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Intracellular mechanisms mediating the anti-apoptotic action of gastrin

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Abstract

We previously reported that gastrin (G17) inhibits apoptosis of AR4-2J pancreatic adenocarcinoma cells, through the activation of Akt. We dissected the mechanisms responsible for this effect. D2, a CCK_B receptor antagonist, inhibited G17 induction of Akt phosphorylation, measured by Western blots with anti-phospho-Akt antibodies. The intracellular calcium chelator BAPTA-AM, but not the PKC inhibitor GF109203X, blocked G17 induction of Akt. G17 stimulated BAD phosphorylation, measured by both Western blots with anti-phospho-BAD antibodies and by in vitro Akt kinase assays using recombinant BAD as substrate. G17 also induced FOXO3 phosphorylation assessed by Western blots with anti-phospho-FOXO3 antibodies, and BAPTA-AM inhibited this effect. Gastrin inhibited luciferase activity in cells transfected with FOXO1 together with a vector containing insulin-responsive sequences upstream of the luciferase reporter gene. In conclusion, G17 induces Akt through activation of CCK_B receptors and of intracellular calcium-dependent, PKC-independent, pathways. This effect leads to BAD phosphorylation and to forkhead transcription factors inactivation.

Keywords: Cellular apoptosis; Protein kinases; Forkhead/winged-helix transcription factors; Akt kinase

Although characterized as a stimulant of gastric acid secretion, the peptide hormone gastrin also exerts growth-promoting effects on normal and malignant gastrointestinal tissues [1–4]. In addition, gastrin has been shown to induce the growth of colonic, gastric, and pancreatic carcinomas both in vivo and in vitro, underscoring the importance of gastrin as a growth factor for gastrointestinal neoplasms [3,4].

The intracellular signal transduction pathways activated by gastrin to induce cellular proliferation have been the focus of numerous investigations. Gastrin induces protein tyrosine kinase activity, stimulates phosphoinositide 3-kinase (PI3K), and activates the extracellular-signal regulated protein kinases (ERKs) or mitogen activated protein kinases (MAPKs) [3,4]. In particular, we and others have reported that gastrin stimulates the growth of rat pancreatic adenocarcinoma cells (AR4-2J) through induction of the ERKs and of the early response gene c-fos through PKC-dependent and -independent mechanisms [5–7].

In addition to this well-established effect on the regulation of cellular growth and proliferation, gastrin has also been shown to be a potent inhibitor of cellular apoptosis. Indeed, work from our laboratory has demonstrated that gastrin inhibits AR4-2J cell apoptosis through a signal transduction pathway that requires the activation of Akt [8], a protein kinase known to induce cellular growth and survival [9–14]. Akt has been shown to phosphorylate several cellular proteins that are known to play an important role in the induction of apoptosis. In particular, Akt phosphorylates the pro-apoptotic proteins BAD [10–12] and caspase-9 in vivo [10]. It also phosphorylates and inactivates forkhead transcription factors such as FOXO1, FOXO3, and FOXO4 that are involved in the regulation of cellular apoptosis [13,14].

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Interestingly, a recent report has indicated that inhibition of the proliferative and anti-apoptotic actions of gastrin in the human pancreatic cancer cell line PAN1 leads to increased sensitivity of the cells to the action of cytotoxic agents, suggesting that the ability of gastrin to inhibit pancreatic cancer cell apoptosis might be of considerable clinical relevance and significance [15].

The signal transduction pathways that regulate the activation of Akt in response to gastrin stimulation have been only partially characterized. Accordingly, we undertook studies to further dissect the intracellular mechanisms that mediate the anti-apoptotic actions of gastrin in the AR4-2J cells. Our data indicate that gastrin, binding to specific CCK_B receptors, induces Akt through the activation of intracellular calcium-dependent, PKC-independent signal transduction pathways. In addition, gastrin appears to stimulate the phosphorylation of the pro-apoptotic protein BAD and the transcriptional inactivation of FOXO forkhead transcription factors.

Materials and methods

Plasmids. The plasmid expressing 3× IRS-Luc [13] was a gift of K. Guan (Ann Arbor, MI). The FOXO1 (FKHR) expressing vector was a gift from F.G. Barr (Philadelphia, PA) [13]. Gal4-ElkC [16] was a gift from R. Treisman (London, UK). pCMV-βGal was a gift from M. Uhler (Ann Arbor, MI). Recombinant BAD [12] was a gift from G. Nunez (Ann Arbor, MI).

