NITRIC OXIDE-INDUCED IL-8 EXPRESSION IS MEDIATED BY NF-κB AND AP-1 IN GASTRIC EPITHELIAL AGS CELLS

Inducible nitric oxide synthase (iNOS) and interleukin-8 (IL-8) mediate gastric inflammation. Nitric oxide (NO) produced by iNOS may activate oxidant-sensitive transcription factors. There are the binding sites for NF-κB, AP-1, and C/EBP (CCAAT/enhancer binding protein) in the promoter regions of IL-8 gene. The present study aims to investigate whether NO donors, SIN-1 and NOC-18, activate oxidant-sensitive transcription factors NF-κB and AP-1 as well as C/EBP to induce IL-8 expression in gastric epithelial AGS cells. Gastric epithelial AGS cells were treated with NO donors, SIN-1 and NOC-18. mRNA expression and protein level of IL-8 in the medium were determined. Nitrite level in the medium and DNA binding activities of NF-κB, AP-1, and C/EBP were assessed. NO donors induced the increase in the levels of IL-8 and nitrite in the medium as well as mRNA expression of IL-8 in AGS cells time-dependently. The induction of IL-8 by NO donors was accompanied with the activation of NF-κB and AP-1 but not C/EBP in AGS cells. Conclusion: Large amount of NO, which may be produced by iNOS, may induce the activation of NF-κB and AP-1 and the expression of IL-8 in gastric epithelial cells.

Key words: nitric oxide donor, nuclear factor-κB, AP-1, interleukin-8, AGS cells, inducible nitric oxide synthase, nitrite

INTRODUCTION

Nitric oxide (NO) as a free radical easily reacts with oxygen, and other radicals. Nitric oxide synthase (NOS) is responsible for NO synthesis. Based on the calcium-dependency, NOS are classified into two groups; an inducible NOS (iNOS) and a constitutive NOS (cNOS). NO synthesized from eNOS is involved in maintaining mucosal integrity through modulation of gastric mucosal blood flow, epithelial secretion, and barrier function (1). However, large amounts of NO produced by iNOS are associated with the development of a number of autoimmune diseases, the initiation and progression of the immune response (2). We previously demonstrated that iNOS expression was elevated in H. pylori-infected gastric epithelial cells which was mediated by the activation of oxidant-sensitive transcription factors NF-κB and AP-1 (3, 4). High level of iNOS expression was shown in the area of the stomach which was significantly diminished after the eradication of H. pylori (5). Gastric inflammation and epithelial cell damage were induced by prolonged production of nitric oxide by iNOS (6). During chronic inflammation such as H. pylori gastritis, constant nitric oxide production may lead to tissue and DNA damage, thus increase the risk for incidence of cancer (7). Since severity of gastric inflammation and damage is related to the gene expression and protein levels of iNOS in gastric tissues (8-11), inhibition of the expression of this inflammatory enzyme may be beneficial for the treatment of gastric diseases.

Transcription factor, NF-κB, is a key mediator in innate immune response and inflammation (12). It is localized in the cytoplasm as a dimer form until inhibitory protein, IκB, is phosphorylated and degraded from NF-κB components in the response of stimuli such as viruses, bacteria, mitogens, reactive oxygen species, and inflammatory cytokines (13). AP-1 is a transcriptional regulator composed of members of the Fos and Jun families of DNA binding proteins. It regulates a broad variety of genes regarding inflammation responses, control of cell proliferation, regulation of bone formation and tumor formation (14, 15). There are studies showing that the expression of inflammatory cytokine IL-8 requires the activation of the combination of NF-κB and AP-1 or that of NF-κB and C/EBP. The binding sites for NF-κB, AP-1, and C/EBP are present in the promoter regions of IL-8 genes (16-21). IL-8 expression could be modulated by fine tones of various transcription factors (22, 23).

NO donor, spermine NONOate suppressed TNF-α-induced secretions of IL-8, MCP-1, and RANTES in human dermal microvascular endothelial cells (24). NO induced 5.7-fold increase of IL-8 and 2.2-fold increase of TNF-α at mRNA level in THP-1 cells and human primary monocytes. Study using actinomycin D showed that NO increased the stabilization of IL-8 mRNA but did not change that of TNF-α mRNA (25). SIN-1 reduced IL-8 accumulation in both LPS-treated human neutrophils and unstimulated neutrophils. In contrast, NO donor, 1,2,3,4-oxatriazolium 5-amino chloride, increased IL-8 accumulation in lipopolysaccharide-treated cells and unstimulated cells. In contrast, inhaled nitric oxide reduced primary graft dysfunction after lung transplantation by limiting neutrophil adhesion and lowering serum IL-6, IL-8, IL-10, suggests beneficial effects of NO (26). The
evidences showing that NO has different effects on the IL-8 production depending on the source and concentration of NO and cell type, indicates that relationship between NO and IL-8 needs to be investigated more in detail.

