TNF-α and interleukin 1 activate gastrin gene expression via MAPK- and PKC-dependent mechanisms

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Suzuki, T., E. Grand, C. Bowman, J. L. Merchant, A. Todisco, L. Wang, and J. Del Valle. TNF-α and interleukin 1 activate gastrin gene expression via MAPK- and PKC-dependent mechanisms. Am J Physiol Gastrointest Liver Physiol 281: G1405–G1412, 2001.—Helicobacter pylori and proinflammatory cytokines have a direct stimulatory effect on gastrin release from isolated G cells, but little is known about the mechanism by which these factors regulate gastrin gene expression. We explored whether tumor necrosis factor (TNF)-α and interleukin (IL)-1 directly regulate gastrin gene expression and, if so, by what mechanism. TNF-α and IL-1 significantly increased gastrin mRNA in canine G cells to 181 ± 18% and 187 ± 28% of control, respectively, after 24 h of treatment. TNF-α and IL-1 stimulated gastrin promoter activity to a maximal level of 285 ± 12% and 415 ± 26% of control. PD-98059 (a mitogen-activated protein kinase kinase inhibitor), SB-202190 (a p38 kinase inhibitor), and GF-109203 (a protein kinase C inhibitor) inhibited the stimulatory action of both cytokines on the gastrin promoter. In conclusion, both cytokines can directly regulate gastrin gene expression via a mitogen-activated protein kinase- and protein kinase C-dependent mechanism. These data suggest that TNF-α and IL-1 may play a direct role in Helicobacter pylori-induced hypergastrinemia.

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Isolated cells were separated by centrifugal elutriation and then cultured on Matrigel-coated plates in Ham's F-12/DMEM containing 10% heat-inactivated dog serum, insulin (1 mg/ml), hydrocortisone (1 mg/ml), and gentamicin (100 mg/ml) for 40 h in a humidified atmosphere of 5% CO₂-95% air at 37°C. The fraction used in these experiments consisted of 20–25% G cells based on immunohistochemical staining (9, 31).

The AGS cell line derived from a human gastric adenocarcinoma was cultured in DMEM supplemented with 10% fetal bovine serum (GIBCO-BRL, Grand Island, NY), 100 µg/ml penicillin, and 100 µg/ml streptomycin in a humidified atmosphere of 5% CO₂-95% air. AGS cells were stably transfected as described previously (12). In brief, the 240 GasLuc AGS stable cell line was developed through stable transfection of AGS cells with the expression construct 240 GasLuc, which contains the 240 bp of the human gastrin promoter ligated upstream of the luciferase reporter gene in the pGLO2B vector (Promega, Madison, WI). In some experiments, the mutant 240 GasLuc AGS stable cell line, which expresses a mutated epidermal growth factor (EGF)-responsive element (gERE) within the 240 GasLuc construct, was used for luciferase assays as previously described (12).

Gastrin release and gastrin gene expression in canine G cells. After 40 h, enriched canine G cells were washed to remove dead and nonadherent cells and then incubated with ligands in Earle's balanced salt solution for 2 h. Radioimmunoprecipitation was used to measure gastrin cell content and gastrin released into the media as previously described (9, 31).

For Northern blot analysis, cells were cultured as described above and exposed to TNF-α (10 ng/ml) and IL-1α (10 ng/ml) for 24 h, at which time total RNA was isolated from cells with Trizol (GIBCO-BRL) according to the manufacturer's instructions. Denatured RNA samples (20 µg) were analyzed by gel electrophoresis in a 1.25% agarose gel and transferred to a nylon membrane by capillary blotting in 20× standard saline citrate (3 M NaCl and 0.3 M sodium citrate). The RNA was ultraviolet cross-linked to the membrane. The nylon membrane was hybridized overnight at 65°C with a 32P-labeled human gastrin cDNA probe. After autoradiography, the membrane was reprobed with a radiolabeled cDNA encoding glyceraldehyde phosphate dehydrogenase (Repligene, the membrane was reprobed with a radiolabeled cDNA from this reaction was mixed with PCR buffer, MgCl2, (GIBCO, Gaithersburg, MD) protocol. The cDNA obtained from the 240 GasLuc construct, was used for luciferase assays as previously described (12).