Cell culture, transient transfection, and luciferase assays. For our experiments we used the rat exocrine pancreatic cell line AR4-2J (obtained from American Type Culture Collection, Rockville, MD), which is known to express receptors for gastrin [17]. The AR4-2J cells were grown at 37 °C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% sodium pyruvate. The cells were maintained in 5% CO_2/95% O_2. For the studies, the cells were starved for 24 h in serum-free medium and then treated for different time periods with G17 (10 nM). In some experiments, the intracellular calcium chelator BAPTA-AM (100 μM) and the PKC inhibitor GF109203X (3.5 μM) (Calbiochem, La Jolla, CA), were added 30 min prior to the addition of gastrin. BAPTA-AM and GF109303X were dissolved in dimethyl sulfoxide (DMSO, Sigma, St. Louis, MO). All other test substances were dissolved in water. Control experiments were performed by incubating the cells in either incubation buffer or vehicle without the test substances. Subconfluent AR4-2J cells were transfected with 0.5 μg of the luciferase reporter plasmid, 0.05 μg of the β-galactosidase expressing vector and, where indicated, with 0.5 μg of the vectors expressing either FOXO1 or Gal4-ElkC. Transfections were carried out using Lipofectamine (Gibco-BRL, Grand Island, NY) as previously described [6]. At the end of the incubation period, the cells were washed, lysed, and used for luciferase and β-galactosidase assays according to previously described methods [6]. Luciferase activity was expressed as RLU (relative light units) and normalized for β-galactosidase activity.

Immunoprecipitations and Akt assays. Immunoprecipitations and Akt assays were performed according to previously described techniques [7]. The AR4-2J cells were lysed in 500 μl lysis buffer (50 mM Hepes, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM EDTA, 1.5 mM $MgCl_2$, 1 mM Na_3VO_4, 10 mM NaF, 10 mM $Na_4P_2O_7{\cdot}10\ H_2O$, 1 mM 4-(2-aminoethyl)benzenesulfonylfluoridehydrochlorine [AEBSF, ICN-Biomedicals, Aurora, OH], 1 μg/ml leupeptin, and 1 μg/ml aprotinin). The lysates were transferred into microfuge tubes and spun at 16,000*g* for 20 min at 4 °C. Equal amounts of protein from each treatment group (600 μg) were incubated with an anti-Akt antibody (Cell Signaling, Beverly, MA). Samples were mixed on a rotating platform for 16–18 h at 4 °C. Protein concentrations were measured by the Bradford method [18]. Aliquots of protein A–Sepharose (50 μl) (Pharmacia Biotech, Piscataway, NJ) were then added and the solutions were mixed for one additional hour. After centrifugation the pellets were washed once with lysis buffer and twice with kinase buffer. Immunoprecipitated Akt was used to phosphorylate recombinant BAD according to previously published techniques [12].

Western blots. Eighty micrograms of AR4-2J cell lysates was loaded on a 10% SDS–polyacrylamide mini-gels and run at 20 A for 8 h. The gels were transferred on an Immobilon-P transfer membrane (Millipore, Bedford, MA) in 25 mM Tris, 150 mM glycine, and 20% methanol. After transfer the membranes were blocked in 10 ml TBST (20 mM Tris, 0.15 M NaCl, and 0.3% Tween), 5% dried milk for 1 h and then incubated for 16–18 h at 4 °C in 10 ml TBST, 5% dried milk, containing either a specific anti-phospho-Akt antibody which recognizes phosphorylated serine 473 of Akt kinase (1:1000) (Cell Signaling, Beverly, MA) or a specific anti-phospho FOXO3 antibody (1:500) (Upstate Biotechnology, Lake Placid, NY). In some experiments the membranes were incubated with a specific anti-phospho-BAD antibody (1:1000) (Cell Signaling, Beverly, MA). At the end of the incubation period the membranes were washed in TBST for 30 min at room temperature and then incubated for 1 h in TBST, 5% dried milk, containing either protein A, directly conjugated to horseradish peroxidase (HRP) (Amersham Life Science, Arlington Heights, IL) (1:2500). The membranes were washed in TBST for 30 min at room temperature and then exposed to the Amersham ECL detection system according to the manufacturer's instructions.

Data analysis. Data are presented as means ± SE. Statistical analysis was performed using Student's *t* test. P values < 0.05 were considered to be significant.