Therefore, the present study aims to investigate whether NO donors, SIN-1 and NOC-18, activate transcription factors NF-κB, AP-1 and C/EBP to induce IL-8 expression in gastric epithelial AGS cells.

MATERIALS AND METHODS

Cell line and chemicals

Human gastric epithelial AGS cells (gastric adenocarcinoma, ATCC CRL 1739) were obtained from the American Type Culture Collection (Rockville, Maryland, USA) and cultured in RPMI-1640 medium (pH 7.4; Sigma, St. Louis, Missouri, USA) supplemented with 10% fetal bovine serum, 4 mM glutamine (GIBCO-BRL, Grand Island, New York, USA) and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin). The cells were seeded in 12-well cell culture plates at 10^4 cells per well in a volume of 1 ml and cultured to reach 80% confluency for the determination of mRNA expression and the levels of IL-8 and nitrite in the medium. For the activation of transcription factors, AGS cells were seeded in 10 cm culture plates at 10^6 cells and cultured to reach 80% confluency. 3-Morpholinosydnonimine (SIN-1) and DETA NO (NOC-18) were purchased from Calbiochem (San Diego, CA). NOC-18 was freshly dissolved in 0.1 M NaOH while SIN-1 was suspended in medium. The stock solutions were filtered through 0.22-µm membranes (27).

Experimental protocol

300 µM of NOC-18 or SIN-1 was treated to the cells for 24 h and the supernatants were collected for the determination of IL-8 and nitrite at the indicated time points. IL-8 level was assessed by enzyme-linked immunosorbent assay (ELISA) and nitrite level was determined by colorimetric analysis. IL-8 mRNA expression was determined using whole cell extracts by Northern blot analysis while nuclear extracts were used for DNA binding activities of NF-κB, AP-1, and C/EBP by electrophoretic mobility shift assay (EMSA). To determine the involvement of NF-κB and AP-1 on NO-induced IL-8 expression, the cells were transfected with each luciferase vector and the transfected cells were treated with 300 µM of SIN-1 or NOC-18. After 24 h incubation, the luminescence intensities of whole cell extracts were determined.

Determination of IL-8

IL-8 levels was determined in the medium by ELISA kits (R&D System, Minneapolis, MN, USA). Purified human recombinant IL-8 was used as the standard. The detection limit for IL-8 was 10 pg/ml. IL-8 level was expressed as pg/ml medium.

Determination of nitrite

Nitrite in the medium was quantitated colorimetrically after reaction with the Griess reagents (28). Samples (0.5 ml) were mixed with 0.25 ml of 1% sulfanilic acid in 5% phosphoric acid and 0.25 ml of 0.1% naphthalene diamine dihydrochloride and allowed to stand for 30 min at room temperature. The absorbance was measured at 550 nm in an Ultraspec 3000 spectrophotometer (Pharmacia Biotech, Cambridge, England).

Northern blot analysis for IL-8

Total RNA (10 µg) from the cells in a volume of 10 µl was added to an equal volume of denaturing buffer (50% deionized formamide, 6% formaldehyde, 10 mM sodium phosphate buffer, 0.5 mM EDTA, pH 7.4) and heated at 70°C for 10 min. The samples were mixed with 5 µl of loading buffer (50% glycerol, 1 mM EDTA (pH 8.0), 0.25% bromophenol blue and 0.25% xylene cyanol) and loaded on a 1% agarose gel containing 1.1 M formaldehyde in 3-(N-morpholino)propanesulfonic acid (MOPS). After completion of electrophoresis, total RNA was transferred to Hybond-N (Amersham, Arlington Heights, IN, USA) membrane by a positive pressure blotter (Stratagene, La Jolla, CA, USA) and fixed by UV cross-linking (Stratagene). The prehybridization and hybridization solution consisted of 0.25 M Na2HPO4, 7% sodium dodecyl sulfate (SDS) and 1 mM EDTA (pH 8.0) at a volume of 20 µl per membrane. After 30 min of prehybridization at 60°C, hybridization was carried out overnight in the same solution at 60°C with 50 ng of a human IL-8 cDNA probe labeled with 2 x 10^6 cpm/ml [32P] dCTP (Amersham) by a random primer method (rediPrime; Amersham). Blots were washed in 2 x standard sodium citrate (SSC)/0.1% SDS at 60°C for 15 min. The membranes were exposed to BioMax film (Eastman Kodak, Rochester, NY, USA) at ~80°C with intensifying screens. The concentration and loading of RNA in each lane was standardized by hybridization with 25 ng of cDNA probe for the constitutively expressed β-actin (Clontech, Palo Alto, CA, USA).

