

[原著]

Neutrophil elastase stimulates IL-10 production by monocytes via the Ca^{2+} /phospholipase C/protein kinase C signaling pathway

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Abstract

Neutrophils have an important role in the development of tissue injury associated with acute pancreatitis. In this study, changes of IL-10 messenger RNA (mRNA) and protein levels in monocytes were investigated after stimulation of these cells with human neutrophil elastase (HNE).

Adherent monocytes were obtained from primary cultures of human mononuclear cells. Reverse transcription polymerase chain reaction (RT-PCR) showed that HNE enhanced IL-10 mRNA expression by monocytes, while western blotting revealed that IL-10 protein levels were increased in cell culture medium. A phospholipase C inhibitor (U73122) blunted the induction of IL-10 mRNA by HNE. IL-10 mRNA expression in monocytes was significantly reduced by Rottlerin (a protein kinase C inhibitor), but not by SB 203580 (a p38 mitogen-activated protein kinase inhibitor).

A calcium chelator (TMBC-8) inhibited the response of IL-10 mRNA to HNE. These results indicate that HNE stimulates IL-10 mRNA expression and protein production in monocytes via Ca^{2+} /phospholipase C /PKC-mediated signal transduction.

Key words: neutrophil elastase, monocytes, interleukin 10, phospholipase C, protein kinase C

I. Introduction

Neutrophil elastase is a 29 KDa serine endoprotease from the proteinase S1 family that exists as a single 238-amino acid peptide chain with four disulfide bonds. Elastase released from activated neutrophils can cause tissue destruction^{1), 2), 3)} and neutrophil elastase is an important mediator of inflammatory tissue damage since it is involved in the degradation of extracellular matrix components such as elastin, fibronectin, proteoglycan, and collagen⁴⁾.

Acute pancreatitis is associated with significant morbidity and a mortality rate that can be as high as 30–50%, with death being caused by dysfunction of multiple organs, including the pancreas itself and the lungs, liver, kidneys, and heart⁵⁾. Elastase released from polymorphonuclear leukocytes is considered to be one of the most important mediators of inflammatory tissue damage in the setting of acute pancreatitis⁶⁾.

Levels of pro-inflammatory and anti-inflammatory cytokines are significantly increased in the early stage of acute pancreatitis, and the

circulating cytokine profiles are consistent with the systemic inflammatory response syndrome (SIRS) and the compensatory anti-inflammatory response syndrome (CARS) in patients who have acute pancreatitis complicated by sepsis. These pro- and anti-inflammatory cytokine responses to acute pancreatitis associated with sepsis have been termed the SIRS/CARS continuum and may be of predictive value for the severity of disease⁷⁾. Interleukin-10 (IL-10) has an immunosuppressive role in the inflammatory process. Lipopolysaccharide (LPS) stimulation of a human monocytic cell line has been reported to lead to rapid upregulation of the expression of both the pro-inflammatory cytokine tumor necrosis factor (TNF) and the anti-inflammatory cytokine IL-10⁸⁾. Therefore, the present study was undertaken to investigate the kinetics and production of IL-10 by human peripheral blood monocytes after stimulation with human neutrophil elastase (HNE).

II. Materials and Methods

1. Ethics statement

Human peripheral blood samples were obtained from healthy volunteers and this study was approved by the Institutional Review Board of Kumamoto Health Science University.

2. Reagents

Human neutrophil elastase (HNE) with 81 U/

mg of activity was purchased from SERVA Electrophoresis (Heidelberg, Germany). Elastase inhibitor III (Merck Millipore, Bedford, MA), Rottlerin ((Merck Millipore), SB203580 (Wako, Osaka, Japan), PD98059 (Cell Signaling Technology, Boston, MA), U73122 (Merck Millipore), and TMB-8 (Sigma-Aldrich, Ontario, Canada) were employed to study the intracellular signal transduction pathways involved in IL-10 mRNA expression. Each reagent solution was negative for endotoxin, as determined by the Endospecy test⁹⁾. The characteristics of these reagents are summarized in Table 1.