Results

We previously observed that gastrin (G17) inhibits apoptosis of the AR4-2J rat acinar cell line through the phosphorylation and activation of protein kinase B/Akt [8]. In this study, we further dissected the mechanisms that mediate the anti-apoptotic action of gastrin in the AR4-2J cells. We confirmed the involvement of specific CCK_B receptors in gastrin induction of Akt activation. D2 (10 nM), a specific CCK_B receptor antagonist [5], inhibited gastrin (10 nM)-induced phosphorylation of Akt (Fig. 1).

Mobilization of intracellular calcium is a well-established signal transduction pathway activated by gastrin in the AR4-2J cells [3,4,7]. Accordingly, we investigated the effects of the intracellular calcium chelator BAPTA-AM (100 μM) on gastrin induction of Akt phosphorylation. As shown in Fig. 2A, BAPTA-AM potently inhibited gastrin stimulation of Akt activation.

Gastrin is known to exert growth-promoting effects in the AR4-2J cells through the activation of PKC [3–7]. Thus, we examined the action of the PKC inhibitor GF109203X (3.5 μM) on gastrin-stimulated Akt phosphorylation. Although GF109203X inhibits gastrin-stimulated AR4-2J cell proliferation [5,7], it failed to

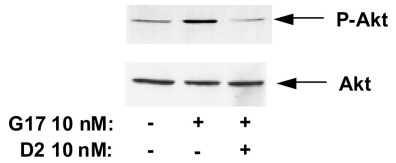

Fig. 1. Effect of the specific CCK_B receptor antagonist D2 on gastrin (G17) induced phosphorylation of Akt. Phosphorylation of Akt kinase in lysates from AR4-2J cells stimulated for 5 min with gastrin (G17) (10 nM), alone or in association with the specific CCK_B receptor antagonist D2 (10 nM), was studied by Western blots using an antibody that recognizes Ser 473 of Akt. Total Akt kinase levels were monitored by western blots with an antibody recognizing Akt kinase independent of its phosphorylation state. Identical results were obtained in at least three other separate experiments.

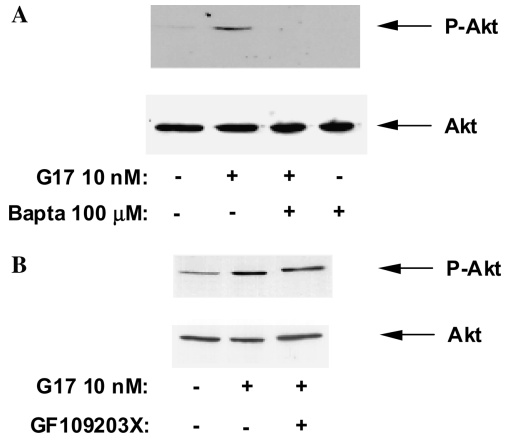

Fig. 2. Effect of the intracellular Ca^{2+} chelator BAPTA-AM and the PKC inhibitor GF109203X on gastrin (G17) induced phosphorylation of Akt. Phosphorylation of Akt kinase in lysates from AR4-2J cells stimulated for 5 min with gastrin (G17) (10 nM), alone or in association with the intracellular Ca^{2+} chelator BAPTA-AM (100 μM) (A) or the PKC inhibitor GF109203X (3.5 μM) (B), was studied by Western blots using an antibody that recognizes Ser 473 of Akt. Total Akt kinase levels were monitored by western blots with an antibody recognizing Akt kinase independent of its phosphorylation state. Identical results were obtained in at least two other separate experiments.

block the stimulatory action of gastrin on Akt phosphorylation (Fig. 2B). Thus, gastrin targets Akt through intracellular calcium-dependent, PKC-independent, pathways.

Akt phosphorylates and inactivates the pro-apoptotic protein BAD [10–12]. Accordingly, we investigated if gastrin induction of Akt leads to BAD phosphorylation. Akt was immunoprecipitated from the AR4-2J cells and its activity was measured by vitro kinase assays using recombinant BAD as substrate. As shown in Fig. 3A, gastrin stimulated BAD phosphorylation through an Akt-dependent mechanism. We examined if gastrin stimulates BAD phosphorylation in vivo, using Western blots with an anti-phospho-BAD antibody. In agreement with the kinase assay data, gastrin potently stimulated BAD phosphorylation (Fig. 3B).