Preparation of nuclear extracts

The cells were washed with ice-cold PBS, harvested by scraping with cell scraper into PBS, and pelleted by centrifugation at 1500 g for 5 min. The cells were extracted in the buffer containing 10 mM Hepes, 10 mM KCl, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 1.5 mM MgCl2, 0.2% Nonidet P-40, 1 mM dithiothreitol (DTT), and 0.5 mM phenylmethylsulfonylfluoride (PMSF). The nuclear pellet was resuspended on ice in nuclear extraction buffer containing 20 mM Hepes, 420 mM NaCl, 1.5 mM EDTA, 1.5 mM MgCl2, 25% glycerol, 1 mM DTT, and 0.5 mM PMSF (29) and the concentration of nuclear protein was determined by the method of Bradford (30).

Electrophoretic mobility shift assay (EMSA)

NF-κB gel shift oligonucleotide (5’-AGTTGAGGGGACTT TCCCAGGC-3’), AP-1 gel shift oligonucleotide (5’-CGTTG ATGAGTCAGCCGGAAA-3’), and C/EBP gel shift oligonucleotide (5’-GTACCACTGCAAACT TTA-3’) (Promega Corp, Madison, WI, USA) were labelled with [32P] dATP (Amersham) using T4 polynucleotide kinase (GIBCO-BRL). End-labelled probe was purified from unincorporated [32P] dATP using a Bio-Rad purification column (Bio-Rad Laboratories, Hercules, CA, USA) and recovered in tris-EDTA buffer (TE). Nuclear extracts (5 µg) were preincubated in the buffer containing 12% glycerol; 12 mM Hepes, pH 7.9; 4 mM Tris-HCl, pH 7.9; 1 mM EDTA; 1 mM DTT; 25 mM KCl; 5 mM MgCl2; 0.04 µg/ml poly[d(I-C)] (Boehringer Mannheim, Indianapolis, IN, USA); 0.4 mM PMSF; and TE. The labeled probe was added and nuclear extracts were incubated for 10 min.
on ice. Samples were subjected to electrophoretic separation at
room temperature on a nondenaturing 5% acrylamide gel at 30
mA using 0.5 x Tris borate EDTA buffer. The gels were dried at
80°C for 1 hr and exposed to the radiography film for 6-18 h at
-70°C with intensifying screens (31).

Luciferase reporter assay

The 5'-flanking region of the IL-8 gene spanning from base
pairs (bp) -133 to +144 (-133 bp wt) was subcloned into a
luciferase expression vector as previously described (32). Site-
directed mutagenesis of the IL-8 NF-κB, AP-1, and C/EBP
binding sites was carried out (33) and designated as mt-NF-κB,
mt-AP-1, and mt-C/EBP. For the luciferase assay, 2 x 10⁵ AGS
cells were transfected with 2 µg of each luciferase vector and 40
ng of pRL-TK vector (Promega) as an internal control using
transfection reagent DOTAP (N-[-(2,3-Dioleoyloxy) propyl]-N,N,N-trimethyl ammonium methylsulfate) (Boehringer-
Mannheim, Pentzberg, Germany). After 24 hr, the transfected
cells were treated with 300 µM of SIN-1 or NOC-18. After
another 24-hr incubation, cell lysates were prepared using
passive lysis buffer (Promega), and the light intensities were
measured using a Biolumat LB9500C Luminescence Reader
(EG & G Berthold, Bad Wildbad, Germany).

Statistical analysis

Results are expressed as means±standard error (SE) of four
separate experiments. Analysis of variance (ANOVA) followed
by Newman-Keul's test was used for statistical analysis. P<0.05
was considered statistically significant.

RESULTS

Time-dependent mRNA expression of IL-8 and increase in the
levels of IL-8 and nitrite in the medium by NO donors

To determine whether NO donors induce IL-8 production in
AGS cells, the cells were treated with NO donors, SIN-1 or NOC-
18, and mRNA expression of IL-8 and the levels of IL-8 and nitrite
in the medium were determined. mRNA expression of IL-8 (Fig.
1A) and protein levels of IL-8 (Fig. 1B) increased time-
dependently by treatment of NO donors, NOC-18 and SIN-1.
Interestingly NOC-18 produced relatively more IL-8 than SIN-1 in
the cells (Fig. 1B) even though the levels of nitrite released from
SIN-1 and NOC-18 were similar in the cells, determined by the
levels of nitrite in the medium (Fig. 1C). At 4 h, mRNA
expression of IL-8 was higher in NOC-18 - treated cells compared
to that of SIN-1 - treated cells. Nitrite levels in the medium sharply
increased and reached to the maximum at 1 h.

