

Metformin Disrupts Crosstalk between G Protein–Coupled Receptor and Insulin Receptor Signaling Systems and Inhibits Pancreatic Cancer Growth

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Abstract

Recently, we identified a novel crosstalk between insulin and G protein–coupled receptor (GPCR) signaling pathways in human pancreatic cancer cells. Insulin enhanced GPCR signaling through a rapamycin-sensitive mTOR-dependent pathway. Metformin, the most widely used drug in the treatment of type 2 diabetes, activates AMP kinase (AMPK), which negatively regulates mTOR. Here, we determined whether metformin disrupts the crosstalk between insulin receptor and GPCR signaling in pancreatic cancer cells. Treatment of human pancreatic cancer cells (PANC-1, MIA-PaCa-2, and BxPC-3) with insulin (10 ng/mL) for 5 minutes markedly enhanced the increase in intracellular $[Ca^{2+}]_i$ induced by GPCR agonists (e.g., neurotensin, bradykinin, and angiotensin II). Metformin pretreatment completely abrogated insulin-induced potentiation of Ca^{2+} signaling but did not interfere with the effect of GPCR agonists alone. Insulin also enhanced GPCR agonist–induced growth, measured by DNA synthesis, and the number of cells cultured in adherent or non-adherent conditions. Low doses of metformin (0.1–0.5 mmol/L) blocked the stimulation of DNA synthesis, and the anchorage-dependent and anchorage-independent growth induced by insulin and GPCR agonists. Treatment with metformin induced striking and sustained increase in the phosphorylation of AMPK at Thr¹⁷² and a selective AMPK inhibitor (compound C, at 5 μ mol/L) reversed the effects of metformin on $[Ca^{2+}]_i$ and DNA synthesis, indicating that metformin acts through AMPK activation. In view of these results, we tested whether metformin inhibits pancreatic cancer growth. Administration of metformin significantly decreased the growth of MIA-PaCa-2 and PANC-1 cells xenografted on the flank of nude mice. These results raise the possibility that metformin could be a potential candidate in novel treatment strategies for human pancreatic cancer. [Cancer Res 2009; 69(16):6539–45]

Introduction

Ductal adenocarcinoma of the pancreas is a devastating disease, with an overall 5-year survival rate of only 3% to 5%. The incidence of this disease in the United States has increased recently to

>37,000 new cases each year, and is now the fourth leading cause of cancer mortality in both men and women. As the current therapies offer very limited survival benefits, novel therapeutic strategies are urgently required to treat this aggressive disease.

G protein–coupled receptors (GPCR) and their cognate agonists are increasingly implicated as autocrine/paracrine growth factors for multiple solid tumors, including small cell lung cancer, colon, prostate, breast, and pancreas (1–3). Pancreatic cancer cell lines express multiple functional GPCRs using a Ca^{2+} mobilization assay as indicators of productive ligand-receptor interactions (4). A variety of GPCR agonists, including neurotensin, angiotensin II, and bradykinin, stimulated DNA synthesis in pancreatic cancer cell lines, including PANC-1 and MIA-PaCa-2 (4–7). Furthermore, a broad-spectrum GPCR antagonist inhibited the growth of pancreatic cancer cells either *in vitro* or xenografted into *nu/nu* mice (8). Other studies showed increased expression of angiotensin II and neurotensin GPCRs in pancreatic cancer tissues (9–12). More recently, we identified a novel crosstalk between insulin/insulin-like growth factor-I receptors and GPCR signaling systems in pancreatic cancer cells, leading to the enhancement of GPCR-induced early signaling (13), including Ins(1,4,5) P_3 generation and increase in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). Insulin-induced potentiation of GPCR signaling was mediated through the phosphatidylinositol-3-kinase/Akt/mTOR signaling module (13), a key pathway in insulin/insulin-like growth factor action (14). These findings assume an added importance in view of the large number of epidemiologic studies linking long-standing type 2 diabetes, obesity, and metabolic syndrome, characterized by peripheral insulin resistance and compensatory overproduction of insulin, with increased risk for developing pancreatic cancer (see ref. 15 for review). Given the complexity of the pancreatic microcirculation (16), and the close topographical relationship between the islets and small ducts in these diseases (17), the locally overproduced insulin is thought to act directly on ductal pancreatic cancer cells.

Metformin (1,1-dimethylbiguanide hydrochloride), one of the most widely prescribed drugs for the treatment of type 2 diabetes, stimulates AMP kinase (AMPK) in intact cells (18). AMPK activity is switched on by phosphorylation on Thr¹⁷² in the activation loop of the catalytic subunit by LKB-1/STK11, the product of the Peutz-Jegher syndrome tumor-suppressor gene (19). Major downstream targets of AMPK include TSC2 and raptor (20, 21). The AMPK-mediated phosphorylation of these targets leads to the inhibition of mTOR complex 1 activity. Interestingly, a recent epidemiologic report linked the administration of metformin with a 62% reduced risk of pancreatic cancer in patients with type 2 diabetes mellitus (22). In addition, metformin prevented carcinogen-induced pancreatic cancer induction in hamsters maintained on high-fat diets (23) and inhibited the growth of breast and colon cancer cells (24, 25). Despite its potential clinical implications, there is no

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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doi:10.1158/0008-5472.CAN-09-0418