Luciferase assays. 240 GasLuc AGS cells were plated at a density of 0.5 × 10⁶ cells/well, cultured for 24 h in DMEM, and then placed in serum-free media for 24 h before ligand treatment. In some experiments, the mitogen-activated protein kinase (MAPK) kinase inhibitor PD-98059 (New England Biolabs, Beverly, MA), p38 kinase inhibitor SB-202190 (Calbiochem-Novabiochem, San Diego, CA), protein kinase C (PKC) inhibitor bisindolylmaleimide I (GP-109203; Calbiochem-Novabiochem), myristoylated protein kinase A inhibitor (PKI; Calbiochem-Novabiochem), anti-TNF receptor antibody (R&D Systems, Minneapolis, MN), or IL-1 receptor antibody (IL-1ra) (Sigma, St. Louis, MO) were added 30 min before addition of cytokines. Luciferase assays were carried out using luciferin, ATP, and coenzyme A, in a Lumat LB9501 Luminometer (Lumat, Berthold, Germany) as described previously (12, 30). Luciferase assays were normalized to protein determined by the Bradford method (6).

Immune complex kinase assays. AGS cells were lysed in 400 µl of lysis buffer (50 mM HEPES, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM EDTA, 1.5 mM MgCl2, 1 mM Na3VO4, 10 mM NaF, 10 mM Na2P2O7·10H2O, 1 mM 4-(2-aminoethyl)-benzenesulfonylfluoride hydrochloride, 1 µg/ml leupeptin, and 1 µg/ml aprotinin). Lysate was clarified by centrifugation at 16,000 g for 10 min at 4°C, and the supernatant was used for the assay after protein normalization by the Bradford method (6). The lysate samples were diluted with lysis buffer (final volume of 600 µl and a final protein content of 300 µg) and incubated with either an anti-extra-cellular signal-regulated kinase (ERK)-2 or anti-p38 specific antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and mixed on a rotating platform overnight at 4°C. Fifty percent protein A-Sepharose beads (50 µl; Pharmacia Biotech, Piscataway, NJ) were added and rotated for 1 h, then centrifuged for 2 min at 7,000 rpm at 4°C, and pellets were washed once in 500 µl lysis buffer and twice in 500 µl kinase buffer (18 mM HEPES, 10 mM MgAc, 50 µM ATP). Kinase reactions were carried out by resuspending 20 µl kinase reaction buffer (18 mM HEPES, 10 mM MgAc, 50 µM ATP, 2 µg/sample glutathione S-transferase-activating transcription factor 2 or myelins basic protein (Sigma), and 2 µCi/sample [γ32P]ATP (Amersham)) and incubated for 30 min at 30°C. The reaction products were electrophoresed on 10% acrylamide-SDS gels, stained, dried, and autoradiographed. Phosphoprotein activity was quantitated by scanning densitometry.

Western blot analysis of PKC and measurement of PKC activity. Cell membrane samples were prepared and analyzed as previously described (37). Briefly, stimulated AGS cells were suspended in 62.5 mM Tris·HCl (pH 7.4), 2 mM

**Fig. 1.** Effect of interleukin (IL)-1α on gastrin release from canine G cells. IL-1α at concentrations of 1 and 10 ng/ml stimulated gastrin release to 138 ± 12% and 192 ± 27% of control, respectively. Data are expressed as %control and represent means ± SE of 4 separate animal preparations. *P < 0.05.
EDTA, and 2 mM dithiothreitol and sonicated. Sonicates were centrifuged at 1,000 g for 5 min at 4°C. Supernatants were harvested and centrifuged at 120,000 g and were resuspended in 10 mM Tris-HCl (pH 7.4) containing 1 mM dithiothreitol. Membrane samples (50 μg) were resolved by electrophoresis on 10% acrylamide SDS gels. Proteins were transferred to nitrocellulose blocked with 5% nonfat dry milk for 1 h. Blots were incubated with the primary antibody [anti-PKC-] and IL-1α for 24 h, and RNA was isolated using standard techniques. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. Bottom: TNF-α and IL-1α stimulated gastrin mRNA to 181 ± 18% and 187 ± 28% of control, respectively. Data are expressed as %control and represent means ± SE of 3 separate animal preparations. *P < 0.05.

