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Metformin inhibition of mTORC1 activation, DNA synthesis and proliferation in pancreatic cancer cells: dependence on glucose concentration and role of AMPK

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Abstract

Metformin, a widely used anti-diabetic drug, is emerging as a potential anticancer agent but the mechanisms involved remain incompletely understood. Here, we demonstrate that the potency of metformin-induced AMPK activation, as shown by the phosphorylation of its substrates acetyl-CoA carboxylase (ACC) at Ser⁷⁹ and Raptor at Ser⁷⁹², was dramatically enhanced in human pancreatic ductal adenocarcinoma (PDAC) cells PANC-1 and MiaPaCa-2 cultured in medium containing physiological concentrations of glucose (5mM), as compared with parallel cultures in medium with glucose at 25 mM. In physiological glucose, metformin inhibited mTORC1 activation, DNA synthesis and proliferation of PDAC cells stimulated by crosstalk between G protein-coupled receptors and insulin/IGF signaling systems, at concentrations (0.05–0.1 mM) that were 10 to 100-fold lower than those used in most previous reports. Using siRNA-mediated knockdown of the α_1 and α_2 catalytic subunits of AMPK, we demonstrated that metformin, at low concentrations, inhibited DNA synthesis through an AMPK-dependent mechanism. Our results emphasize the importance of using medium containing physiological concentrations of glucose to elucidate the anticancer mechanism of action of metformin in pancreatic cancer cells and other cancer cell types.

Keywords

crosstalk mitogenic signaling; insulin; IGF-1; neurotensin; Raptor; acetyl-CoA carboxylase; PANC-1; MiaPaCa-2; ribosomal S6 protein kinase

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC), which comprises 90% of all human pancreatic cancers, is one of the most lethal human diseases, with overall 5-year survival rate of only 3–5% and a median survival period of 4–6 months. The incidence of this disease in the US

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has increased to more than 44,000 new cases in 2011 and is now the fourth leading cause of cancer mortality in both men and women [1]. As the current therapies offer very limited survival benefits, novel molecular therapeutic targets and strategies are urgently needed to treat this aggressive disease.

G protein-coupled receptors (GPCRs) 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 [2–4]. We showed that PDAC cell lines express multiple functional GPCRs [5] and that a variety of GPCR agonists, including neurotensin, stimulated DNA synthesis and proliferation in PDAC cells, such as PANC-1 cells [5–7]. Furthermore, a broad-spectrum GPCR antagonist inhibited the growth of PDAC cells either *in vitro* or xenografted into nu/nu mice [8]. More recently, we identified crosstalk between insulin/IGF1 receptors and GPCR signaling systems in PDAC cells, leading to enhancement of GPCR-induced mitogenic signaling [7, 9, 10]. Insulin-induced potentiation of GPCR signaling was mediated through the phosphatidylinositol 3-kinase (PI3K)/Akt/mTORC1 signaling module [7, 9], a key pathway in insulin/IGF action [11]. These findings assume an added importance in view of the large number of epidemiological studies linking obesity and long standing type-2 diabetes mellitus (T2DM), characterized by peripheral insulin resistance and compensatory overproduction of insulin, with increased risk for developing PDAC [see [12] for review].