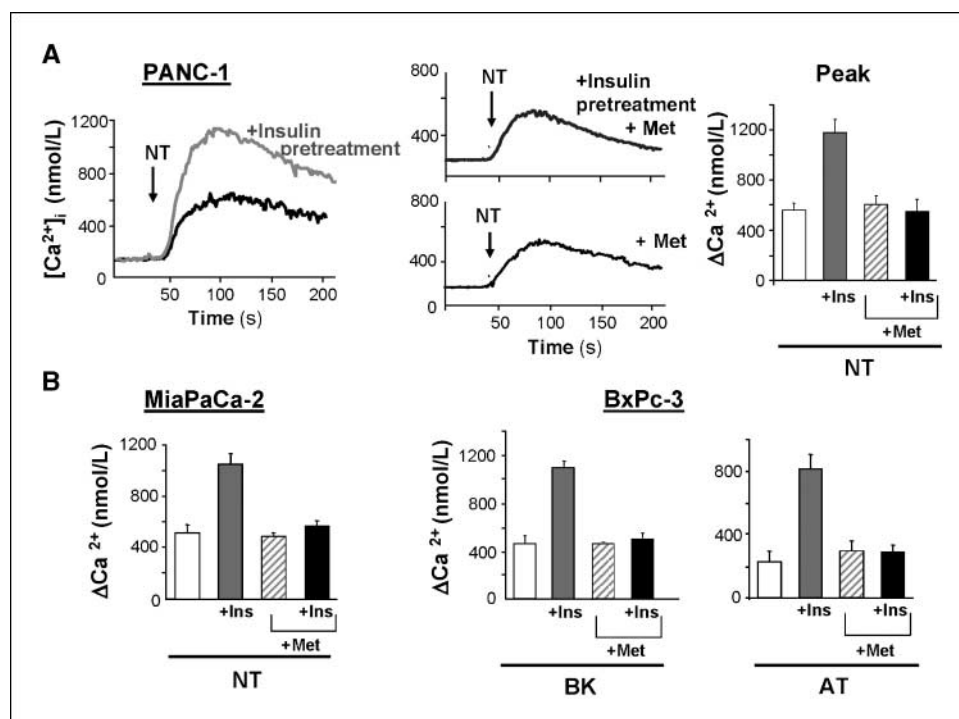


Figure 1. Metformin blocks enhanced Ca^{2+} mobilization induced by neurotensin, bradykinin, or angiotensin in insulin-treated human pancreatic cancer cells. **A**, effect of metformin on Ca^{2+} mobilization induced by neurotensin in PANC-1 cells. PANC-1 cells were treated with or without 10 ng/mL of insulin for 5 min and then stimulated with 5 nmol/L of neurotensin (NT; left). $[Ca^{2+}]_i$ was monitored as described in Materials and Methods. Pretreatment with 5 mmol/L of metformin for 1 h prevented insulin-induced potentiation of the increase in $[Ca^{2+}]_i$ (middle-top) but did not interfere with neurotensin-induced $[Ca^{2+}]_i$ (middle-bottom). Columns (right), peak, maximal increments in $[Ca^{2+}]_i$ in response to 5 nmol/L of neurotensin (NT) in cells pretreated with or without 5 mmol/L of metformin (Met) for 1 h and then incubated with or without 10 ng/mL of insulin for 5 min (Ins) before neurotensin stimulation. Columns, mean; bars, SE ($n = 25$ for each condition). **B**, effect of metformin on $[Ca^{2+}]_i$ induced by neurotensin in MIAPaCa-2 cells and by bradykinin and angiotensin II in BxPC-3 cells. Left, MIAPaCa-2 cells were incubated for 1 h in the absence or presence of 5 mmol/L of metformin (Met) and then treated with or without 10 ng/mL of insulin for 5 min (Ins) prior to stimulation with 5 nmol/L of neurotensin (NT). Right, BxPC-3 cells were incubated for 1 h in the absence or presence of 5 mmol/L of metformin (Met) and then treated with or without 10 ng/mL of insulin for 5 min (Ins) prior to stimulation with 5 nmol/L of bradykinin (BK) or 100 nmol/L of angiotensin II (AT). Columns, mean MIAPaCa-2 and BxPC-3 values from at least four independent experiments each performed by quadruplicate; bars, SE ($n = 16$ for each condition).

understanding of the precise mechanism(s) by which metformin inhibits the proliferation of cancer cells, and it is not known whether metformin has any direct effect on pancreatic cancer growth.

In this study, we show that metformin disrupts the crosstalk between insulin receptor and GPCR signaling systems in pancreatic cancer cells. Specifically, metformin prevented insulin-induced augmentation of Ca^{2+} signaling, DNA synthesis, and anchorage-independent proliferation in response to stimulation with GPCR agonists in PANC-1 and MIAPaCa-2 pancreatic cancer cells. Metformin inhibited these signaling systems through AMPK. Furthermore, our results show that metformin administration inhibits the growth of PANC-1 and MIAPaCa-2 tumor xenografts *in vivo*.

Materials and Methods

Cells and culture conditions. The human pancreatic cancer cell lines PANC-1, MIAPaCa-2, BxPC-3, and AsPC-1 were obtained from the American Type Culture Collection. PANC-1 and MIAPaCa-2 cells were grown in DMEM with 2 mmol/L of glutamine, 1 mmol/L of sodium pyruvate, 100 units/mL of penicillin, and 100 μ g/mL of streptomycin, and 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere containing 10% CO_2 . BxPC-3 cells and AsPC-1 cells were grown in RPMI 1640 containing 100 units/mL of penicillin, 100 μ g/mL of streptomycin, and 10% FBS at 37°C in a humidified atmosphere containing 5% CO_2 .

Measurement of $[Ca^{2+}]_i$. Ca^{2+} signaling was determined in cells grown on glass coverslips for 4 to 5 d, as previously described (13).

