Gastrin increases mcl-1 expression in type I gastric carcinoid tumors and a gastric epithelial cell line that expresses the CCK-2 receptor

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Pritchard DM, Berry D, Przemeck SM, Campbell F, Edwards SW, Varro A. Gastrin increases mcl-1 expression in type I gastric carcinoid tumors and a gastric epithelial cell line that expresses the CCK-2 receptor. Am J Physiol Gastrointest Liver Physiol 295: G798–G805, 2008. First published August 21, 2008; doi:10.1152/ajpgi.00015.2008.—Elevated serum concentrations of the hormone gastrin are associated with the development of gastric carcinoid tumors, but the mechanisms of tumor development are not fully understood. We hypothesized that the antia apoptotic effects of gastrin may be implicated and have therefore investigated the role of antia apoptotic members of the bcl-2 family of proteins. AGS-GR human gastric carcinoma cells stably transfected with the CCK-2 receptor were used to assess changes in the expression of bcl-2 family members following gastrin treatment and the function of mcl-1 during apoptosis was investigated by use of small-interfering RNA (siRNA). Treatment of AGS-GR cells with 10 nM gastrin for 6 h caused maximally increased mcl-1 protein abundance. Gastrin-induced mcl-1 expression was inhibited by the transcription inhibitor actinomycin D and by the protein synthesis inhibitor cycloheximide. Downstream signaling of mcl-1 expression occurred via the CCK-2 receptor, protein kinase C, and MAP kinase pathways, but not via PI 3-kinase. Transfection with mcl-1 siRNA significantly suppressed mcl-1 protein expression and abolished the antia apoptotic effects of gastrin on serum starvation-induced apoptosis. Mcl-1 protein expression was also specifically increased in the type I enterochromaffin-like cell carcinoid tumors of 10 patients with autoimmune atrophic gastritis and hypergastrinemia. Gastrin therefore signals via the CCK-2 receptor, protein kinase C, and MAP kinase pathways, but not via PI 3-kinase. Transfection with mcl-1 siRNA significantly suppressed mcl-1 protein expression and abolished the antia apoptotic effects of gastrin on serum starvation-induced apoptosis.

Apoptosis or programmed cell death is a tightly regulated cellular process and several families of proteins are thought to be involved. Gastrin has previously been shown to exert antia apoptotic effects in a number of cell lines including cells of pancreatic (e.g., AR42J) and gastric (e.g., IMGE-5) origin, and gastrin-induced alterations in the expression of several members of the bcl-2 family of proteins have also previously been demonstrated (10, 11, 16, 31–33). Increased immunohistochemical expression of antia apoptotic bcl-2 has also been demonstrated in biopsies obtained from human subjects with atrophic gastritis (23) and type I gastric carcinoid tumors (1).

We have therefore hypothesized that gastrin may regulate apoptosis by specifically altering the expression of individual members of the bcl-2 family of proteins. Mcl-1 is one antia apoptotic member of this family of proteins and it is thought to inhibit apoptosis by inhibiting mitochondrial cytochrome c release (reviewed in Ref. 24). To assess whether gastrin can alter the expression of antia apoptotic members of the bcl-2 family of proteins we used a human gastric epithelial cell line that has been stably transfected with the CCK-2 receptor and in which gastrin has previously been shown to exert multiple effects (AGS-GR) (28, 29, 38). This cell line is derived from a gastric adenocarcinoma and is not of neuroendocrine origin. Significantly increased expression of antia apoptotic mcl-1, a protein not previously demonstrated to be gastrin regulated, was demonstrated and subsequent experiments were therefore...
performed to investigate the signaling mechanisms involved and the functional consequences of this observation in the cell line. We subsequently investigated whether mcl-1 expression was also increased in gastric epithelial cells that express the CCK-2 receptor in vivo. To do this we used endoscopic biopsies obtained from human patients with hypergastrinemia-associated type I gastric ECL cell carcinoid tumors.

**MATERIALS AND METHODS**

*Materials.* Ro-32-0432, PD-98059, LY-294002, and wortmannin were all from Calbiochem (Nottingham, UK), YM022 was from Tocris Bioscience (Bristol, UK), and gastrin-17 was from Bachem (St. Helens, UK). All other routine chemicals were from Sigma (Poole, UK), unless stated.

*Tissue culture.* The AGS human gastric carcinoma cell line and a transfected stably expressing the CCK-2 receptor (AGS-GR) were used as previously described (35, 38). Cells were cultured in Ham’s F12 medium supplemented with 10% fetal calf serum (GBICO, Paisley, UK), 2 mM l-glutamine, and 1% penicillin-streptomycin at 37°C in a water-saturated atmosphere of 95% air and 5% CO2.