Metformin (1,1-dimethylbiguanide hydrochloride), the most widely prescribed drug for treatment of T2DM worldwide, is emerging as a potential anticancer agent. At the cellular level, metformin indirectly stimulates AMP-activated protein kinase (AMPK) activation [13, 14], though other mechanisms of action have been proposed, especially using high concentrations of this biguanide [15]. Major downstream targets of AMPK include the tumor suppressor TSC2 (tuberin) and the substrate binding subunit Raptor of mTORC1 [16, 17], the phosphorylation of which inhibits mTORC1 activity [18]. Converging epidemiological, clinical and preclinical studies support the use of metformin in cancer prevention and therapeutics [10, 19]. Strikingly, T2DM patients who had taken metformin had a 62% lower adjusted incidence and better survival of PDAC compared with those who had not taken this drug [20, 21]. Conversely, T2DM patients who received insulin or insulin secretagogues had a significantly higher risk of PDAC compared with diabetic patients who had not taken these drugs [20, 22]. In preclinical studies, metformin disrupted crosstalk between insulin/IGF receptor and GPCR signaling systems in PDAC cells and inhibited the growth of PDAC xenografts [7]. Other studies showed that metformin inhibited the proliferation of breast, colon, lung and prostate cancer cells [10, 19]. All these reports suggest that the antidiabetic drug metformin may provide a novel approach for the prevention and treatment of PDAC and other malignancies. Although the effects of metformin on cancer cells *in vitro* suggest a direct mechanism, most previous studies used very high concentrations of this agent to demonstrate inhibitory outcomes (e.g. 5–30 mM [15, 23–27]) and consequently, their significance has been questioned.

We noticed that many studies examining effects of metformin *in vitro* were carried out with cancer cells cultured in media supplemented with supra-physiological concentrations of glucose. Here, we demonstrate that PDAC cells cultured in medium containing physiological concentrations of glucose (5 mM) display a marked increase in their sensitivity to metformin. The biguanide inhibited stimulation of mTORC1, DNA synthesis and cell proliferation in PDAC cells, at concentrations 10 to 100-fold lower than those used in most previous reports. At low concentrations, metformin prevented stimulation of DNA synthesis, at least in part, through an AMPK-dependent pathway.

MATERIALS and METHODS

Cell culture

The human pancreatic cancer cell lines PANC-1, MiaPaCa-2 and the immortalized ductal cell line hTERT-HPNE were obtained from the American Type Culture Collection (ATCC, Manassas, VA). PANC-1 and MiaPaCa-2 cells were grown in Dulbecco's modified Eagle Medium (DMEM) and 10% fetal bovine serum (FBS). hTERT-HPNE cells were grown in the medium containing 75% DMEM without glucose, 25% Medium M3 Base (Incell Corp. Cat# M300F- 500), 5% FBS, 10 ng/ml human recombinant EGF, 5.5 mM D-glucose (1g/L) and 750 ng/ml puromycin.

Assays of [³H]-thymidine incorporation

Measurements of [³H]-thymidine incorporation into DNA and cell proliferation were performed as previously described [7]. Metformin, neurotensin and insulin were added for 16 h at 37°C prior to the addition of [³H]-thymidine for 6 h.

Western blot analysis

The cultures of PANC-1 or MIA PaCa-2 cells, treated as describe in individual experiments, were lysed in 2× SDS-PAGE sample buffer [200 mM Tris-HCl (pH 6.8), 2 mM EDTA, 0.1 M Na₃VO₄, 6% SDS, 10% glycerol, and 4% 2-mercaptoethanol], the lysates subjected to SDS-PAGE on 10% gels and transferred to Immobilon-P membranes (Millipore, Billerica, MA). Western blots were then performed in PBS containing 0.1% Tween-20. The immunoreactive bands were detected with enhanced chemiluminescence (ECL) reagents (GE Healthcare Bio-Sciences Corp, Piscataway, NJ). The phosphospecific antibodies used were polyclonal antibodies that detect the phosphorylated state of acetyl-CoA carboxylase (ACC) at Ser⁷⁹, Raptor at Ser⁷⁹², 40S ribosomal protein subunit S6 kinase (S6K), at Thr³⁸⁹ and 40S ribosomal S6 protein at Ser^{235/236}. Quantification was performed using Multi Gauge V3.0.