$[^3H]$ Thymidine incorporation into DNA. PANC-1, MIAPaCa-2, BxPC-3, and AsPC-1 cells (5×10^4) were plated and grown in 3.5 cm tissue culture plates in DMEM with 2 mmol/L of glutamine, 1 mmol/L of sodium pyruvate (PANC-1 and MIAPaCa-2 cells) or RPMI 1640 (BxPC-3 and AsPC-1 cells), and 10% FBS. After 5 d, the cultures were washed twice with PBS and incubated in serum-free medium. After 24 h, the cultures were transferred to fresh medium containing the specified concentration of agonist and/or inhibitor for 17 h and then pulse-labeled for 6 h with $[^3H]$ thymidine (0.25 μ Ci/mL). The acid-insoluble radioactivity was determined as previously described (7).

Western blot analysis. Confluent cultures of PANC-1 cells grown on 6 cm dishes were washed twice with PBS and incubated with serum-free medium for 3 h. Metformin was added at 5 mmol/L and the cultures were incubated at 37°C for the specified times (0 min–24 h). To detect the activation of AMPK α , the cultures were washed in cold PBS and directly lysed in 2 \times SDS-PAGE sample buffer [200 mmol/L Tris-HCl (pH 6.8), 2 mmol/L EDTA, 0.1 mol/L Na_3VO_4 , 6% SDS, 10% glycerol, and 4% 2-mercaptoethanol]. The lysates were subjected to SDS-PAGE on 10% gels and separated proteins were transferred to Immobilon-P membranes (Millipore). Western blots were then performed on membranes incubated overnight with phosphorylated AMPK α (Thr¹⁷²) or phosphorylated ERK1/2 (Thr²⁰² and Tyr²⁰⁴) monoclonal antibodies at a dilution of 1:1,000 in PBS containing 5% nonfat dried milk and 0.1% Tween 20. The immunoreactive bands were detected with enhanced chemiluminescence reagents (Amersham). The same membranes were subsequently stripped and probed in a similar fashion with anti-AMPK α polyclonal antibody or anti-ERK at a dilution of 1:2,000 in PBS containing 5% nonfat dried milk and 0.1% Tween 20.

Anchorage-dependent cell proliferation. PANC-1 and MIAPaCa-2 cells (2×10^4) were plated on tissue culture 12-well plates in DMEM containing

1% FBS. After 24 h of incubation at 37°C, 5 nmol/L of neurotensin, in the absence or presence of 10 ng/mL of insulin, was added with or without 1 mmol/L of metformin. The cultures were then incubated for 7 to 9 d, and the total cell count was determined from a minimum of four wells per condition using a Coulter counter, after cell clumps were disaggregated by passing the cell suspension 10 times through a 19-gauge, and subsequently, a 21-gauge needle.

Anchorage-independent cell proliferation. Cells were plated on tissue culture 12-well plates coated with polyhydroxyethylmethacrylate [poly-(HEMA)] as previously described (7). Under these conditions, cells are denied attachment to the substratum. Cultures of PANC-1 or BxPC-3 cells, 3 to 5 d after passage, were washed and suspended in DMEM (PANC-1) or RPMI 1640 (BxPC-3 cells). Cells were then disaggregated by two passes through a 19-gauge needle into an essentially single-cell suspension as judged by microscopy. Cell numbers were determined using a Coulter counter, and 2×10^4 cells were seeded in either DMEM containing 1% FBS on the poly-(HEMA)-coated dishes (PANC-1 cells) or in RPMI containing 1% FBS (BxPC-3 cells). After 24 h of incubation at 37°C, 5 nmol/L of neurotensin (PANC-1 cells) or 5 nmol/L of bradykinin (BxPC-3 cells), in the absence or in the presence of 10 ng/mL of insulin, was added with or without 1 mmol/L of metformin. The cultures were then incubated in a humidified atmosphere containing 10% CO₂ at 37°C for 7 to 9 d, and the total cell count was determined from a minimum of four wells per condition using a Coulter counter, after cell clumps were disaggregated by passing the cell suspension 10 times through a 19-gauge, and subsequently, a 21-gauge needle.

Mice xenografts. Early passage PANC-1 or MIAPaCa-2 cells were harvested, and 2×10^6 cells were implanted into the right flanks of male *nu/nu* mice. The male *nu/nu* mice were maintained in a specific pathogen-free facility at the University of California at Los Angeles. The UCLA Chancellor's Animal Research Committee approved all the animal experiments.

The animals were randomized into control and treated groups (10 mice per group). Treatment was initiated when the tumors reached a mean diameter of 2 mm, and the first day of treatment in both cases was designated as day 0. For injection, metformin was dissolved in sterile saline and was given once daily i.p. at 250 mg/kg (50 μ L/mouse). The control group received vehicle only (50 μ L saline). Tumor volume (*V*) was measured

with an external caliper every 4 d and it was calculated as $V = 0.52$ (length \times width²). The treatment was continued until any of the tumors reached ~ 1.5 cm in length, when all the animals were sacrificed and the tumors removed. The volume of the excised tumors was calculated as $V = 0.52$ (length \times width \times depth).