Time-dependent activation of NF-κB and AP-1 by NO donors

The activation of redox-sensitive transcription factors NF-
κB and AP-1 were determined by DNA binding activities in
the cells treated with NO donors by EMSA (Fig. 2A,B). Maximum
activation of NF-κB and AP-1 was shown at 2 h for SIN-1 (left
panel) while NOC-18 showed the maximal activation of both
transcription factors from 1 h and continued to 2 h (right panel).
The results supported that NOC-18 induced more IL-8
expression at mRNA level well as protein level shown in Fig. 1A
and 1B. The level of C/EBP was not changed by NO donors in
AGS cells (Fig. 2C). The results demonstrate the possible
involvement of NF-κB and AP-1, not C/EBP on IL-8 expression
in NO donors - treated cells.

Involvement of NF-κB and AP-1 on NO donors-induced IL-8
transcription

The effects of NO donors on IL-8 transcription were
investigated using luciferase assay. After transfection with point
mutated NF-κB, AP-1 and C/EBP reporter genes into the cell, the
cells were treated with SIN-1 (Fig. 3A) or NOC-18 (Fig. 3B).
Increased luciferase activity by NO donors was completely
inhibited in the cells transfected with mutant NF-κB gene and
those with mutant AP-1. NO donors-induced increases in
luciferase activity were not changed in the cells transfected with
mutant C/EBP. The results demonstrate that the activation of both NF-κB and AP-1 mediates IL-8 transcription but C/EBP is not involved in NO donors-induced IL-8 transcription.

**DISCUSSION**

The present study demonstrates that NO donors induced the activation of NF-κB and AP-1 and the expression of IL-8 in AGS cells. Chemokine gene transcription requires the activation of the combination of transcription factor NF-κB and AP-1 or that of NF-κB and C/EBP (CCAAT/enhancer binding protein), depending on the types of cell or stimuli since there are the binding sites for NF-κB, AP-1, and C/EBP in the promoter regions of IL-8 gene (16-21). However, the present study shows that the similar activation of both NF-κB and AP-1 mediates IL-8 expression in AGS cells treated with SIN-1 and NOC-18. The results suggest that involvement of the transcription factors on L-8 expression may be different depending on cell types and the kinds of NO donors.

IL-8, a prototype CXC chemokine, seems to play an important role in recruiting and activating neutrophils (34) in the gastric mucosa. Several reports suggest that gastric epithelial cells represent an important source of IL-8 (35). In addition to chemotactic potential, IL-8 is capable of activating polymorphonuclear leukocyte degranulation, the respiratory burst, and the 5-lipoxygenase pathway (34). Therefore, IL-8 may be a component of the inflammatory cascade. Indeed, gastric mucosal levels of IL-8 correlate with histological severity in the patients with *H. pylori*-induced gastritis (34). Prolonged IL-8 production by gastric epithelial cells could result in the recruitment of leukocytes to infected tissues and therefore IL-8 may be important in the regulation of inflammatory and immune processes (36, 37). Endogenous NO produced by mainly iNOS and partly endothelial NOS contributes to the healing of dextran sulfate sodium-induced colonic lesions, through the upregulation of vascular endothelial growth factor (VEGF) expression and enhancement of angiogenesis (38). In relation to anticancer effect of NO, NO-releasing aspirin caused induction of apoptosis and growth inhibition in Barrett's adenocarcinoma cell line, suggesting that this compound may represent a promising chemopreventive agent for Barrett's adenocarcinoma (39). In general, low concentrations of nitric oxide produced by cNOS inhibit adhesion molecule expression, cytokine and chemokine synthesis and leukocyte adhesion. Large amounts of NO, produced by iNOS, may be toxic and pro-inflammatory. However, actions of nitric oxide are not dependent primarily on the enzymatic source, but rather on the cellular context, NO concentration (dependent on the distance from NO source) and initial priming of immune cells (40).

NOC-18 is one of the well known NO releasing compounds which directly release NO in neutral solution even though it is
stable in alkaline solution. SIN-1 produces NO and peroxynitrite formed by the reaction of superoxide anion and nitric oxide (41). In our previous study, the same amount of SIN-1 and NOC-18 showed similar pattern of cell death in AGS cells (42). The present study demonstrates that the nitrite levels released into the medium were similar between the cells treated with SIN-1 and NOC-18. However, NOC-18 activated NF-kB and AP-1 earlier and produce more IL-8 than SIN-1 did. The results were supported by the previous study showing that NOC-18 induced more DNA damage than SIN-1 did in porcine chondrocytes (43). The difference of activation mechanism between SIN-1 and NOC-18 has not been clarified yet. Since half life of SIN-1 to produce NO is approximately 230 min while that of NOC-18 is 3400 min (44, 45), this discrepancy may contribute to the different activation pattern between SIN-1 and NOC-18. Further study should be performed to investigate the relation of half-lives of NO donors and the activation of signaling molecules and transcription factors to induce cytokines and inflammatory mediators in gastric epithelial cells in association with NO-induced gastric inflammation.

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