EDTA, and 2 mM dithiothreitol and sonicated. Supernatants were harvested and centrifuged at 120,000 g for 5 min at 4°C and were resuspended in 10 mM Tris-HCl (pH 7.4) containing 1 mM dithiothreitol. Membrane samples (50 μg) were resolved by electrophoresis on 10% acrylamide SDS gels. Proteins were transferred to nitrocellulose blocked with 5% nonfat dry milk for 1 h. Blots were incubated with the primary antibody [anti-PKC-α or anti-PKC-β rabbit polyclonal antibody (Santa Cruz Biotechnology)] for 5 h. After washing with Tris-buffered saline containing 0.25% dry milk, blots were incubated with peroxidase-linked secondary antibody (goat anti-rabbit horseradish peroxidase; Zymed) for 60 min. Immunoreactive bands were visualized using the standardized EC-like immunoblotting detection system (Amer sham). PKC activity in AGS cell membranes was measured by using an Amer sham PKC assay kit according to the manufacturer’s instructions as previously described (37).

Data analysis. Data are presented as means ± SE. Statistical analysis was performed using Student’s t-test. P < 0.05 was considered significant.

RESULTS

Effect of TNF-α and IL-1 on gastrin release and mRNA in G cells. We previously reported that treatment of G cells with TNF-α for 24 h led to a concentration-dependent increase in gastrin release (5). In this study, we observed that IL-1α at concentrations of 1 and 10 ng/ml stimulated gastrin release by 138 ± 12% (P < 0.05) and 192 ± 27% (P < 0.05) of control, respectively (Fig. 1). Of note, the stimulatory effect of IL-1 was lost at high ligand concentration. The reason for the loss in stimulatory effect is not clear but may involve downregulation or desensitization of the corresponding receptor. In identical cell preparations, bombesin (10−9 M) stimulated gastrin release by 331 ± 87% (P < 0.05). We also examined the effect of these cytokines on gastrin mRNA in G cells. TNF-α and IL-1α (10 ng/ml) significantly increased gastrin mRNA in canine G cells to 181 ± 18% and 187 ± 28% of control levels, respectively, after 24 h of treatment (Fig. 2).

TNF-α and IL-1α-mediated regulation of 240 GasLuc activity in AGS cells. In view of the limited number of G cells obtained in our canine preparation, the heterogeneous population of cells, and the inherent difficulty with manipulating primary cells, we chose to examine cytokine-mediated gastrin regulation in the well-characterized AGS cell line. We first confirmed by PCR that AGS cells expressed receptors for IL-1 and TNF-α (data not shown). Next, we explored the effect of TNF-α and IL-1α on gastrin promoter activity in AGS cells stably transfected with the 240 GasLuc construct (Fig. 3). TNF-α and IL-1α dose-dependently stimulated 240 GasLuc activity to maximal levels of 285 ± 28% of control, respectively. Stimulation of the gastrin promoter was maximal 3 h after agonist treatment. After 3 h, the effect of cytokines on 240 GasLuc activity gradually decreased. Monoclonal anti-human TNF receptor antibody was used to examine the specificity of the observed response. Anti-TNF receptor antibody inhibited the effect of TNF-α (10 ng/ml, 3 h) in a dose-dependent manner with a half-maximal neutralization dose of ~1 μg/ml (Fig. 4A). In contrast, the naturally occurring IL-1 antagonist IL-1ra (0.1–50 ng/ml) did not alter the stimulatory effect of TNF-α (data not shown). IL-1ra dose-dependently inhibited the effect of IL-1α (10 ng/ml, 3 h) with an IC50 of ~9 ng/ml (Fig. 4B), whereas the anti-human TNF receptor antibody (0.1–10 μg/ml) did not alter the stimulatory effect of IL-1α (data not shown).
Effect of kinase inhibitors on TNF-α- and IL-1-mediated activity on 240 GasLuc activity. We next examined the signal transduction pathways by which TNF-α (10 ng/ml) and IL-1α (10 ng/ml) regulate 240 GasLuc activity. For these experiments, we used a series of well-characterized kinase inhibitors. The stimulatory action of both cytokines on 240 GasLuc activity was dose-dependently inhibited by PD-98059, SB-202190, and GF-109203 (data not shown). These inhibitors blocked the action of both cytokines but did not affect basal promoter activity. In contrast, PKI at concentrations as high as 1 μM did not inhibit 240 GasLuc activity stimulated by both cytokines. Cotreatment with PD-98059, SB-202190, and GF-109203 led to an inhibitory effect, which was greater than that observed with the individual antagonist (Fig. 5).