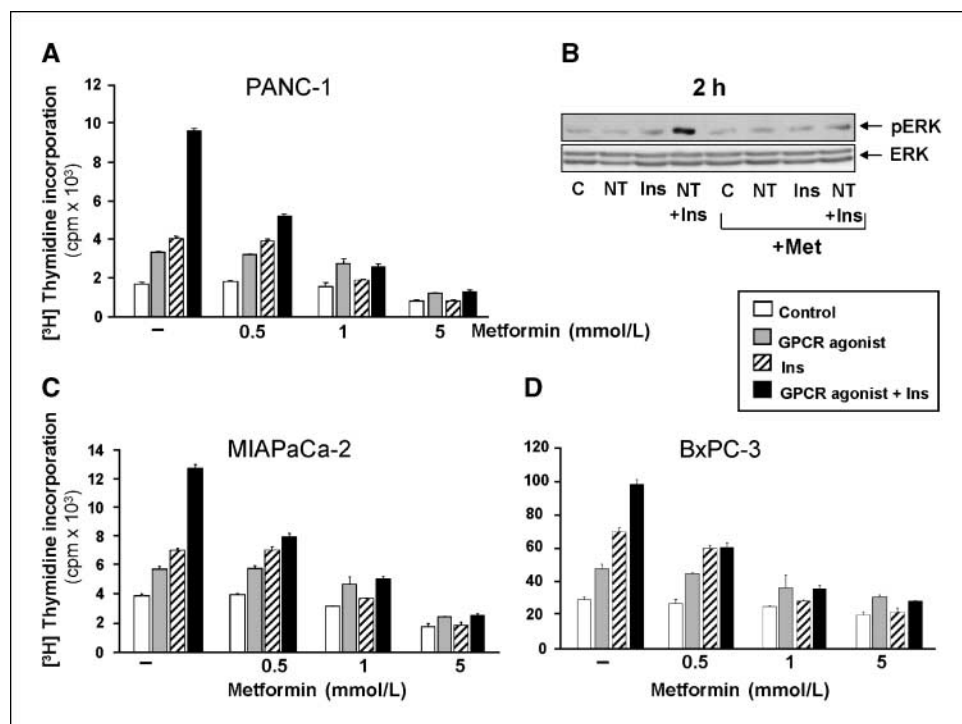
Chemicals and reagents. DMEM was obtained from Life Technologies. Neurotensin, angiotensin, bradykinin, and insulin were obtained from Sigma Chemical. Metformin was obtained from Sigma-Aldrich. The AMPK inhibitor, compound C, was purchased from Calbiochem. An antibody that detects the phosphorylated state of Thr¹⁷² of AMPK α and an antibody against AMPK α were purchased from Millipore. An antibody that detects the phosphorylated state of Thr²⁰² and Tyr²⁰⁴ of ERK1/2 and an antibody against ERK1/2 were purchased from Cell Signaling Technology. Horseradish peroxidase-conjugated antirabbit IgG and antimouse IgG were from Amersham Biosciences. All other reagents were of the highest grade available.

Statistical analysis. The values obtained are presented as the mean \pm SE and analyzed with Student's *t* test, using SigmaPlot 2000 (SPSS).

Results

Metformin blocks the crosstalk between insulin and GPCR receptor signaling on intracellular Ca²⁺ mobilization. As a first step to investigate whether metformin disrupts the crosstalk between the insulin/insulin-like growth factor and GPCR signaling systems, we determined whether it prevents the stimulatory effect of insulin on GPCR-induced increase in [Ca²⁺]_i in pancreatic cancer cells. As shown in Fig. 1A, the addition of neurotensin (5 nmol/L) to PANC-1 cells induced a rapid increase in [Ca²⁺]_i from a basal level of 142 ± 15 nmol/L (mean \pm SE; *n* = 20) to a peak value of 735 ± 75 nmol/L (*n* = 20) at 25 to 35 seconds, which subsequently declined toward a plateau phase (Fig. 1A, left, black trace and right, open column). In contrast, the addition of insulin (10 ng/mL) to PANC-1 cells did not produce any detectable change in [Ca²⁺]_i. However, treatment of PANC-1 cells with 10 ng/mL of insulin for 5 minutes prior to stimulation markedly enhanced neurotensin-induced increase in

Figure 2. Metformin inhibits DNA synthesis induced by GPCR agonists and insulin in PANC-1, MIAPaCa-2, and BxPC-3 cells. A, C, and D, dose-response effect of metformin on DNA synthesis induced by neurotensin, insulin, or both in PANC-1 (A), MIAPaCa-2 (C), or BxPC-3 cells (D). PANC-1 and MIAPaCa-2 cells were incubated without (open columns, control) or with 5 nmol/L of neurotensin (NT, gray columns), 10 ng/mL of insulin (striped columns), or the combination of neurotensin and insulin (black columns) in the presence of increasing concentrations of metformin (0.5–5 mmol/L) for 17 h prior to the addition of [³H]thymidine for 6 h. BxPC-3 cells were treated identically, except that they were stimulated with bradykinin instead of neurotensin. Columns, mean obtained in three independent experiments (radioactivity incorporated into acid-insoluble pools); bars, SE. B, metformin prevents prolonged ERK signaling. PANC-1 cells were incubated with or without 5 nmol/L of neurotensin (NT), 10 ng/mL of insulin, or the combination of neurotensin and insulin in the absence or presence of 5 mmol/L of metformin (Met), as indicated. Cell lysates were subjected to Western blot analysis using antibodies that detect dually phosphorylated ERK1/2 (pERK) or total ERK.



$[Ca^{2+}]_i$ that now reached a peak value of $1,185 \pm 134$ nmol/L ($n = 25$; Fig. 1A, *gray trace* and *black column*).

Pretreatment with 5 mmol/L of metformin for 1 hour did not have any significant effect on the $[Ca^{2+}]_i$ increase induced by neurotensin alone (Fig. 1A, *bottom-middle* and *striped column*), but completely blocked the enhancing effect of insulin on neurotensin-induced Ca^{2+} mobilization (peak value 559 ± 102 nmol/L, *top-middle* and *gray column*). Similar results were obtained with either MIAPaCa-2 cells stimulated with neurotensin (Fig. 1B, *left*) or BxPC-3 cells stimulated with either bradykinin or angiotensin (Fig. 1B, *middle* and *right*). These results indicate that exposure to metformin abrogates the crosstalk between insulin and GPCR signaling systems in a variety of pancreatic cancer cell lines.