*Western blotting.* Protein extracts were prepared and electrophoresed on 10% SDS-polyacrylamide gels followed by transfer onto nitrocellulose membrane (Protran, Schleicher & Schuell). Nonspecific antibody binding was blocked by incubating the membrane in 1% nonfat milk in PBS-Tween-20. Membranes were incubated with the following primary antibodies: mouse monoclonal anti-mcl-1 antibody (Calbiochem) at a dilution of 1:200, mouse monoclonal anti-bcl-2 (DakoCytomation, Cambridge, UK) at a dilution of 1:100, mouse monoclonal anti-bcl-xL (Calbiochem) at a dilution of 1:25, or mouse monoclonal anti-actin antibody (Neomarkers, Freemont, CA) at a dilution of 1:1,000. The secondary antibody was horseradish peroxidase (HRP)-conjugated rabbit anti-mouse immunoglobulins from Dianova (H and E), chromogranin A, and mcl-1 immunohistochemistry and were assessed by an experienced specialist gastrointestinal pathologist (F. Campbell) for the presence of H. pylori, atrophic gastritis, ECL cell hyperplasia, and carcinoid tumor. The study was approved by the local ethics committee and all participants gave written, informed consent.

*Analysis of plasma gastrin concentrations.* Radioimmunoassay for amidated gastrin was performed on serum samples using antibody L2 as previously described (34).

*Immunohistochemistry.* Tissue sections were deparaffinized and antigen retrieval was performed by microwaving in 10 mM citric acid buffer (pH 6) for 20 min. The primary antibodies used were a rabbit polyclonal anti-mcl-1 antibody from Santa Cruz Biotechnology (Santa Cruz, CA) at a dilution of 1:1,000, a mouse monoclonal anti-chromogranin antibody from DakoCytomation at a dilution of 1:500, a rabbit anti-cleaved caspase 3 antibody from Cell Signaling Technology at a dilution of 1:100, and a mouse monoclonal anti-bcl-2 antibody from DakoCytomation at a dilution of 1:500. The signal was developed by using the appropriate species-specific Dako EnVision+ system HRP (DAB) kits (DakoCytomation) and the slides were counterstained with hematoxylin.

*Statistical analyses.* Differences were assessed by two-tailed Student’s t-test assuming unequal variance of the groups being tested. A level of P < 0.05 was interpreted as significant.

**RESULTS**

Gastrin increases mcl-1 protein abundance in AGS-GR gastric epithelial cells. Treatment of AGS-GK cells with 10 nM gastrin-17 in the presence of medium containing 10% serum increased mcl-1 protein abundance maximally at 6 h (Fig. 1A). This increased mcl-1 expression was not observed in untransfected AGS cells that lack the CCK-2 receptor (Fig. 1C). A slight increase in antiapoptotic bcl-xL was also observed at 2–4 h (Fig. 1A). No bcl-2 protein could be detected in this cell line, but bcl-2 was detected in two other cell lines, Raji B (a lymphoid cell line) and HCT116 (human colorectal carcinoma), which were used as positive controls (Fig. 1A). A dose-response analysis showed that mcl-1 protein was induced in AGS-GK cells by concentrations of gastrin-17 ≥2.5 nM (Fig. 1B). A dose of 10 nM gastrin-17 for 6 h caused maximally increased mcl-1 protein expression, hence this dosing schedule was used for all subsequent experiments.

Signaling pathways involved in gastrin-induced mcl-1 induction in AGS-GK gastric epithelial cells. Specific inhibitors were used to investigate the signaling pathways responsible for the gastrin-induced increase in mcl-1 expression in AGS-GK cells. Pretreatment of AGS-GK cells with the transcriptional inhibitor actinomycin D or the protein synthesis inhibitor cycloheximide for 20 min significantly inhibited gastrin-induced mcl-1 expression (Fig. 2). Pretreatment of AGS-GK cells with the specific CCK-2 receptor antagonist YM022 virtually abolished the gastrin-induced increase in mcl-1 expression (fig. 3, A and C). The absence of gastrin-induced mcl-1 up-regulation in AGS cells that lack the CCK-2 receptor (Fig. 1C) and in YM022-treated AGS-GK cells (Fig. 3) suggests that the gastrin-induced increase in mcl-1 expression occurs via gastrin binding to the CCK-2 receptor. The gastrin-induced increase in mcl-1 protein abundance observed in AGS-GK cells was also significantly inhibited by the protein kinase C inhibitor Ro-32-0432 and by the MAP kinase inhibitor PD-98059 (Fig. 3, A and C). However, pretreatment of AGS-GK cells with the phospho-
tidylinositol 3-kinase (PI 3-kinase) inhibitors LY-294002 and wortmannin caused no significant changes in gastrin-induced mcl-1 expression (Fig. 3, B and C). These studies suggest that following binding to the CCK-2 receptor, gastrin signals via protein kinase C and MAP kinase pathways, but not via the PI 3-kinase pathway to cause increased mcl-1 protein abundance.