3. Isolation of adherent monocytes from peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were isolated as described previously¹⁰⁾. Briefly, heparinized blood samples were obtained from nonsmoking healthy volunteers and were diluted 1:1 with pyrogen-free saline. Then PBMCs were isolated by density gradient centrifugation with Ficoll-Hypaque and washed three times. The viability of the cells thus obtained was greater than 95% as determined by trypan blue dye exclusion. PBMCs were resuspended in RPMI-1640 medium supplemented with 25 mM HEPES, 100 mmol/L L-glutamine, 100 U/mL penicillin, 100 μ g/mL streptomycin, and 10% fetal calf serum. For monocyte isolation by plastic adherence, 1×10^6 cells per well were distributed into 12-well

Table. 1. Functional characteristics of chemical agents used

Chemical agents	Functions
Elastase inhibitor III	Elastase inhibitor
Rottlerin	Protein kinase C inhibitor
SB203580	MAPK p38alpha/beta inhibitor
PD98059	ERK inhibitor
U73122	Phospholipase C inhibitor
TAPI-1	Disintegrin and metalloproteinase inhibitor
TMB-8	Intracellular calcium antagonist

MAPK: mitogen-activated protein kinase

ERK: extracellular signal-regulated kinase

plates (Corning Inc. Costar, NY, USA) and allowed to adhere in a 5% CO_2 incubator at 37 °C for 2 hr and washed 3 times with warm phosphate-buffered saline (PBS) to remove nonadherent cells. Then, monocytes were cultured in complete medium consisting of RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (FCS) and $10 \times 10^3 \mu\text{g/L}$ gentamicin at 37 °C in 5% CO_2 humidified air. The adherent monocytes were recovered with a cell scraper. The purity of monocytes was evaluated by fluorescent staining with CD14-phycoerythrin (PE) mouse anti-human monoclonal antibody (Life technologies, Staley Road Grand Island, NY) and Fluorescence Activated Cell Sorting (FACS) analysis. The recovery of monocytes was also evaluated by trypan blue staining and counted using a Zeiss microscope (Jena, Germany). Only isolated CD14⁺ monocytes of > 85% purity were used for each experiment. After monocytes were resuspended in RPMI-1640 medium (Sigma-Aldrich, Oakville, Ontario, Canada) supplemented with 25 mM HEPES (Sigma-Aldrich), 100 mM L-glutamine (Sigma-Aldrich), 100 U/mL penicillin, and 100 $\mu\text{g/mL}$ streptomycin (Sigma-Aldrich), the cells were stimulated with HNE for 6 hr.

4. Extraction of RNA and reverse transcription polymerase chain reaction (RT-PCR)

Monocytes (1×10^6 cells) were extracted with 1 ml of Isogen RNA kit (Nippon Gene, Toyama, Japan). Total RNA was isolated and precipitated according to the manufacturer's instructions, after which 200 ng of total RNA was reverse-transcribed using Oligo dT primer with PrimeScript™ RT reagent Kit (Takara Bio, Shiga, Japan). Then the reverse-transcribed RNA was amplified by PCR using a TaKaRa PCR thermal cycler (Takara Bio). The primer sequences used were as follows: IL-10: forward, 5'- ATGCCCCAAGCTG AGAACCAAGAC-3' and reverse, 5'- TCTCAA GGGGCTGGGTCAGCTATCCCA-3'; beta-actin: forward, 5'-GTGGGGCGCCCCAGGCACCA-3' and reverse, 5'-CTCCTTAATGTCACGCACGATTTC

-3'. The PCR conditions were as follows: IL-10, 35 cycles (94 °C for 60 seconds, 60 °C for 60 seconds, and 72 °C for 30 seconds) ; and beta-actin, 35 cycles (94 °C for 30 seconds, 60 °C for 30 seconds, and 72 °C for 30 seconds) . PCR products were analyzed on agarose gels.