Metformin abolished the crosstalk between insulin and GPCR receptor signaling systems on DNA synthesis and cell proliferation in pancreatic cancer cells. We next determined whether crosstalk between GPCR agonists and insulin in pancreatic cancer cells leads to long-term biological responses, including ERK signaling, DNA synthesis, and cell proliferation. Cultures of PANC-1, MIAPaCa-2, or BxPC-3 cells were incubated in medium containing increasing doses of metformin with neurotensin (PANC-1 and MIAPaCa-2) or bradykinin (BxPC-3) in the absence or presence of insulin, as indicated in Fig. 2. After 17 hours of incubation, the cultures were pulse-labeled with $[^3H]$ -thymidine. The results in Fig. 2 show that either insulin or GPCR agonists stimulated DNA synthesis in PANC-1 (A), MIAPaCa-2 (C), and BxPC-3 (D) cells. The combination of the GPCR agonists and insulin (*black columns*) induced a striking enhancement in DNA synthesis in all three cell lines and induced marked prolongation of ERK signaling, as shown by Western blot analysis of lysates of PANC-1 cells treated with neurotensin and insulin (Fig. 2B).

The salient feature of the results shown in Fig. 2 is that metformin abolished the increase in DNA synthesis induced by the combination of neurotensin and insulin in PANC-1 cells (Fig. 2A), and prevented the prolonged ERK signaling induced by neurotensin and insulin in PANC-1 cells (Fig. 2B). Similarly, metformin prevented the stimulation of DNA synthesis induced by neurotensin and insulin in MIAPaCa-2 cells (Fig. 2C), and bradykinin and insulin in BxPC-3 cells (Fig. 2D).

In order to determine whether metformin inhibits the proliferation of pancreatic cancer cells, single cell suspensions of PANC-1 cells were plated on normal culture dishes (anchorage-dependent growth; Fig. 3A) or on culture dishes coated with poly-(HEMA), which prevents the adhesion of the cells to the substratum (anchorage-independent growth; Fig. 3B). The cells were incubated in medium supplemented with neurotensin, insulin, or neurotensin and insulin in the presence or absence of 1 mmol/L of metformin. As illustrated in Fig. 3, the combination of neurotensin and insulin promoted a marked increase in cell number in both anchorage-dependent and anchorage-independent conditions. The addition of metformin markedly inhibited the stimulation of either anchorage-dependent or anchorage-independent growth induced by the combination of neurotensin and insulin in PANC-1. Metformin inhibited the stimulation of anchorage-dependent growth of MIAPaCa-2 cells induced by neurotensin and insulin (Supplementary Fig. S1), and the anchorage-independent growth of BxPC-3 cells induced by bradykinin and insulin (Supplementary Fig. S2). The results show that metformin directly inhibits DNA synthesis and proliferation induced by insulin and GPCR agonists in pancreatic cancer cells.

Metformin inhibits crosstalk between insulin and GPCR signaling systems through AMPK. AMPK activity is switched on by phosphorylation on Thr¹⁷² in the activation loop of the catalytic subunit by LKB-1/STK11. In order to determine whether metformin stimulates AMPK activation in human pancreatic cancer cells, we used Western blot analysis with an antibody that detects the phosphorylated state of the α subunit of AMPK on Thr¹⁷². As shown in Fig. 4A, treatment of PANC-1 cells with metformin induced striking and sustained increase in AMPK phosphorylation on Thr¹⁷², indicating that metformin treatment induces AMPK activation in PANC-1 cells.

Next, we determined whether a specific inhibitor of AMPK (compound C; ref. 26) reverses the inhibitory effects of metformin on insulin-induced augmentation of GPCR-mediated Ca^{2+} signaling. As shown in Fig. 4B, treatment with compound C (5 μ mol/L) reversed the inhibitory effect of metformin on neurotensin-induced Ca^{2+} signaling in insulin-pretreated PANC-1 cells. A similar reversal of metformin inhibition was obtained with BxPC-3 cells treated with insulin and stimulated with bradykinin (Supplementary Fig. S3).

We also determined whether the selective inhibitor of AMPK counteracts the inhibitory effects of metformin on DNA synthesis induced by neurotensin and insulin in PANC-1. As shown in Fig. 4C, compound C (5 μ mol/L) reversed the inhibitory effect of metformin on DNA synthesis induced by either insulin or by the

