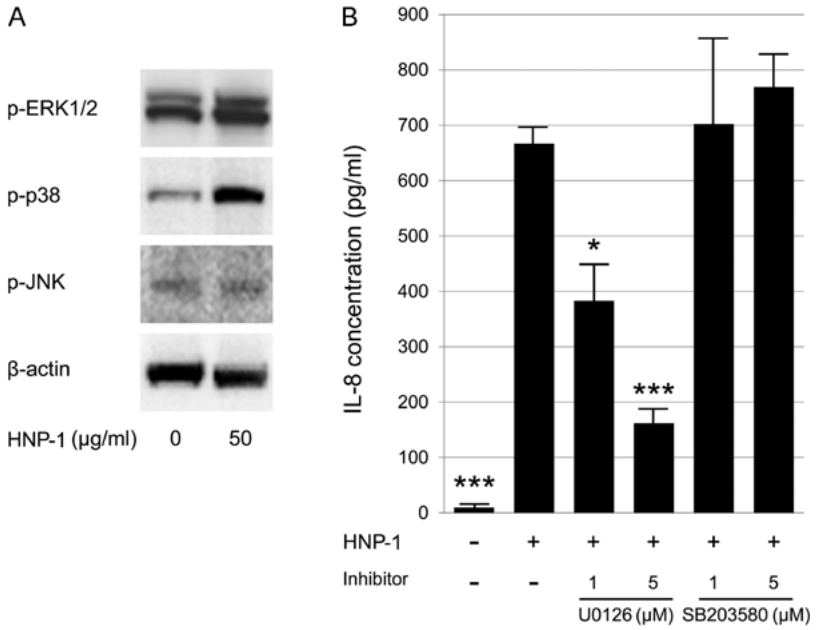

Figure 5. Extracellular signal-regulated kinase 1/2 (ERK1/2) activation is involved in the human neutrophil peptide-1 (HNP-1)-induced production of interleukin-8 (IL-8) in HT-29 cells. (A) Expression of phosphorylated ERK1/2 (p-ERK1/2), phosphorylated p38 mitogen-activated protein kinase (MAPK; p-p38) and phosphorylated JNK (p-JNK) following treatment with HNP-1 in HT-29 cells. HT-29 cells were stimulated by the addition of 50 μg/ml HNP-1 for 30 min. The immunoblots shown are representative of three independent experiments. β-actin was used as a loading control. (B) HT-29 cells were incubated with 50 μg/ml HNP-1 with or without U0126 or SB203580 at the indicated concentrations for 24 h. The IL-8 levels in culture supernatants were measured using ELISA. Data are the means ± SE from three experiments. Significant differences relative to the HNP-1 treated cells without an inhibitor are indicated as follows: *P<0.05; ***P<0.001.

HNP-1-induced production of IL-8 by HT-29 cells is dependent on ERK1/2 activation. In the Caco-2/15 cells, the increased production of IL-8 downstream of $P2Y_6$ activation is dependent on the ERK1/2 signaling pathway (29). ERK1/2 activation is involved in the HNP-induced production of IL-8 in lung epithelial cells and monocytes, whereas p38 MAPK activation is required for IL-8 production only in monocytes (13). Moreover, it was recently reported that the HNP-1-induced production of IL-8 in rheumatoid fibroblast-like synoviocytes is regulated by the JNK and ERK signaling pathways (16). Thus, we sought to determine which MAPK signaling pathways are involved in the HNP-1-induced production of IL-8 by HT-29 cells. Using western blot analysis, we found that HNP-1 induced the phosphorylation of ERK1/2 and p38 MAPK, while no significant changes were observed in JNK activity in the HT-29 cells (Fig. 5A). To identify the relevant signaling pathway involved in the HNP-1-induced production of IL-8, we treated the HT-29 cells with specific inhibitors of ERK1/2 (U0126) and p38 MAPK (SB203580). Treatment with U0126 significantly reduced the HNP-1-induced production of IL-8; however, the addition of SB203580 did not have any significant inhibitory effects on IL-8 production (Fig. 5B). These results suggest that the HNP-1-induced production of IL-8 is dependent on ERK1/2 activation in intestinal epithelial cells.

Discussion

In the present study, to the best of our knowledge, we demonstrate for the first time the induction of IL-8 by HNP-1 in intestinal epithelial cells. Our results suggest that HNP-1 released from infiltrated neutrophils induces IL-8 production by the intestinal mucosa. As a result of the increase in IL-8 expression, neutrophils are recruited to the site of inflammation, where they contribute to the extended tissue damage observed in patients with IBD.