5. Western blotting for interleukin 10 (IL-10) in supernatant of cell culture.

Monocytes (1×10^6 cells/well) were stimulated with (0 or $5 \times 10^3 \mu\text{g/L}$) for 6hr and the cells were washed. Then fresh media was added. After further incubation on day 7, IL-10 protein levels in supernatant of cell culture was measured by western blotting. Equivalent amounts of whole-cell lysates were subjected to electrophoresis and the products were transferred to polyvinylidene difluoride membranes. The membranes were incubated with $0.2 \times 10^3 \mu\text{g/L}$ rabbit anti-human IL-10 IgG (Santa Cruz Biotechnology, Santa Cruz, CA), washed, and incubated with alkaline phosphatase-conjugated anti-rabbit IgG (Santa Cruz Biotechnology) diluted to 1:4000. Then the membranes were incubated with chemiluminescent enhancer (Immun-Star, Bio-Rad, Hercules, California) and exposed to XAR film (Kodak, Rochester, NY). After the film was developed, bands were quantified with a densitometer and ImageQuant software (Molecular Dynamics, Sunnydale, CA).

6. Statistical analysis

Data are expressed as the mean+SE. Analysis of variance and the *t*-test of independent means were used to determine differences between multiple group and two groups, respectively. When the F ratio was significant, means were compared by using a post hoc Bonferroni's test. A P value<0.05 was considered significant in all analyses.

III. Results

IL-10 mRNA expression by monocytes

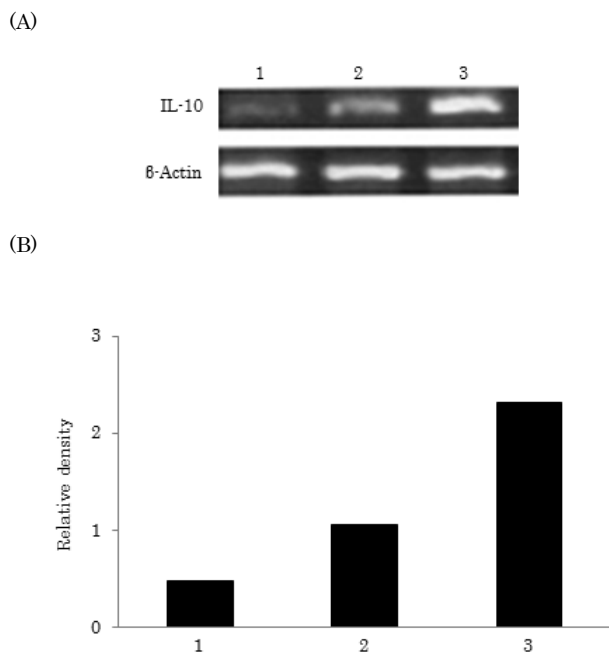


Figure 1. Representative results of RT-PCR for detection of IL-10 mRNA expression by monocytes stimulated with HNE.

When monocytes were stimulated with HNE (0 , 1 , or $5 \times 10^3 \mu\text{g/L}$) for 6hr, IL-10 mRNA expression was increased in a concentration-dependent manner, as shown by RT-PCR (A) and by densitometry of the PCR products normalized for beta-actin (B).

1. HNE ($0 \times 10^3 \mu\text{g/L}$)
2. HNE ($1 \times 10^3 \mu\text{g/L}$)
3. HNE ($5 \times 10^3 \mu\text{g/L}$)

increased in a concentration-dependent manner after stimulation with HNE (Fig. 1). The IL-10 mRNA level increased in monocytes after exposure to HNE, while addition of a neutrophil elastase inhibitor prevented this increase (Fig. 2). IL-10 mRNA expression was significantly reduced by Rottlerin (a protein kinase C inhibitor), but not by SB 203580 (a p38 mitogen-activated protein kinase inhibitor) or by PD98059 (an extracellular signal-regulated kinase inhibitor) (Fig. 3). A phospholipase C inhibitor U73122 reduced the response of IL-10 mRNA to HNE in monocytes (Fig. 4). Addition of TMB-8 (a calcium chelator) inhibited the increase of IL-10 mRNA in response