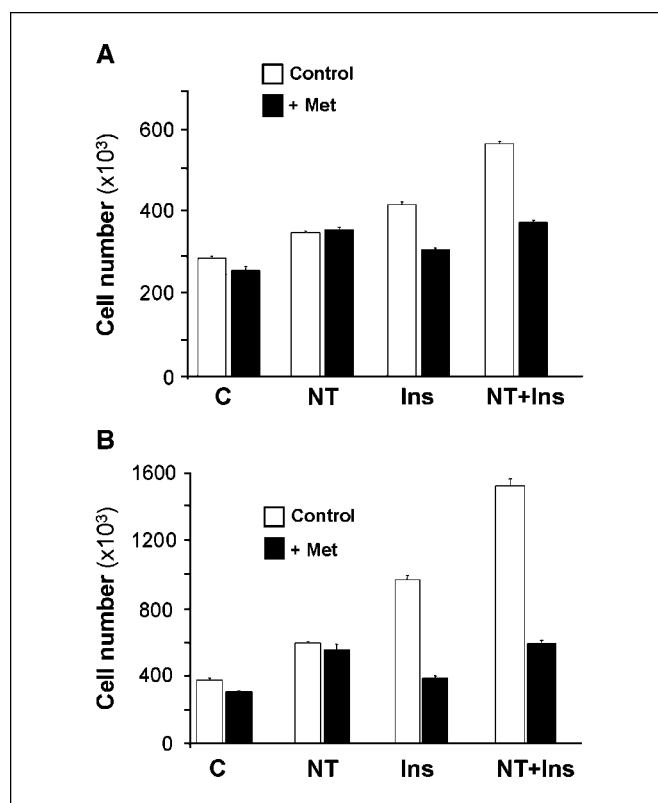
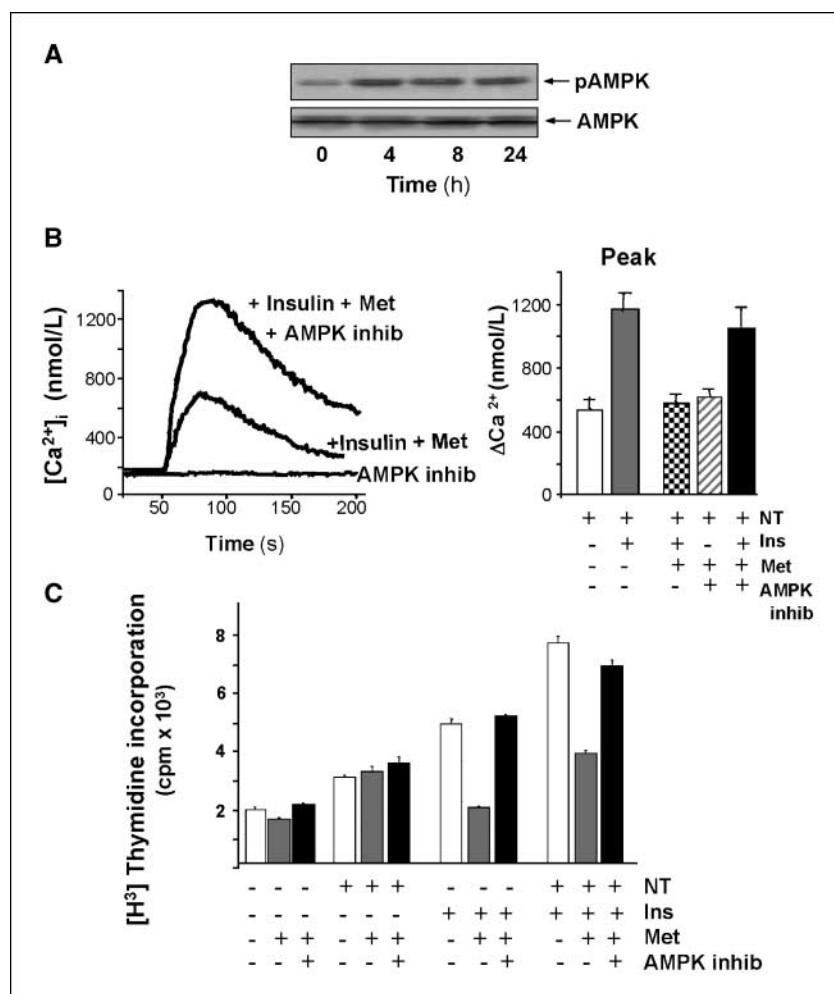


Figure 3. Metformin inhibits anchorage-dependent and anchorage-independent proliferation of PANC-1 cells in response to neurotensin, insulin, and their combination. Single-cell suspension of PANC-1 cells was plated either on tissue culture dishes (A) or on poly-(HEMA)-coated dishes (B). After 24 h, the cultures were shifted to DMEM containing 1% serum without (C) or with 5 nmol/L of neurotensin (NT), 10 ng/mL of insulin (Ins), or a combination of neurotensin and insulin in the absence (*open columns*) or presence (*closed columns*) of 1 mmol/L of metformin. The cultures were incubated for 7 days as described in Materials and Methods. *Columns*, mean cell counts (determined from four to six wells per condition); *bars*, SE. Similar results were obtained in two independent experiments.

Figure 4. Metformin disrupts the crosstalk between insulin receptor and GPCR signaling systems through AMPK.

A, metformin activates AMPK in PANC-1 cells. Lysates of PANC-1 cells treated with 5 mmol/L of metformin for various time periods (0–24 h) were analyzed for AMPK phosphorylation at Thr¹⁷² as indicated in Materials and Methods (autoluminogram). Similar results were obtained in three independent experiments. **B**, AMPK inhibitor reverses the inhibitory effect of metformin on Ca²⁺ mobilization induced by neurotensin in PANC-1 cells. PANC-1 cells were pretreated with or without the AMPK inhibitor compound C (5 μmol/L) for 1 h. Then, 5 mmol/L of metformin was added to the medium for 1 h and subsequently 10 ng/mL of insulin for 5 min. The cells then were stimulated with 5 nmol/L of neurotensin and [Ca²⁺]_i was monitored as described in Materials and Methods. *Columns (right)*, peak, maximal increment in [Ca²⁺]_i in response to neurotensin in cells incubated with or without 10 ng/mL of insulin for 5 min before stimulation with neurotensin (NT). Cultures were pretreated with or without 5 μmol/L Compound C (AMPK *inhib*) for 1 h followed by 5 mmol/L of metformin for 1 h prior to the experiment (Met), as indicated. *Columns (right)*, peak, maximal increment in [Ca²⁺]_i in response to neurotensin in cells incubated with or without 10 ng/mL of insulin for 5 min before stimulation with neurotensin (NT). Cultures were pretreated with or without 5 μmol/L Compound C (AMPK *inhib*) for 1 h followed by 5 mmol/L of metformin for 1 h prior to the experiment (Met), as indicated. **C**, the selective AMPK inhibitor compound C reverses the inhibitory effect of metformin on DNA synthesis induced by neurotensin and insulin in PANC-1 cells. Cultures of PANC-1 cells were treated with or without 5 mmol/L of metformin (Met) and some of the cultures exposed to metformin were also treated with 5 μmol/L of compound C (AMPK *inhib*). DNA synthesis was assayed as described in Materials and Methods.



combination of insulin and neurotensin in PANC-1 cells. The addition of compound C in the absence of metformin did not induce any significant effect [the levels of DNA synthesis were similar to those shown in Fig. 4C (*open columns*)]. Treatment with compound C (5 μmol/L) also reversed the inhibitory effect of metformin on DNA synthesis induced by bradykinin and insulin in BxPC-3 cells (Supplementary Fig. S4).