Knockdown of AMPK protein levels

The pooled siRNA duplexes were purchased from either Dharmacon (Lafayette, CO) or Santa Cruz (Santa Cruz, CA) and designed to target the α_1/α_2 subunits of human AMPK. For siRNA transfection the reverse transfection method was used, the siRNA pool was mixed with Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. PANC-1 cells were then plated on top of the siRNA/ Lipofectamine RNAiMAX complex at a density of 1×10^5 cells/35 mm dish. Control transfections were carried out with Dharmacon siCONTROL non-targeting siRNA four-oligo pool (Sc) (catalog number D-001206-13). Three days after transfection, cells were used for western blot analysis and DNA synthesis.

Materials

Neurotensin and insulin were obtained from Sigma Chemical (St. Louis, MO). Metformin was obtained from Sigma-Aldrich (St. Louis, MO). All antibodies were purchased from Cell Signaling Technology (Danvers, MA). Horseradish peroxidase-conjugated anti-rabbit IgG and anti-mouse IgG were from GE Healthcare Bio-Sciences Corp (Piscataway, NJ). All other reagents were of the highest grade available.

RESULTS

Stimulation of AMPK by metformin in PDAC cells: dependence on glucose concentration in the culture medium

In order to determine whether AMPK activation in response to metformin in PDAC cells depends on the ambient glucose concentration, cultures of MiaPaCa-2 and PANC-1 cells were incubated in medium containing glucose at 5 mM (a physiological concentration) or 25 mM (as in regular DMEM) and then treated with increasing concentrations of metformin. Cell lysates were analyzed by immunoblotting using antibodies that detect the phosphorylated state of acetyl-CoA carboxylase (ACC) at Ser⁷⁹ or Raptor at Ser⁷⁹², residues directly phosphorylated by AMPK and used as markers of AMPK activity within intact cells. As shown in Fig. 1, metformin-induced phosphorylation of ACC at Ser⁷⁹ and Raptor at Ser⁷⁹² was dramatically enhanced in PDAC cells cultured in medium containing a physiological concentration of glucose (5mM), as compared with cells in medium containing glucose at a supra-physiological concentration (25 mM). AMPK activation in cells incubated in 5 mM glucose was detected at concentrations of metformin as low as 0.05–0.1 mM, which are orders of magnitude lower than those used in most previous studies.

Metformin inhibits mTORC1 activation in PDAC cells cultured in medium containing physiological concentration of glucose

We next determined the influence of ambient glucose concentration on the ability of metformin to inhibit mTORC1 activation. MiaPaCa-2 or PANC-1 cells in 5 mM or 25 mM glucose were treated with or without 1 mM metformin and then stimulated with insulin, the GPCR agonist neurotensin or their combination to elicit positive crosstalk [7, 10]. mTORC1 activity was determined by phosphorylation of 40S ribosomal protein subunit S6 kinase (S6K) at Thr³⁸⁹, a direct target of mTORC1, and phosphorylation of S6 (Ser^{235/236}), a substrate of S6K. Metformin virtually abolished mTORC1 signaling induced by insulin, neurotensin or their combination in PDAC cells incubated in 5 mM glucose (Fig. 2, A). In sharp contrast, metformin inhibited only slightly mTORC1 activation in cells incubated in medium containing 25 mM glucose. Using the same lysates, we verified that metformin stimulated AMPK, as shown by ACC phosphorylation at Ser⁷⁹, in cells incubated in medium containing 5 mM glucose but caused only a small increase in ACC phosphorylation in cultures exposed to 25 mM glucose (Fig. 2, A).

Next, we examined whether metformin exerts cellular effects at concentrations close to those found in the plasma of T2DM patients treated with this drug. As shown in Fig. 2 B, metformin added at 0.05 mM or 0.1 mM inhibited mTORC1 (S6K Thr³⁸⁹ phosphorylation) in MiaPaCa-2 cells in a dose-dependent manner. In the same cell lysates, metformin induced phosphorylation of ACC at Ser⁷⁹ and Raptor at Ser⁷⁹² at the same concentrations that inhibited mTORC1 activity (Fig. 2 B, **left**). The effects of metformin at 0.05 mM or 0.1 mM were statistically significant, as revealed by quantification of the immunoreactive bands obtained in 6 independent experiments (Fig. 2 B, **right**).