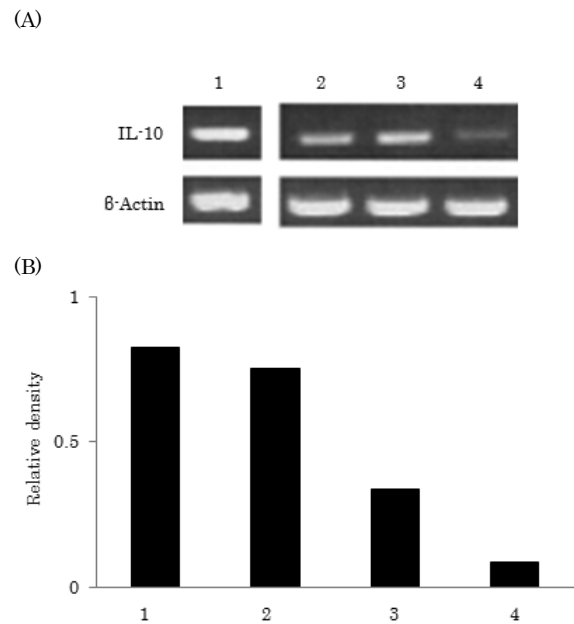


Figure 2. Effect of neutrophil elastase inhibitors on IL-10 mRNA expression.

IL-10 mRNA expression by monocytes increased in response to stimulation with HNE ($5 \times 10^3 \mu\text{g/L}$) for 6hr, whereas elastase inhibitors blocked this increase, as shown by RT-PCR (A) and by densitometry of the PCR products normalized for beta-actin (B). A representative result is shown.

1. Elastase inhibitor III ($0 \mu\text{M}$) + HNE ($5 \times 10^3 \mu\text{g/L}$)
2. Elastase inhibitor III ($100 \mu\text{M}$) + HNE ($5 \times 10^3 \mu\text{g/L}$)
3. Elastase inhibitor III ($200 \mu\text{M/L}$) + HNE ($5 \times 10^3 \mu\text{g/L}$)
4. Elastase inhibitor III (300 M) + HNE ($5 \times 10^3 \mu\text{g/L}$)

to HNE (Fig. 5). The protein level of IL-10 increased in supernatant of cell culture after stimulation with HNE (Fig. 6).

IV. Discussion

The present study demonstrated that IL-10 production is significantly enhanced at both the mRNA and protein levels after stimulation of

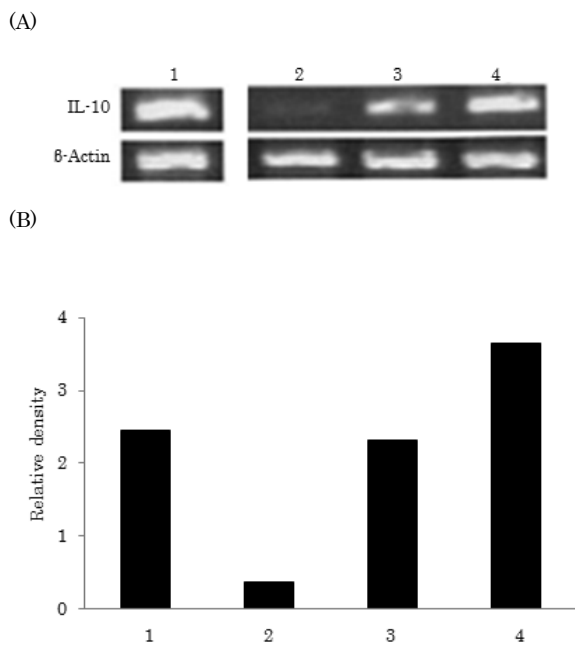


Figure 3. Effect of Rottlerin, SB302580, and PD98059 on IL-10 mRNA expression.

IL-10 mRNA expression in response to stimulation with HNE ($5 \times 10^3 \mu\text{g/L}$) was reduced by Rottlerin (a protein kinase C inhibitor), but not by SB 203580 (a p38 mitogen-activated protein kinase inhibitor) or PD98059 (an extracellular signal-regulated kinase inhibitor). A representative result is shown by RT-PCR (A) and by densitometry of the PCR products normalized for beta-actin (B).