In line with the results presented above, LKB-1/STK11 is expressed in many pancreatic cancer cells, including PANC-1, MIAPaCa-2, and BxPC-3. However, LKB-1 is silenced by promoter hypermethylation in the pancreatic cancer cell line AsPC-1 (27). If the effects of metformin are mediated by AMPK, AsPC-1 cells (in contrast with PANC-1, MIAPaCa-2, and BxPC-3) should be refractory to metformin because these cells do not express LKB-1/STK11, the upstream kinase that phosphorylates and activates AMPK in response to metformin. Treatment of AsPC-1 cells with 1 mmol/L of metformin did not prevent the stimulation of DNA synthesis induced by neurotensin and insulin in these cells, in contrast to results obtained in parallel cultures of PANC-1 cells (Supplementary Fig. S5). Taken together, these results indicate that metformin disrupts the crosstalk between insulin and GPCR signaling systems through AMPK in human pancreatic cancer cells.

Metformin inhibits the growth of PANC-1 or MIAPaCa-2 xenografts in nude mice. Given our results showing the inhibitory effects of metformin on signaling and proliferation in pancreatic cancer cells, we next examined whether this compound could

inhibit pancreatic cancer growth using PANC-1 and MIAPaCa-2 tumor xenografts in nude mice. The animals were randomized into control and metformin-treated groups (10 mice per group). Treatment was initiated when the tumors reached a mean diameter of 2 mm. Metformin was given once daily i.p. at 250 mg/kg for the duration of the experiment. As shown in Fig. 5, administration of metformin strikingly decreased the growth of either PANC-1 or MIAPaCa-2 cells xenografted in nude mice. For example, the tumor volumes of PANC-1 xenografts at the end of the experiment (day 41) were $286.11 \pm 50.95 \text{ mm}^3$ in the control and $98.47 \pm 29.20 \text{ mm}^3$ in the metformin-treated group ($P = 0.0057$; Fig. 5A, *inset*). MIAPaCa-2 xenografts at the end of the experiment (day 25) were $228.35 \pm 40.99 \text{ mm}^3$ in the control and $92.67 \pm 20.10 \text{ mm}^3$ in the metformin-treated group ($P = 0.0072$; Fig. 5B, *inset*). These results show, for the first time, that metformin inhibits the growth of human pancreatic cancer cells xenografted into nude mice.

Discussion

Recently, we identified a novel crosstalk between insulin/insulin-like growth factor-I receptors and GPCR signaling systems in pancreatic cancer cells, leading to the enhancement of GPCR-induced early signaling (13). Insulin-induced potentiation of Gq signaling was prevented by either inhibitors of phosphatidylinositol-3-kinase or by rapamycin, a specific inhibitor of mTOR

complex 1. These findings indicated that in addition to its well-established role in the regulation of protein synthesis, the rapamycin-sensitive phosphatidylinositol-3-kinase/TORC1/S6K pathway mediates a novel crosstalk between insulin receptors on GPCR signaling systems (13). The results presented in this study showed that insulin also enhances long-term biological responses induced by GPCR agonists in pancreatic cancer cells, including DNA synthesis, proliferation, and anchorage-independent growth. We conclude that potentiation of Gq signaling by insulin through an mTOR-dependent pathway provides a crosstalk mechanism by which insulin enhances the mitogenic responsiveness of pancreatic cancer cells to Gq-coupled receptor agonists.

The biguanide metformin is the most widely prescribed drug for the treatment of type 2 diabetes, worldwide. The primary systemic effect of metformin is the lowering of blood glucose levels through reduced hepatic gluconeogenesis and increased glucose uptake in skeletal muscles and adipose tissue (28). Metformin not only lowers blood glucose but also reduces the hyperinsulinemia associated with insulin resistance. At the cellular level, metformin is known to stimulate the activation of AMPK (18), a conserved regulator of the cellular response to low energy that is activated when ATP concentrations decrease and 5'-AMP concentrations increase in response to nutrient deprivation, hypoxia, and metformin administration (19). AMPK directly binds to the AMPK γ -subunit, causing a conformational change that exposes Thr¹⁷² in the activation loop of the α -subunit, allowing it to be phosphorylated by LKB-1/STK11, the product of the Peutz-Jegher syndrome tumor-suppressor gene (19). Interestingly, the Peutz-Jegher syndrome is characterized by predisposition to various gastrointestinal neoplasms, including pancreatic adenocarcinoma (29) and intraductal papillary-mucinous neoplasms (30).