Effect of TNF-α and IL-1 on ERK and p38 kinase activity in AGS cells. There are inherent limitations associated with the interpretation of data obtained by using pharmacological agents to inhibit kinase pathways. Therefore, we determined whether the cytokines of interest were capable of directly activating the pathways proposed to be important in mediating their action in AGS cells. Both cytokines led to a time-dependent increase in ERK and p38 kinase activity. Maximal increase in activity (8- to 9-fold) for both kinases was achieved within 15 min, with activity returning to basal after 60 min (data not shown). As shown in Fig. 6, TNF-α and IL-1α were potent activators of ERK activity. The effects of TNF-α and IL-1α were abolished by preincubation with PD-98059 (10⁻⁵ M) and also inhibited by the TNF receptor antibody (10 μg/ml) and IL-1ra (50 ng/ml), respectively (Fig. 6). Both cytokines activated p38 activity in a reversible manner (Fig. 7). SB-202190 (10⁻⁷ M) inhibited the effect of both cytokines on p38 kinase activity. Anti-human TNF receptor antibody (10 μg/ml) and IL-1ra (50 ng/ml) inhibited TNF-α and IL-1α induction of p38 kinase activity, respectively (Fig. 7). PD-98059 (10⁻⁵ M) and SB-202190 (10⁻⁷ M) did not alter basal ERK and p38 kinase activity.

Effect of TNF-α and IL-1α on PKC in the AGS cell. In view of the inhibitory effect of GF-109203 on TNF-α- and IL-1-mediated regulation of 240 GasLuc, we determined whether these cytokines promote translocation of the conventional PKC isoforms (α and β) to AGS membranes. As shown in Fig. 8, AGS cells express both PKC isoforms, and treatment with phorbol 12-myristate 13-acetate (TPA), TNF-α, and IL-1α for 5 min promoted translocation of these into membranes. We also examined the effects of TPA, TNF-α, and IL-1α on PKC activity in AGS cells. As shown in Fig. 9, TNF-α (10 ng/ml), IL-1α (10 ng/ml), and TPA (10⁻⁶ M) led to an increase in PKC activity in AGS cells.

Fig. 4. A: effect of TNF-α receptor antibody (Anti TNF R) on 240 GasLuc activity stimulated by TNF-α (10 ng/ml, 3 h) in AGS cells. Luciferase activity was dose-dependently inhibited by anti-TNF-α antibody. The half-maximal neutralization dose was ~1 μg/ml. In contrast, the antibody did not alter the stimulatory effect of IL-1α. B: effect of IL-1 receptor antagonist IL-1ra on 240 GasLuc activity stimulated by IL-1α (10 ng/ml, 3 h). IL-1ra dose-dependently inhibited the effect of IL-1α. IC_{50} was ~9 ng/ml. In contrast, IL-1ra did not alter the stimulatory effect of TNF-α. Results are expressed as a %TNF-α- or %IL-1α-stimulated 240 GasLuc activity in absence of TNF-α receptor antibody or IL-1α, respectively, and represent the means ± SE of 6 experiments. *P < 0.05.
Effect of mutating the gERE on cytokine-mediated activation of GasLuc. We initiated characterization of the segment within the gastrin promoter responsible for mediating the effect of TNF-α and IL-1 on gastrin gene transcription by examining the effect of both cytokines on cells transfected with a construct mutated at the gERE. As shown in Fig. 10, mutation of the gERE did not inhibit the effect of either TNF-α (10 ng/ml) or IL-1α (10 ng/ml), even though this mutation significantly inhibited luciferase induction by EGF (10^{-8} M; data not shown).