Metformin potently inhibits DNA synthesis in PDAC cells cultured in medium containing physiological concentration of glucose

Subsequently, we examined the effect of increasing concentrations of metformin on the stimulation of DNA synthesis induced by insulin and neurotensin in PDAC cells incubated in either 5 or 25 mM glucose. Metformin potently inhibited DNA synthesis in MiaPaCa-2 cells incubated in the presence of 5 mM glucose in a dose-dependent manner (Fig. 3 A). In contrast, metformin only attenuated DNA synthesis in cells incubated in 25 mM glucose. Similar shifts in dose-response relationships were seen with PANC-1 cells. We did not

detect any substantial inhibitory effect of 1 mM metformin on DNA synthesis stimulated by IGF-1 in immortalized human pancreatic duct-derived epithelial cells incubated in 5 mM glucose, implying that metformin preferentially inhibits DNA synthesis in cancer cells (Fig. 3, B). In additional experiments, we corroborated that metformin inhibited DNA synthesis (Fig. 3, C) and cell proliferation (Fig. 3, D) at 0.05 mM ($p<0.05$) and 0.1 mM ($p<0.001$) in MiaPaCa-2 or PANC-1 cells incubated in 5 mM glucose, but did not inhibit DNA synthesis or proliferation at comparable concentrations in these cells incubated in medium containing 25 mM glucose (results not shown).

Knockdown of the α subunits of AMPK reverses metformin-induced inhibition of DNA synthesis

Next, we determined whether knockdown of the α_1 and α_2 catalytic subunits of AMPK prevents the inhibitory effects of metformin on DNA synthesis by insulin and GPCR agonists in PDAC cells incubated in 5 mM glucose. Transfection of PANC-1 cells with siRNA targeting the α_1 and α_2 catalytic subunits of AMPK caused a striking knockdown (>90%) of their protein expression, as compared with cells transfected with non-targeting siRNA (Fig. 4 A). Down-regulation of AMPK α_1 and α_2 dramatically decreased metformin-induced phosphorylation of either ACC at Ser⁷⁹ or Raptor at Ser⁷⁹², confirming the specificity of our assays of AMPK activation within PDAC cells. The salient feature in Fig. 4 is that knockdown of α_1 and α_2 catalytic subunit expression of AMPK substantially reversed the inhibitory effect of metformin (1 mM) on the stimulation of DNA synthesis in PDAC cells (Fig. 4 B). Similar results were obtained with siRNAs directed to a different sequence of the catalytic subunits of AMPK.

DISCUSSION

Many studies have demonstrated that metformin inhibits proliferation of cells in culture, suggesting that the anticancer action of this drug could be mediated, at least in part, through a direct effect on cancer cells. However, an important shortcoming of most previous studies *in vitro* is that metformin was used at very high concentrations. Consequently, the clinical significance of inhibitory effects obtained using metformin at ~10 mM has been questioned. In order to understand the anticancer mechanism of metformin, it is important to elucidate whether this antidiabetic drug can exert direct effects on cancer cells at concentrations close to the therapeutic range.