Because metformin-induced activation of AMPK inhibits mTOR function via TSC2 and raptor phosphorylation (20, 21, 31, 32), we hypothesized that metformin disrupts the crosstalk between insulin receptor and Gq signaling via AMPK. Here, we present several lines of evidence supporting this hypothesis: (a) metformin completely blocked the enhancing effect of insulin on neurotensin-induced Ca²⁺ mobilization in either PANC-1 or MIAPaCa-2 cells stimulated with neurotensin or in BxPC-3 cells stimulated with either bradykinin or angiotensin. (b) Metformin abolished the increase in DNA synthesis induced by neurotensin and insulin in PANC-1 or MIAPaCa-2 cells. Similarly, metformin prevented the stimulation of DNA synthesis promoted by bradykinin and insulin in BxPC-3 cells. (c) Pancreatic cancer cell treatment with metformin produced a marked and sustained increase in AMPK activation, as judged by Thr¹⁷² phosphorylation. (d) Treatment with compound C, a specific inhibitor of AMPK, reversed the inhibitory effect of metformin on neurotensin-induced Ca²⁺ signaling in insulin-pretreated PANC-1 cells. A similar reversal of metformin inhibition was obtained with BxPC-3 cells treated with insulin and stimulated with bradykinin. (e) Compound C also reversed the inhibitory effect of metformin on DNA synthesis induced by the combination of insulin and neurotensin in PANC-1 cells or by the combination of bradykinin and insulin in BxPC-3 cells. (f) Metformin did not disrupt crosstalk between insulin and GPCR signaling systems in the pancreatic cancer AsPC-1 cells which do not express LKB-1/STK11 (27), the upstream kinase that phosphorylates and activates AMPK. These results indicate that metformin disrupts crosstalk between insulin and GPCR signaling systems through AMPK in human pancreatic cancer cells.

Recent epidemiologic reports linked the administration of metformin with a reduced incidence and improved prognosis in

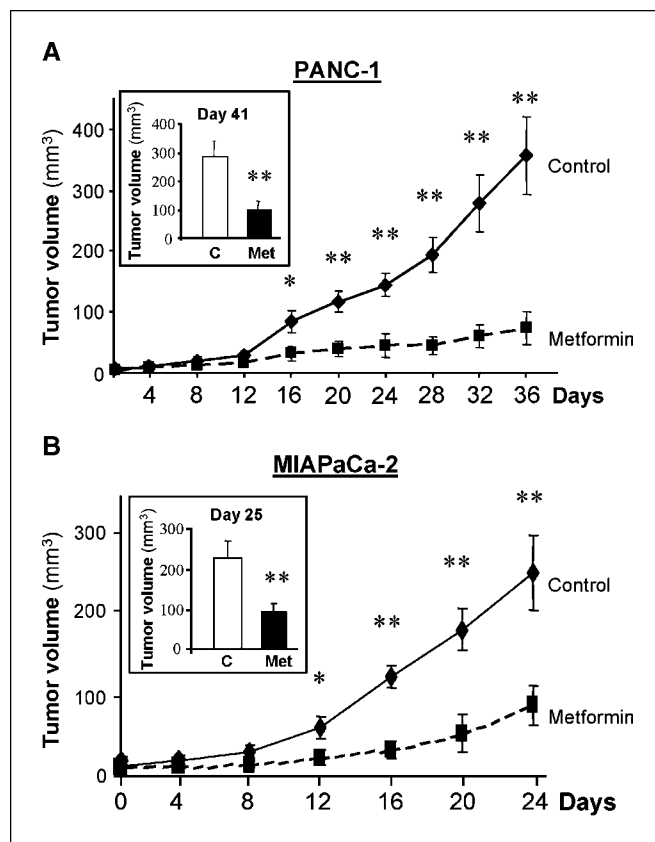


Figure 5. Metformin inhibits the growth of PANC-1 and MIAPaCa-2 tumor xenografts. Xenografts were generated by implantation of 2×10^6 cells of PANC-1 (A) or MIAPaCa-2 (B) cells into the right flanks of male *nu/nu* mice. When the tumors reached a mean diameter of 2 mm, the animals were randomized into control and treated groups (10 mice per group). Metformin (250 mg/kg) was given once daily i.p. for the duration of the experiment (squares). The first day of treatment was designated as day 0. Control animals received saline. Tumor volumes were measured every 4 days as described in Materials and Methods. On day 41, PANC-1 tumors were removed, measured, and tumor volumes estimated as $V = 0.52$ (length \times width \times depth). The results are shown in the inset (mean \pm SE). On day 25, the MIAPaCa-2 tumors were removed, measured, and tumor volumes were estimated as described for PANC-1. Points, mean; bars, SE (*, $P < 0.05$; **, $P < 0.01$ versus control, Student's *t* test).

patients with cancer (33, 34). A recent study showed a statistically significant association between metformin therapy and reduced risk of pancreatic cancer in patients with type 2 diabetes mellitus (22). In addition to these epidemiologic associations, carcinogen-induced pancreatic cancer in hamsters maintained on high-fat diets was prevented by metformin (23) and metformin inhibited the growth of breast and colon cancer cells (24, 25, 35). However, the precise mechanisms involved remain incompletely understood. In view of the direct inhibitory effects of metformin on signaling and proliferation of pancreatic cancer cells shown in the present study, we examined whether this compound inhibits pancreatic cancer growth using PANC-1 and MIAPaCa-2 tumor xenografts in nude mice. Our results show, for the first time, that metformin markedly inhibits the growth of human pancreatic cancer cells xenografted into nude mice.

In conclusion, our results raise the attractive possibility that treatment with metformin, a widely used agent for counteracting metabolic syndrome and type 2 diabetes, directly inhibits pancreatic cancer cell proliferation. The results provide a basis

for novel therapeutic strategies for the treatment of pancreatic cancer, a devastating disease with limited survival options.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Acknowledgments

Received 2/5/09; revised 5/20/09; accepted 6/17/09.

Grant support: NIH grants R21CA137292, RO1DK56930, RO1DK55003, and P30DK41301 (E. Rozengurt).

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