**Mcl-1 siRNA transfection of AGS-GR gastric epithelial cells abolishes the antiapoptotic effects of gastrin.** Transfection of AGS-GR cells with siRNA targeted against human mcl-1 caused a significant reduction in basal mcl-1 protein expression, and no increase in mcl-1 expression was observed following treatment with 10 nM gastrin for 6 h (Fig. 4, A and B). By contrast, transfection of AGS-GR cells with control siRNA had no significant effect on basal or gastrin-induced mcl-1 protein abundance compared with untransfected AGS-GR cells (Fig. 4, A and B). Following transfection, cells were transferred into serum-free medium for 6 h to induce apoptosis. Treatment with 10 nM gastrin significantly inhibited serum starvation-induced apoptosis in control siRNA-transfected AGS-GR cells. However, 10 nM gastrin treatment of mcl-1 siRNA-transfected AGS-GR cells did not result in inhibition of serum starvation-induced apoptosis but actually caused a significant increase in the percentage of apoptotic cells (Fig. 4C). Apoptosis was also assessed by another independent technique, namely assessment of effector caspase activity using the Caspase-Glo 3/7 assay. Using this method, we demonstrated a significant 25% decrease in caspase 3/7 activity in control siRNA-transfected cells following 6 h treatment with 10 nM gastrin in the presence of serum-free medium (P < 0.01, n = 8), whereas mcl-1 siRNA-transfected cells conversely showed a significant 16% increase in caspase 3/7 activity following the same treatment (P < 0.05, n = 8).

**Mcl-1 is expressed in type I ECL cell gastric carcinoid tumors.** We hypothesized that the gastrin-induced increase in mcl-1 protein abundance observed in AGS-GR cells in vitro also occurred in vivo in the stomach of human patients with hypergastrinemic states. We obtained endoscopic biopsies from the gastric corpus and nodules of 10 patients (8 women, mean age 57.7 yr, range 43–80 yr) who all had atrophic gastritis, achlorhydria, marked hypergastrinemia, and evidence of type I gastric carcinoid tumor development (Table 1). In all cases (although not absolutely confirmed in case 10), the gastric atrophy was thought to have an autoimmune cause, because of the presence of positive antigastric parietal cell antibodies and/or vitamin B₁₂ deficiency, with absence of *H. pylori* by histological analysis in all cases and in 8 of the 10 cases by serology. No patients were taking acid-suppressing medications. In addition, gastric corpus biopsies were also obtained from four control patients (2 women, mean age 44.8 yr, range 27–60 yr), who all had normal esophagogastroduodenoscopy, normal gastric histology, normogastrinemia (fasting serum gastrin < 20 pM in each case), and absent *H. pylori* infection by histological and serological criteria and who were not taking acid-suppressing medications.

Tissue sections were stained with H and E and immunostained for mcl-1 and chromogranin A and were then assessed.
by an experienced specialist gastrointestinal pathologist (F. Campbell). No positive signal was detected in control slides processed without the addition of primary antibody. Weak diffuse cytoplasmic mcl-1 expression was observed in a few cells in the upper portion of the gastric corpus glands of the control normogastrinemic patients (see Fig. 5A with chromogranin A immunohistochemistry of a serial section shown in Fig. 5B) as described in previous reports (18). Six of the 10 hypergastrinemic patients had microscopically confirmed carcinoid tumor in the biopsy specimens obtained. In five of these six cases there was distinct cytoplasmic mcl-1 expression in the gastric carcinoid tumor cells (see Fig. 5C for representative mcl-1 staining, with chromogranin A immunohistochemistry of a serial section shown in Fig. 5D), and one case showed weak cytoplasmic mcl-1 expression in the carcinoid tumor. All 10 cases additionally showed the presence of ECL cell hyperplasia in both linear and nodular forms. Eight of the 10 patients showed distinct cytoplasmic mcl-1 expression in areas of nodular ECL cell hyperplasia (see Fig. 5, E and G, for representative mcl-1 staining and Fig. 5, F and H, for chromogranin A staining of serial histological sections). The remaining two patients had weak mcl-1 staining in areas of nodular ECL cell hyperplasia.