DISCUSSION

Accumulating evidence indicates that gastrin secretion and gene expression are directly regulated by a...
host of physiological factors, including feeding, fasting, gastric acid, EGF, and somatostatin. Despite these observations, little is known regarding the pathways impacting gastrin regulation in pathological states such as during infection with the organism \textit{H. pylori}. We attempted to address this issue in the present study by examining the impact of only two of the factors thought to be involved in \textit{H. pylori}-mediated pathogenesis, TNF-\(\alpha\) and IL-1, on gastrin gene expression. Through the utilization of two cell models, primary canine G cells and AGS cells, we demonstrated that both TNF-\(\alpha\) and IL-1 can directly activate gastrin expression. In addition, it appears that the MAPK and PKC pathways play a role in mediating the action of TNF-\(\alpha\) and IL-1 on gastrin regulation.

It has previously been demonstrated that TNF-\(\alpha\) can stimulate gastrin release from primary cells (3, 38). Moreover, our work is consistent with the studies by Weigert and co-workers (38) demonstrating that IL-1 can lead to gastrin release from isolated rabbit G cells. We extended these observations by exploring the mechanism by which TNF-\(\alpha\) and IL-1 impact gastrin regulation. Our work with G cells demonstrates that, in addition to stimulating release of the peptide, both TNF-\(\alpha\) and IL-1 \(\alpha\) can increase gastrin mRNA, suggesting that these proinflammatory factors may regulate gastrin expression at a transcriptional level.

Elucidation of the mechanism by which the cytokines of interest mediate transcription of the gastrin gene is virtually impossible in primary G cells. We utilized AGS cells, a well-characterized gastric carcinoma cell line that resembles gastric epithelial cells, as a model for studying cytokine-mediated gastrin transcription. This model allowed us to look at the effects of TNF-\(\alpha\) and IL-1 on transcriptional regulation of the gastrin gene and provided a system in which to explore cytokine-mediated signal transduction. Using this model, we have successfully demonstrated that both TNF-\(\alpha\) and IL-1 can directly activate transcription of the gastrin gene through regulation of the 240-bp 5'-flanking region examined.

The promoter of the gastrin gene contains several regulatory sequences including gERE, Sp1, and AP2 (23). Previous studies have shown that the gERE element is regulated in a positive manner by EGF, phorbol ester, and cAMP (23, 29). These observations prompted us to examine whether TNF-\(\alpha\)- or IL-1-mediated activity was also dependent on the gERE element. As shown in our studies, mutation of the gERE element did not alter TNF-\(\alpha\)- or IL-1\(\alpha\)-mediated 240 GasLuc activity, suggesting that the action of both cytokines is independent of this promoter element.

![Fig. 8. Effect of TNF-\(\alpha\) and IL-1\(\alpha\) on PKC translocation in AGS cells. AGS cells express both \(\alpha\) (A) and \(\beta\) (B) isoforms of PKC. In addition, treatment of AGS cells with TNF-\(\alpha\) (10 ng/ml), IL-1\(\alpha\) (10 ng/ml), and phorbol 12-myristate 13-acetate (TPA, 10$^{-6}$ M) for 5 min promoted translocation of both isoforms to cell membranes. Data are expressed as %untreated cells (control) and represent means \pm SE of 3 experiments. $^*P < 0.05$; $^{**}P < 0.01$. A representative assay is depicted.](http://ajpgi.physiology.org/)

![Fig. 9. Effect of TNF-\(\alpha\) (10 ng/ml), IL-1\(\alpha\) (10 ng/ml), and TPA (10$^{-6}$ M) on PKC activity in AGS cells. TNF-\(\alpha\), IL-1\(\alpha\), and TPA led to an increase in PKC activity in AGS cells after 5 min. Data are expressed as %control and represent means \pm SE of 4 experiments. $^*P < 0.05$.](http://ajpgi.physiology.org/)