1. HNE ($5 \times 10^3 \mu\text{g/L}$)
2. Rottlerin ($10 \mu\text{M}$) + HNE ($5 \times 10^3 \mu\text{g/L}$)
3. SB203580 ($10 \mu\text{M}$) + HNE ($5 \times 10^3 \mu\text{g/L}$)
4. PD98059 ($10 \mu\text{M}$) + HNE ($5 \times 10^3 \mu\text{g/L}$)

monocytes with human neutrophil elastase (HNE). Gresnigt et al. reported that neutrophil elastase degrades recombinant human IL-1 β and TNF- α , but not IL-10, with this reaction being inhibited by addition of α 1-antitrypsin¹¹. Therefore, IL-10 protein produced by monocytes after stimulation with HNE was presumably not degraded.

The mechanism(s) by which HNE stimulates IL-10 expression in monocytes have not been investigated before. It is known that enzymatic cleavage of membrane phosphatidyl inositols by

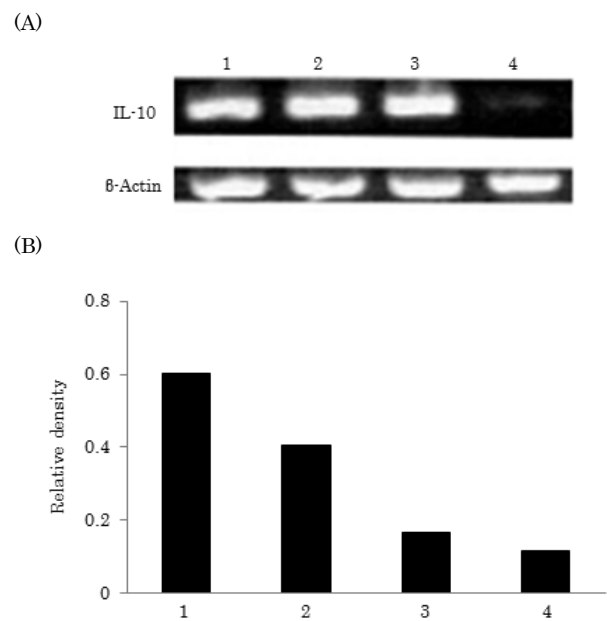


Figure 4. Effect of TAPI-1 or U73122 on IL-10 mRNA expression.

U73122 (a phospholipase C inhibitor) reduced HNE-induced expression of IL-10 mRNA by monocytes. A representative result is shown by RT-PCR (A) and by densitometry of the PCR products normalized for beta-actin (B).

1. HNE ($5 \times 10^3 \mu\text{g/L}$)
2. TAPI-1 ($10 \mu\text{M}$) + HNE ($5 \times 10^3 \mu\text{g/L}$)
3. U73122 ($1 \mu\text{M}$) + HNE ($5 \times 10^3 \mu\text{g/L}$)
4. U73122 ($10 \mu\text{M}$) + HNE ($5 \times 10^3 \mu\text{g/L}$)

receptor-G protein-phospholipase C complexes forms the second messengers diacylglycerol and inositol 1,4,5-triphosphate (IP3). A transient increase of diacylglycerol has been observed in aortic smooth muscle cells after HNE stimulation¹². Hydrolysis of inositol phospholipids by phospholipase C is initiated either by receptor stimulation or through the opening of Ca^{2+} channels^{13, 14}. An increase of the intracellular IP3 and Ca^{2+} concentrations can be blocked by the phospholipase C inhibitor U73122¹⁵. HNE enhances Ca^{2+} influx, resulting in the activation of phospholipase C (PLC). These observations suggest that the increase of IL-10 expression in response to stimulation with HNE may be related to elevation of intracellular Ca^{2+} and activation of