HNP-1 induced IL-8 expression partly through $P2Y_6$ in Caco-2 cells. The involvement of $P2Y_6$ in IBD has previously been suggested. $P2Y_6$ is highly expressed in T-cells infiltrating active IBD (30). The mRNA expression levels of the $P2Y_6$ and $P2Y_2$ receptors have been shown to be upregulated in the colonic epithelium of patients with IBD and DSS-treated mice (29). The activation of $P2Y_6$ by its natural ligand, UDP, stimulates the sustained NaCl secretion in rat colonic enterocytes (31). In addition to $P2Y_6$, we considered the involvement of other P2 receptors in HNP-1-induced IL-8 expression. Thus, we used HT-29 cells, which do not express $P2Y_6$, in order to investigate non-$P2Y_6$-mediated mechanisms. As was the case with the Caco-2 cells, HNP-1 significantly induced IL-8 production in the HT-29 cells, and this production was suppressed by suramin and PPADS. We hypothesized that $P2Y_2$ is responsible for the HNP-1-induced production of IL-8 in HT-29 cells, as the $P2Y_2$-mediated release of IL-8 by other epithelial cell lines has been previously reported (25), and the inhibitory effects on IL-8 production by PPADS were much weaker than those exerted by suramin. However, the silencing of $P2Y_2$ had no effect on the induction of IL-8. It has been reported that P2 receptors expressed by HT-29 cells are those for $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_{11}$ (24,27,32). The selective $P2Y_1$ antagonist, MRS2179, did not exert any significant inhibitory effects on the HNP-1-induced production of IL-8 by HT-29 cells (data not shown). The involvement of $P2Y_4$ is unlikely as its receptor is insensitive to suramin. Since PPADS is completely inactive at the human $P2Y_{11}$ receptor (33), this also does not appear to be a dominant pathway involved in the HNP-1-induced production of IL-8.

Further studies on the identification of P2 receptors involved in the HNP-induced production of IL-8 are required in order to better understand the role of HNPs in intestinal epithelial cells.

Three MAPK pathways, the ERK1/2, JNK and p38 MAPK cascades, contribute to the downstream activation of transcription factors, including nuclear factor-κB (NF-κB) and activator protein-1 (AP-1), both of which upregulate IL-8 transcription. The involvement of particular MAPK signaling pathways in the induction of IL-8 is dependent on the cell type and the stimulus (34). A previous study demonstrated that the ERK1/2 and p38 MAPK pathways contribute to the secretion of IL-8 by HT-29 cells in response to tumor necrosis factor (TNF)-α (35). Our results revealed that HNP-1 activated ERK1/2 and p38 MAPK in HT-29 cells. On the other hand, the HNP-1-induced production of IL-8 was inhibited by the blockade of ERK1/2 activation, but not by that of p38 MAPK, indicating that ERK1/2 plays a pivotal role in IL-8 production. Notably, $P2Y_6$ receptor activation by UDP has been shown to increase IL-8 production by Caco-2/15 cells through a mechanism that is ERK1/2-dependent, but p38 MAPK-independent (29). Therefore, the HNP-1-induced production of IL-8 appears to occur through the ERK1/2-dependent signaling pathway in intestinal epithelial cells regardless of the expression of $P2Y_6$.

It has been shown that ERK1/2, p38 MAPK and JNK are activated in the inflamed colonic mucosa of patients with IBD (36). Mesalamine, a drug effective in the treatment of IBD, has been shown to inhibit the TNF-α-induced activation of ERK1/2 (37). A recent study using gene expression profiling confirmed that the ERK/MAPK pathway is regulated by mesalamine (38). Another study demonstrated that the release of IL-8 triggered by mucosal *E. Coli* isolated from IBD is mediated by the ERK1/2 and p38 MAPK pathways and inhibited by mesalamine, but not by hydrocortisone (39). Hence, the reduction in the HNP-induced production of IL-8 by the inhibition of ERK1/2 may be part of the mechanism of action of mesalamine in the treatment of IBD.

In conclusion, in the present study, we demonstrate that the HNP-1-induced production of IL-8 in intestinal epithelial cells is dependent, not only on $P2Y_6$, but also on P2 receptors other than $P2Y_6$. Moreover, we reveal that the activation of the ERK1/2 pathway is required for the HNP-1-induced production of IL-8 by intestinal epithelial cells. HNPs released by infiltrating neutrophils in the UC intestine may stimulate additional neutrophil accumulation by inducing IL-8. The findings of the present study may lead to the development of novel therapeutic strategies to reduce HNP-induced intestinal inflammation.

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