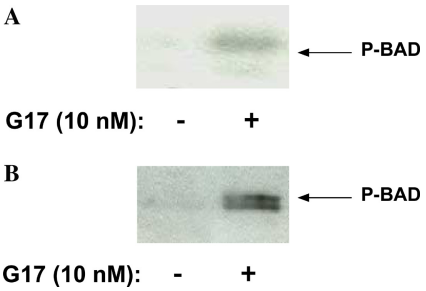

Fig. 3. Effect of gastrin on BAD phosphorylation. Akt in lysates from parietal cells stimulated with gastrin (G17) (10 nM) for 5 min was immunoprecipitated with an anti-Akt antibody and its activity was measured by in vitro kinase assays using recombinant BAD as substrate (A). Phosphorylation of BAD in lysates from AR4-2J cells stimulated for 5 min with gastrin (G17) (10 nM) was studied by Western blots using a specific anti-phospho-BAD antibody (B). Identical results were obtained in one other separate experiment.

In addition to BAD, Akt phosphorylates and inactivates FOXO forkhead/winged-helix transcription factors, nuclear proteins known to regulate the transcription of pro-apoptotic genes [13,14]. Thus, we studied the effect of gastrin on the phosphorylation of the transcription factor FOXO3, using Western blots with an anti-phospho-FOXO3 antibody. Gastrin induced FOXO3 phosphorylation in the AR4-2J cells after 5, 15, and 30 min of incubation and this effect was inhibited by BAPTA-AM (Figs. 4A and B).

In order to define the functional significance of gastrin induced phosphorylation of forkhead/winged-helix transcription factors, we transfected the AR4-2J cells with plasmids expressing FOXO1 together with the plasmid containing three copies of the insulin-responsive sequence (IRS) of the insulin-like growth factor binding

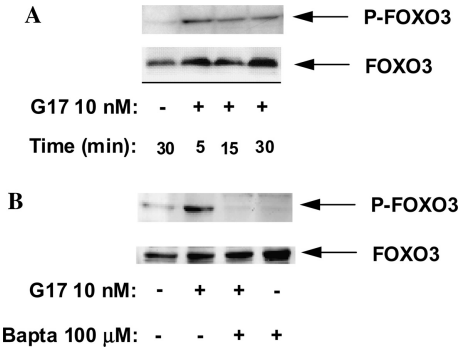

Fig. 4. Effect of gastrin and of the intracellular Ca^{2+} chelator BAPTA-AM on FOXO3 phosphorylation. (A) Phosphorylation of FOXO3 in lysates from AR4-2J cells stimulated for 5, 15, and 30 min with gastrin (G17) (10 nM) was studied by Western blots using a specific anti-phospho-FOXO3 antibody. Phosphorylation of FOXO3 in lysates from AR4-2J cells stimulated for 5 min with G17, alone or in association with the intracellular Ca^{2+} chelator BAPTA-AM (100 μM) is shown in (B). Total FOXO3 levels were monitored by Western blots with an antibody recognizing FOXO3 independent of its phosphorylation state. Identical results were obtained in at least two other separate experiments.

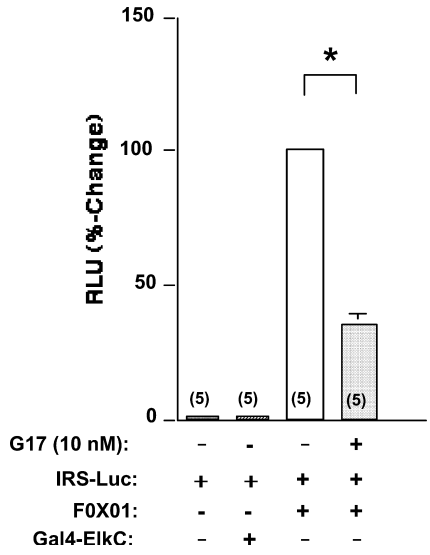

Fig. 5. Effect of gastrin (G17) on FOXO1 transcriptional activity. AR4-2J cells were co-transfected with plasmids expressing either Gal4-ElkC or FOXO1, together with the pCMV-βGal expression vector and the 3× IRS-luciferase reporter plasmid. Cells were either left untreated or stimulated with 10 nM G17 for 5 h. Data are expressed as percentage of FOXO1 stimulated 3× IRS-luciferase activity, means ± SE. *$p < 0.01$. RLU, relative light units.