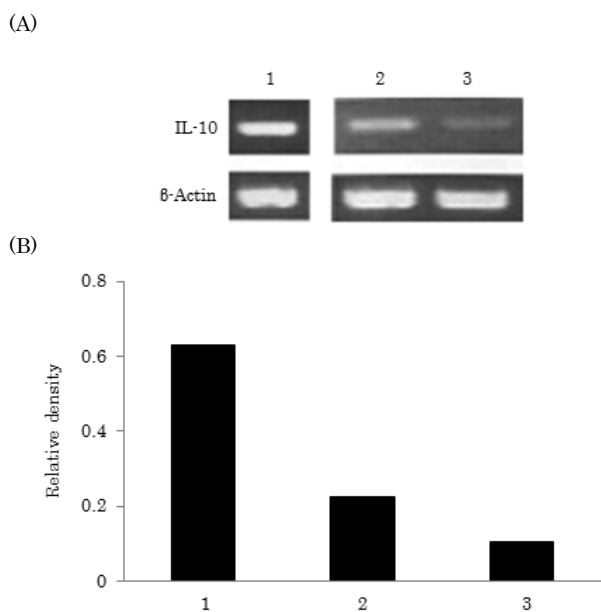


Figure 5. Effects of TMB-8 on IL-10 mRNA expression.

TMB-8 (a calcium chelator) inhibited the response of IL-10 mRNA to HNE in a concentration-dependent manner. A representative result is shown by RT-PCR (A) and by densitometry of the PCR products normalized for beta-actin (B).

1. HNE ($5 \times 10^3 \mu\text{g/L}$)
2. TMB-8 ($100 \mu\text{M}$)+HNE ($5 \times 10^3 \mu\text{g/L}$)
3. TMB-8 ($200 \mu\text{M}$)+HNE ($5 \times 10^3 \mu\text{g/L}$)

phospholipase C. The signal transduction pathway that activates protein kinase C (PKC) involves receptor-mediated activation of PLC, followed by hydrolysis of IP₃ to produce diacylglycerol (DAG), as well as elevation of the intracellular calcium level¹⁶⁾. The present study demonstrated that a PKC inhibitor (Rottlerin) completely prevented IL-10 mRNA expression by monocytes after stimulation with HNE. Interestingly, it has already been demonstrated that treatment with PKC and protease inhibitors can significantly reduce tissue injury due to acute pancreatitis¹⁷⁾.

The onset of acute respiratory distress syndrome in patients with acute pancreatitis is accompanied by an increase of anti-inflammatory cytokines, especially IL-10¹⁸⁾. Activation of the PKC signaling

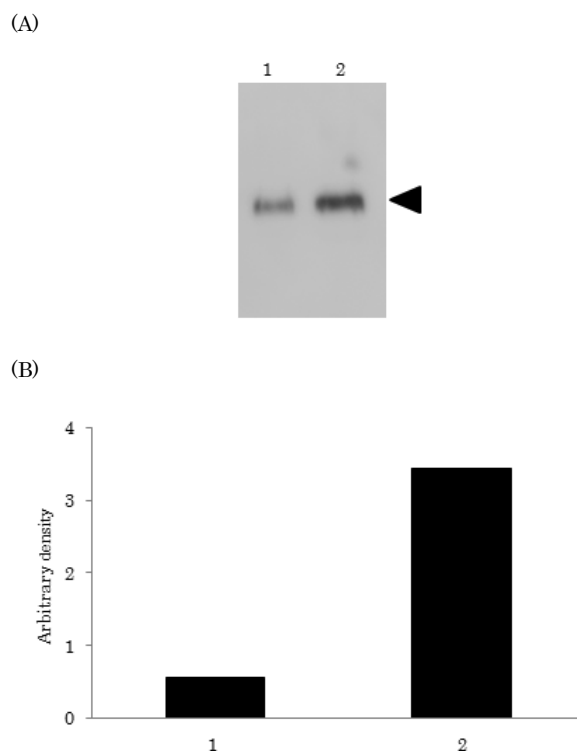


Figure 6. Western blotting for IL-10.

Monocytes were stimulated with HNE ($5 \times 10^3 \mu\text{g/L}$) for 6 hr. After further incubation on day 7, IL-10 protein levels in supernatant of cell culture was determined by western blotting (A). A representative result also is shown by the arbitrary density units (B).

pathway may play an important role in lung injury associated with pancreatitis¹⁹⁾. The present study also demonstrated that IL-10 production was stimulated by HNE through activation of PLC/PKC.