We also immunostained serial sections of each sample for cleaved caspase 3 as a marker of apoptosis. Although occasional cleaved caspase 3-positive cells were seen in the gastric mucosa (Fig. 6, B and F), virtually no cleaved caspase 3-positive cells were seen in areas of nodular ECL cell hyperplasia (Fig. 6E) or of gastric carcinoid tumor (<1 cleaved caspase 3-positive cell per 1,000 cells in all 10 patients).

![Fig. 3. A and B: representative Western blots. C: densitometric analysis of individual Western blots from 3–6 separate experiments showing effects of treatment with the CCK-2 receptor antagonist YM022 (10 nM), the protein kinase C (PKC) inhibitor Ro-32-0432 (1 μM), the MAP kinase (MAPK) inhibitor PD-98059 (20 μM), and the PI 3-kinase (PI3K) inhibitors LY-294002 (20 μM) and wortmannin (500 nM) on mcl-1 expression in AGS-G8 cells following treatment for 6 h with 10 nM gastrin-17. *P < 0.05, **P < 0.01 compared with gastrin treatment alone.](image)

by an experienced specialist gastrointestinal pathologist (F. Campbell). No positive signal was detected in control slides processed without the addition of primary antibody. Weak diffuse cytoplasmic mcl-1 expression was observed in a few cells in the upper portion of the gastric corpus glands of the control normogastrinemic patients (see Fig. 5A with chromogranin A immunohistochemistry of a serial section shown in Fig. 5B) as described in previous reports (18). Six of the 10 hypergastrinemic patients had microscopically confirmed carcinoid tumor in the biopsy specimens obtained. In five of these six cases there was distinct cytoplasmic mcl-1 expression in the gastric carcinoid tumor cells (see Fig. 5C for representative mcl-1 staining, with chromogranin A immunohistochemistry of a serial section shown in Fig. 5D), and one case showed weak cytoplasmic mcl-1 expression in the carcinoid tumor. All 10 cases additionally showed the presence of ECL cell hyperplasia in both linear and nodular forms. Eight of the 10 patients showed distinct cytoplasmic mcl-1 expression in areas of nodular ECL cell hyperplasia (see Fig. 5, E and G, for representative mcl-1 staining and Fig. 5, F and H, for chromogranin A staining of serial histological sections). The remaining two patients had weak mcl-1 staining in areas of nodular ECL cell hyperplasia.

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![Fig. 4. Representative Western blot (A) and densitometric analysis (B) of individual Western blots from 3 separate experiments showing effects of 24 h transfection with mcl-1 or control small-interfering RNA (siRNA) on mcl-1 protein expression in AGS-G8 cells following treatment for 6 h with 10 nM gastrin-17. *P < 0.05 compared with gastrin treatment alone.](image)

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Serial sections of each sample were also immunostained for bcl-2. There appeared to be more bcl-2-positive cells in all 10 hypergastrinemic patients samples compared with the four normogastrinemic control patients (data not shown). However, whereas mcl-1 expression appeared to occur predominantly in areas of ECL cell hyperplasia and of carcinoid tumor (Fig. 6D), bcl-2 expression in all 10 patients was predominantly seen in lymphoid and stromal cells rather than in epithelial cells (Fig. 6, C and G).

**DISCUSSION**

In summary, we have demonstrated that gastrin induces mcl-1 protein expression in AGS-GR cells in a time-, dose-,
and CCK-2-dependent manner (Fig. 1). Signal transduction occurs via protein kinase C and MAP kinase pathways, but not via PI 3-kinase (Fig. 3). Gastrin-induced mcl-1 expression is functionally important, since transfection with mcl-1 siRNA abolished the antiapoptotic effects of gastrin in this cell line (Fig. 4). Finally, we have demonstrated specific mcl-1 expression in the ECL cell nodular hyperplasia and type I carcinoid tumors of human subjects with autoimmune atrophic gastritis, achlorhydria, and hypergastrinemia (Fig. 5, Table 1).

Gastrin has previously been shown to exert antiapoptotic effects in a number of cell lines, and the involvement of various downstream mediators has been demonstrated in these studies (10, 11, 16, 31–33). However, other studies, which initially appear to be contradictory, have demonstrated pro-apoptotic effects of gastrin. For example, increased gastric epithelial apoptosis has been reported in hypergastrinemic INS-GAS mice following both γ-radiation and Helicobacter infection (4, 30), in H2 antagonist-treated hypergastrinemic African rodents Mastomys (Praomys natalensis) (15) and in H. pylori-infected humans with moderate degrees of hypergastrinemia (30). However, most of these reports have studied normal untransformed gastric epithelial cells in vivo. We have previously therefore suggested that the effects of gastrin on apoptosis may be cell type specific and may actually differ between transformed and untransformed cell types (30). It is also possible that the effects of gastrin on apoptosis in the normal gastric epithelium in vivo may not be a direct result of hypergastrinemia but may be due to indirect effects resulting from gastrin-induced alterations in other proapoptotic growth factors. Further studies are required to clarify the precise mechanisms involved. In the present study we have specifically assessed the direct effects of gastrin on transformed CCK-2 receptor-bearing cells in vitro and in vivo.