protein 1 (IGFBP-1) promoter placed upstream of the firefly luciferase reporter gene. The system is based on the observation that insulin inhibits IGFBP-1 gene transcription through a specific insulin-responsive sequence present in the IGFBP-1 promoter. This inhibitory effect involves the activation by insulin of Akt and the subsequent phosphorylation and transcriptional inactivation of forkhead/winged-helix transcription factors which are positive regulators of IGFBP-1 gene transcription [13]. After 24 h in serum-free medium, the cells were either left untreated or stimulated with gastrin for 5 h. In these experiments we observed that FOXO1 stimulated 3× IRS-luciferase activity more then 30-fold and gastrin inhibited this effect by approximately 50% (means ± SE, $n = 5$) (Fig. 5). In order to demonstrate the specificity of this event, we conducted experiments in which we transfected the cells with plasmids expressing the GAL4-ElkC fusion protein together with the plasmid 3× IRS-luc. As shown in Fig. 5, GAL4-ElkC failed to induce luciferase activity (means ± SE, $n = 5$). Thus, gastrin induces both the phosphorylation and the transcriptional inactivation of forkhead/winged-helix transcription factors.

Discussion

The peptide hormone gastrin regulates numerous, complex cellular functions such as growth, proliferation, and secretion [1–4]. In previous studies we reported that gastrin induces the Akt signal transduction pathway and that this effect leads to inhibition of AR4-2J cell apoptosis [8].

In this study, we show that gastrin induction of Akt leads to the phosphorylation, both in vitro and in vivo, of BAD, a well-characterized pro-apoptotic protein, which is phosphorylated and inactivated by Akt in response to growth factor stimulation [10–12]. Thus, one of the mechanisms employed by gastrin to inhibit pancreatic cancer cell apoptosis appears to involve the phosphorylation and inactivation of BAD.

Since gastrin has been shown to interact with receptors other than the CCK_B receptor, we explored the possibility that gastrin might stimulate Akt, in the AR4-2 cells, through interaction with either CCK_A or non-A-non-B-CCK receptors [17,19]. However, when we tested the effect of the highly specific CCK_B receptor antagonist D2, we observed that this compound completely blocked gastrin induction of Akt activation, thus strongly supporting the involvement of CCK_B receptors in the anti-apototic effect of gastrin in the AR4-2J cells.

PKC is known to play an important role in the mediation of the growth promoting effects of gastrin [3–7]. Several reports have demonstrated that activation of this protein kinase in response to gastrin stimulation leads to the induction of cellular proliferation through a signal transduction pathway that involves the activation of the ERKs and of the early response gene c-fos [5–7]. Here, we report that gastrin targets Akt through PKC-independent pathways. Thus, in the AR4-2J cells, gastrin stimulation of PKC activation results in the induction of specific protein kinase cascades, which are involved in the regulation of cellular growth and survival.

Mobilization of intracellular calcium plays an important role in gastrin induction of signal transduction pathways that are involved in the regulation of cellular proliferation [3,4,7]. Gastrin is known to stimulate the hydrolysis of phosphatidylinositol biphosphate by protein lipase C (PLC) leading to the generation of diacylglycerol and inositol triphosphate. These compounds, in turn, are responsible for the activation of PKC and for the mobilization of intracellular Ca^{2+} leading, in the AR4-2J cells, to the phosphorylation of Shc and to the activation of the MAPK pathway [3,4,7]. One intriguing observation of our study is that gastrin-stimulated mobilization of intracellular calcium leads to the activation of Akt and to the phosphorylation of the pro-apoptotic transcription factor FOXO3, since gastrin induction of both Akt and FOXO3 phosphorylation was blocked by the intracellular calcium chelator BAPTA-AM. Accordingly, in the AR4-2J cells mobilization of intracellular calcium, in response to gastrin stimulation, leads to both inhibition of apoptosis and to activation of signal transduction pathways involved in the regulation of cellular proliferation.

In this report, we also demonstrate that gastrin-mediated phosphorylation of FOXO transcription factors leads to their transcriptional inactivation, since gastrin inhibited the activation by FOXO1 of a luciferase reporter construct containing FOXO1-responsive elements. These observations confirm the notion that gastrin activates intracellular signal transduction pathways that inhibit several, well characterized mediators of cellular apoptosis.

In conclusion, we dissected some of the mechanisms that mediate the anti-apoptotic actions of gastrin in a pancreatic cancer cell line expressing gastrin/CCK_B receptors. These novel findings might represent important mechanisms responsible for the growth promoting effect of the hormone on both normal and neoplastic gastrointestinal tissues.

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