![Fig. 10. Effect of TNF-\(\alpha\) (10 ng/ml, 3 h) and IL-1\(\alpha\) (10 ng/ml, 3 h) on wild-type 240 GasLuc and mutant 240 GasLuc activity. The latter contains a mutation within the epidermal growth factor-responsive element (gERE). TNF-\(\alpha\) and IL-1\(\alpha\) stimulated GasLuc activity independently of the gERE. Data are expressed as %activity in untreated cells (control) and represent means \pm SE for 4 experiments performed in duplicate.](http://ajpgi.physiology.org/)
Elucidation of the elements through which TNF-α and IL-1 regulate gastrin expression is the focus of ongoing studies.

The mechanism by which TNF-α or IL-1 regulates gastrin expression and release is unknown. One may consider that the actions of both cytokines are via an interdependent pathway. Specifically, TNF-α is a known stimulant of IL-1 release from several cell systems. Our blocking studies with anti-TNF-α antibody and IL-1ra confirm that each factor leads to activation of 240 GasLuc via different receptors. An additional consideration is that both cytokines are acting on a common factor, which in turn is activating gastrin expression. One potential factor is IL-8, which can be released from AGS cells in response to both TNF-α and IL-1 (1, 4). We explored this possibility by testing the effect of IL-8 on 240 GasLuc activity. Although not shown here, IL-8 at concentrations as high as 10 nM failed to stimulate 240 GasLuc activity. Although this observation does not exclude a modularity role of IL-8 on the regulation of gastrin gene expression, it excludes this segment of the promoter as being an important target for this chemokine.

Although much has been learned regarding the post-receptor events leading to cytokine-mediated cell activation, the specific pathways by which TNF-α and IL-1 regulate many cell types remains unknown. Moreover, it is apparent that gastrin gene transcription is regulated by a host of signaling pathways, including tyrosine kinase, PKC, cAMP, and MAPK (16, 23, 25, 29). Using a series of selective pharmacological tools, we began to examine which pathways may be mediating the action of TNF-α and IL-1 on gastrin expression. Our antagonist studies support that PKC, ERK-2, and p38 kinase in part mediate the action of both cytokines. Of interest, TNF-α and IL-1 appear to share similar mechanisms for regulation of 240 GasLuc activity. Although both factors couple and activate independent cell surface receptors, the postreceptor events for each appear to be quite similar, an observation that has been made in other cell systems.

In view of the known limitations of pharmacological tools for the study of cell signaling, we took our experiments one step further by examining whether TNF-α and IL-1 regulate the specific pathways of interest (PKC, ERK-2, and p38). Consistent with the effect of TNF-α and IL-1 in other cell models (33, 34, 36), both cytokines stimulated PKC activity in AGS cells. Moreover, both cytokines stimulated translocation of two common PKC isoforms, PKC-α and -β, lending further support for the role of PKC in cytokine-mediated 240 GasLuc activation. Both cytokines led to a time- and dose-dependent increase in ERK-2 and p38 kinase activity, findings consistent with observations made in other systems (13, 24). Our data illustrating that the time frame and dose range needed for cytokine-mediated GasLuc activity parallels those required for PKC, ERK-2, and p38 activation supports that these events are linked.

The sequence of events leading to transcriptional activation of the gastrin gene by TNF-α and IL-1 remains unknown. An early event may involve activation of PKC, which subsequently regulates ERK-2 and p38. The fact that PKC inhibition does not abolish the effect of either TNF-α or IL-1 on 240 GasLuc activity suggests that MAPK and PKC are activated in a parallel and complementary fashion. Our studies demonstrating that the different antagonists have added inhibitory effects support this hypothesis. An additional upstream signaling system involved in gastrin gene expression, which we did not explore, includes the Raf-Ras signaling pathway (25).

In summary, we have demonstrated that both TNF-α and IL-1 can directly stimulate gastrin gene expression via a PKC- and MAPK-dependent mechanism. Our findings support the hypothesis that proinflammatory cytokines may play a direct role in H. pylori-associated hypergastrinemia.

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