Mechanistically, metformin is thought to inhibit complex I of the mitochondrial respiratory chain, reduce ATP synthesis and thereby activate AMPK via an increase in the AMP/ATP ratio [13, 14]. However, AMPK-independent mechanisms have been proposed, especially at high concentrations of this biguanide [15]. We noticed that most previous studies examining effects of metformin *in vitro* were carried out with cancer cells cultured in medium supplemented with supra-physiological concentrations of glucose. Cancer cells use aerobic glycolysis to generate ATP when the glucose concentration in the medium is high but retain significant capacity for oxidative phosphorylation [28, 29]. Thus, when cultured in medium with 25 mM glucose (e.g. DMEM), cancer cells derive most of their ATP from glycolysis. In contrast, we reasoned that when the concentration of ambient glucose is within the physiological range (~5 mM) and glucose uptake rates are lower, cells derive part of their ATP from mitochondrial oxidative phosphorylation [30] and hence, become sensitive to mild inhibitors of mitochondrial function, like metformin. In line with this hypothesis, we show here that metformin-induced AMPK activation, as scored by the phosphorylation of its substrates, ACC and Raptor, is strikingly enhanced within PDAC cells cultured in medium containing physiological glucose, as compared with parallel cultures in regular DMEM. AMPK activation was detected at concentrations of metformin as low as 0.05–0.1 mM. These findings identified an experimental condition that allowed the examination of the

mechanism of action of metformin using concentrations of this drug that were close to the therapeutic range and orders of magnitude lower than those used in many other reports. Accordingly, metformin inhibited mTORC1 activation, DNA synthesis and cell proliferation at 0.05–0.1 mM in PDAC cells incubated in medium containing a physiological concentration of glucose (5 mM). Thus, under our conditions, metformin inhibited mitogenic signaling in human pancreatic cancer cells, at concentrations close to those found in the plasma of rodents [31] and humans [32] treated with therapeutic doses of metformin.

As indicated above, the role of AMPK in the mechanism by which metformin inhibits proliferative signals remains controversial. Under the experimental conditions defined in this study, we found that knockdown of the α subunits of AMPK largely reversed the inhibition of DNA synthesis induced by metformin. Consequently, we propose that metformin inhibits mitogenic signaling through an AMPK-dependent pathway when used at low concentrations (<1mM) and at physiological concentration of ambient glucose. This conclusion provides a plausible explanation for apparently contradictory reports on the contribution of AMPK in the mechanism of action of metformin and emphasizes the need of using medium containing physiological concentrations of glucose to define the anticancer mechanism of action of this anti-diabetic drug.

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HIGHLIGHTS

- Metformin inhibits cancer cell growth but the mechanism(s) are not understood.
- We show that the potency of metformin is sharply dependent on glucose in the medium
- AMPK activation was enhanced in cancer cells incubated in physiological glucose.
- Reciprocally, metformin potently inhibited mTORC1, DNA synthesis and proliferation.
- Metformin, at low concentrations, inhibited DNA synthesis through AMPK.

Fig. 1. Enhanced metformin-induced AMPK activation in PDAC cells cultured in medium containing glucose at a physiological concentration

A. Confluent Mia PaCa-2 and PANC-1 cells were incubated with increasing concentrations of metformin for 16 h in DMEM containing either 5 mM or 25 mM glucose, as indicated. Cell lysates were analyzed by immunoblotting for phosphorylation of ACC at Ser⁷⁹ (pACC) and Raptor at Ser⁷⁹² (pRaptor). Total ACC was used to verify equal gel loading. Similar results were obtained in three independent experiments. **B.** Results are expressed as the percentage of maximum mean $\pm$ SEM; n=3.

Fig. 2. Enhanced metformin-induced mTORC1 inhibition in PDAC cells exposed to physiological glucose concentrations

A) Cultures of MiaPaca-2 and PANC-1 cells were incubated without (–) or with 1 mM metformin for 16 h in DMEM containing 5 mM or 25 mM glucose, as indicated. Then, the cells were stimulated for 2 h with 5 nM neurotensin (NT), 10 ng/ml insulin (Ins) or their combination (NT+Ins), lysates were analyzed by immunoblotting with the following phospho antibodies: S6K Thr³⁸⁹ (pS6K), S6 Ser^{235/236} (pS6) and ACC Ser⁷⁹ (pACC). Total ACC was used to verify equal gel loading. Similar results were obtained in 3 independent experiments. **B)** Mia PaCa-2 cells were incubated with DMEM containing 5 mM glucose either in absence or presence of 0.05mM or 0.1 mM metformin for 16 h. Then, the cells were treated with NT+Ins, as in panel A, and lysates were analyzed by immunoblotting. Similar results were obtained in 6 independent experiments. Results are expressed as the percentage of maximum mean $\pm$ SEM, n=6 (S6K) or as the fold increase over basal as indicated. P values were determined using the t-test (Sigma Plot 12.) *p<0.05; **p<0.001; n=6.