A marked increase of the peripheral blood monocyte count is observed in patients with pancreatitis and circulating monocytes also show significant activation²⁰⁾. Nuclear factor kappa B expression has been reported to be higher in the peripheral mononuclear cells of patients with acute pancreatitis than in control subjects²¹⁾. The G protein-coupled receptor gene is reported to be one of those up-regulated in this condition²²⁾. Thus, functional alterations of monocytes occur in the early phase of acute pancreatitis and these

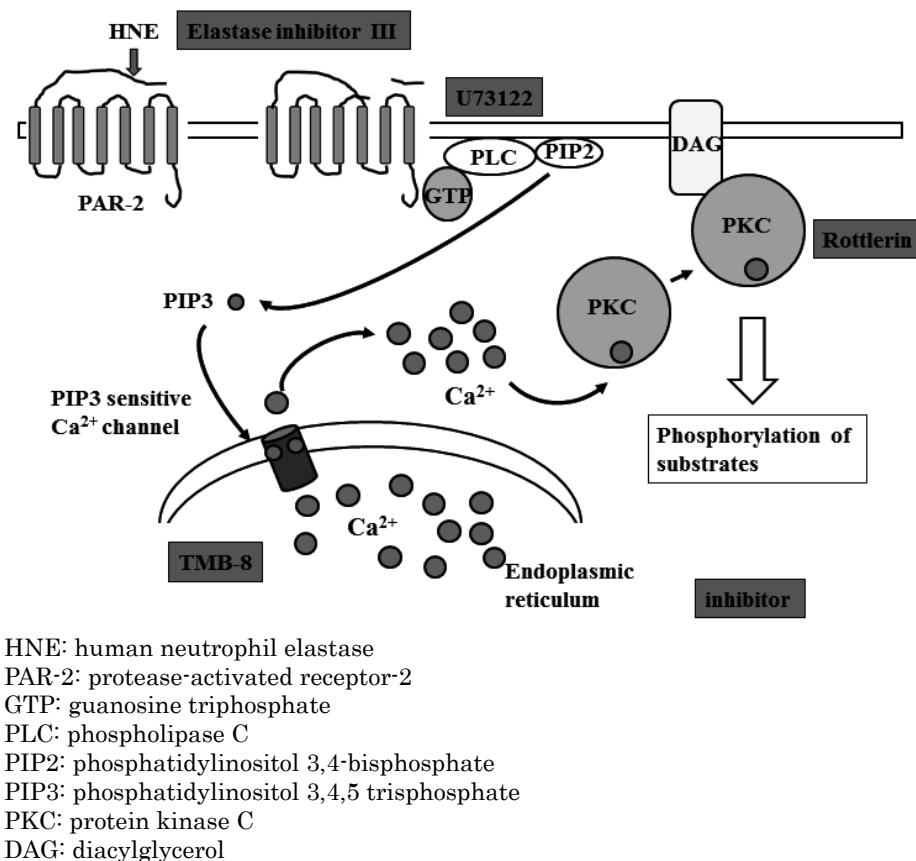


Figure 7. Possible signal transduction pathway of IL-10 mRNA in monocytes stimulated with neutrophil elastase See “Discussion” for description of model.

changes may play an important role in its pathogenesis.

TNF- α - converting enzyme (TACE) cleaves TNF at the Ala-76-Val-77 site and TACE expression has been detected on the surface of alveolar macrophages by flow cytometry²³⁾. It is known that activation of the epidermal growth factor receptor (EGFR) and its downstream signaling cascade are involved in the production of mucin. TACE cleaves pro-transforming growth factor (TGF)- α in airway epithelial cells to release the mature form of TGF- α (soluble TGF- α), which subsequently binds to and activates EGFR²⁴⁾. However, the present study demonstrated that a TACE inhibitor (TAPI-1) did not reduce IL-10 mRNA expression by monocytes.

V. Conclusion

HNE upregulates IL-10 expression in monocytes, probably by promoting intracellular Ca^{2+} influx/phospholipase C/PKC signaling pathway. Acknowledgement: This study was supported by Kumamoto Health Science University special fellowship grant No. 27-A-01. Conflict of Interest Declaration: The authors declare that there are no conflicts of interest.

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(平成28年 2 月10日受理)