Gastrin-induced changes in the expression of several members of the bcl-2 family of proteins have previously been reported. Gastrin-induced inhibition of apoptosis in AR42J pancreatic adenocarcinoma cells has previously been shown to be associated with the phosphorylation and subsequent inhibition of proapoptotic bad (31). A similar mechanism has also been demonstrated in IMGE-5 gastric epithelial cells as a result of gastrin activation of Rac/Cdc42/PAK (11). Gastrin has also been shown to inhibit proapoptotic bax expression and to induce antiapoptotic bcl-xl expression in IMGE-5 cells via a Rho/ROCK pathway (11). Gastrin has also been reported to increase the expression of bcl-2 and survivin in KATO III gastric adenocarcinoma cells (16). Increased immunohistochemical bcl-2 expression has been demonstrated in autoimmune as well as H. pylori-associated atrophic gastritis (23) and in hypergastrinemia-associated ECL cell gastric carcinoid tumors (1, 14). We also demonstrated increased bcl-2 expression in patients with atrophic gastritis and hypergastrinemia, but this bcl-2 expression appeared to occur predominantly in stromal and lymphoid cells rather than in ECL cells (Fig. 6). Increased gastric bcl-2 expression may therefore contribute toward the development of gastric carcinoid tumors, but in view of the lack of bcl-2 expression by ECL cells the effects may not be due to direct inhibition of apoptosis in this cell type. Bcl-2 is also unlikely to contribute toward the antiapoptotic effects of gastrin described in AGS-G8 cells in this paper, since we could detect no expression of bcl-2 protein in this cell type.
tein expression has also previously been detected in human gastric glands (18). Immunohistochemical monitoring of Mcl-1 protein was weakly expressed in the pit region of normal gastric cells. Mcl-1 is an antiapoptotic member of the bcl-2 family of proteins, which acts by inhibiting the release of cytochrome c from mitochondria (reviewed in Ref. 24). It is an unusual protein in view of its rapid turnover (3, 7), and its expression is known to be altered by several growth factors and cytokines (19, 27). In our experimental system the half-life of mcl-1 protein was ~2 h (data not shown), hence the increased mcl-1 expression observed at 6 h was no longer seen at 24 h (Fig. 1). Mcl-1 is weakly expressed in the pit region of normal human gastric glands (18). Immunohistochemical monitoring of Mcl-1 protein expression has also previously been detected in ~70% of patients with gastric adenocarcinoma and expression has been shown to be associated with a poorer prognosis (17, 20). CagA expressing strains of H. pylori have also been shown to increase mcl-1 expression in the gastric pits of infected Mongolian gerbils (25). Interleukin-6 has also been shown to induce mcl-1 expression in A549 gastric carcinoma cells and this was shown to protect cells against apoptosis induced by reactive oxygen species (19). Treatment of gastric carcinoma cell lines with antisense oligonucleotides directed toward mcl-1 has also been shown to significantly increase the apoptosis induced in these cell lines by chemotherapeutic drugs (39). Cultured isolated rat ECL cells have been shown to undergo apoptosis following addition of proinflammatory cytokines such as TNF-α and interleukin-1β (13, 21, 22). This process was inhibited by several growth factors including TGF-α and basic FGF (22). However, in these studies, although gastrin also appeared to partially inhibit cytokine-induced apoptosis, the results did not reach statistical significance and only a low concentration of gastrin (10 pM) was assessed (22). It is, however, possible that although direct effects were not demonstrated in isolated rat ECL cells at a single low concentration of gastrin, significantly elevated serum concentrations of this hormone may regulate ECL cell apoptosis in vivo when other cell types are also present. In conclusion, therefore, we have made the novel observation that gastrin increases the expression of antiapoptotic mcl-1 protein in a gastrin-responsive CCK-2 receptor expressing gastric epithelial cell line. In addition, increased mcl-1 expression has been demonstrated in ECL cell tumors from human hypergastrinemic patients. Gastrin-induced mcl-1 expression may therefore be a factor leading to inhibition of apoptosis in gastric ECL cells, thus promoting the development of gastric carcinoid tumors.

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