Fig. 3. Metformin potently inhibits DNA synthesis and proliferation in PDAC cells incubated in 5 mM glucose

A) Dose-response effect of metformin on DNA synthesis induced by neurotensin and insulin in MiaPaca-2 cells. Cultures in serum-free medium containing 5 mM (*closed circles*) or 25 mM glucose (*open circles*) were stimulated with 5 nM neurotensin and 10 ng/ml insulin in the presence of metformin (0.025–1 mM). Results are expressed as the percentage of maximum mean $\pm$ SEM, $n=6$. **B) Effect of metformin on DNA synthesis induced by IGF in hTERT-HPNE cells.** The hTERT-HPNE cell cultures were stimulated with 10 ng/ml IGF-1 in the absence or presence of 1 mM metformin (as indicated). Results are expressed as the percentage of maximum mean $\pm$ SEM; $n=6$. **C) Low concentrations of metformin inhibit DNA synthesis in MiaPaca-2 and PANC-1 cells cultured in DMEM containing 5 mM glucose.** Cultures were incubated in medium containing 5 mM glucose either in the absence (grey bars) or the presence of 0.05 mM or 0.1 mM metformin (black bars) and 5 nM neurotensin and 10 ng/ml insulin (NT + Ins) as indicated. Results are expressed as the percentage of maximum mean $\pm$ SEM, $n=6$. **D), Low concentrations of metformin inhibit proliferation of MiaPaca-2 and PANC-1 cells cultured in DMEM**

containing 5 mM glucose. Single-cell suspensions of MiaPaca-2 and PANC-1 cells were plated at a density of 2×10^4 cells per dish. After 4 h, the cultures were shifted to media containing 1% FBS without (–) or with 10 nM neurotensin and 10 ng/ml insulin (NT + Ins) the absence (grey bars) or presence (closed bars) of 0.05 mM or 0.1 mM metformin. After 4 days, cell numbers were determined from 6 plates per condition. Results are presented as mean $\pm$ SEM. Similar results were obtained in two independent experiments. P values were determined using the t-test (SigmaPlot 12.) * $p < 0.05$; ** $p < 0.01$; $n = 6$.

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Fig. 4. Knockdown of the α subunits of AMPK abrogates ACC and Raptor phosphorylation and reverses the inhibition of DNA synthesis induced by metformin

A). PANC-1 cells were transfected with either non-targeting negative control (N. Targ siRNA.) or 75 nM AMPK siRNA (AMPK siRNA) in media containing 5 mM glucose. Cells were incubated in the absence or presence of 1 mM metformin for 16h, lysates were analyzed by immunoblotting with the following phospho-antibodies: ACC Ser⁷⁹ (pACC) and Raptor Ser⁷⁹² (pRaptor), α_1/α_2 subunits of AMPK and total ACC. Similar results were obtained in four independent experiments. Results are expressed as the percentage of maximal mean $\pm$ SEM, n=16. **B).** PANC-1 cells were transfected with either transfection reagent alone (open bars) non-targeting negative control (grey bars) or 75 nM AMPK

siRNA (black bars) in DMEM/FBS containing 5 mM glucose. After 3 days the cells were incubated for 6 h in serum-free medium containing 5mM glucose and stimulated with 5nM neurotensin and 10 ng/ml insulin in the absence or presence of 1 mM metformin (Met). Results are expressed as the percentage of maximal mean $\pm$ SEM, n=6.

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