Metformin Decreases Glucose Oxidation and Increases the Dependency of Prostate Cancer Cells on Reductive Glutamine Metabolism

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Abstract

Metformin inhibits cancer cell proliferation, and epidemiology studies suggest an association with increased survival in patients with cancer taking metformin; however, the mechanism by which metformin improves cancer outcomes remains controversial. To explore how metformin might directly affect cancer cells, we analyzed how metformin altered the metabolism of prostate cancer cells and tumors. We found that metformin decreased glucose oxidation and increased dependency on reductive glutamine metabolism in both cancer cell lines and in a mouse model of prostate cancer. Inhibition of glutamine anaplerosis in the presence of metformin further attenuated proliferation, whereas increasing glutamine metabolism rescued the proliferative defect induced by metformin. These data suggest that interfering with glutamine may synergize with metformin to improve outcomes in patients with prostate cancer. Cancer Res; 73(14); 4429–38. ©2013 AACR.

Introduction

Altered cellular metabolism is a hallmark of cancer cells (1–3). Tumor cells adapt their metabolism to provide the needed quantities of ATP, redox equivalents, and biosynthetic precursors necessary for cell growth and uncontrolled proliferation (4–6). Individual cancers appear to have distinct metabolic dependencies to sustain growth and prolif-

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in model systems. While some activity of biguanides in vivo may be attributable to systemic effects such as reduction of insulin levels, there is considerable interest in possible "direct" effects of these compounds under conditions where sufficient intracellular drug concentrations are achieved (26). Indeed, the ability of cancer cells to take up metformin has been challenged (26). Nevertheless, numerous studies have shown antiproliferative effects of metformin in various cancer cell lines, and in prostate cancer cells, these metformin effects appear to be independent of AMPK. Instead metformin appears to activate p53, leading to subsequent-REDD1 mediated mTOR and cyclin D1 inhibition (27–29). Yet, in line with other tumor types, it remains possible that metformin induces antineoplastic activity via direct effects on metabolism. Whether metformin has a direct effect on prostate cancer cell metabolism is unexplored and consequently how best to use the antiproliferative effect of metformin for cancer therapy might be missed.

To understand how to best use metformin in patients with prostate cancer, we sought to understand whether and how metformin affects the metabolism of prostate cancer cells. We found that both glucose and glutamine metabolism are affected by metformin via complex I inhibition. Prostate cancer cells with higher glucose oxidation appear to be the most sensitive to metformin. Moreover, we identified reductive glutamine metabolism as essential for maintaining modest proliferation in the presence of metformin, thus suggesting that interruption of glutamine metabolism may have a synergistic effect with metformin. In agreement with this hypothesis, we found that metformin also increased reductive glutamine metabolism in tumors arising in an SV40-driven mouse model of prostate cancer.

Materials and Methods

Cell lines and cell culture conditions

All cell lines were obtained from American Type Culture Collection. All experiments were carried out in RPMI (Mediatech), with the exception of HuH7 cells which were cultured in Dulbecco’s Modified Eagle’s Media, each supplemented with 10% FBS (Invitrogen) and 1% penicillin/streptomycin (Mediatech). For labeling experiments, FBS was replaced with dialyzed FBS (Invitrogen), and glucose or glutamine was replaced with U-13C glucose (CLM-1396 Cambridge Isotopes), U-13C glutamine (605166 Sigma), or 5–13C glutamine (CLM-1166 Cambridge Isotopes). Cell lines were treated with metformin or any other small molecule for 3 days in all experiments.

Physiology

Growth rates were determined using a hemocytometer or an automated cell counter (Nexcelom). To determine uptake and secretion rates, glucose and lactate concentrations were detected by a Waters Alliance 2695 HPLC (Waters) with a Waters 410 Differential Refractometer and a Bio-Rad HPX-87H column (Bio-Rad). The column was eluted at 50°C with 14 mM of sulfuric acid at a flow rate of 0.7 mL/min. Glutamate consumption and glutamate secretion were measured with an YSI 7100MBS (YSI Life Sciences) according to the manufacturer’s protocol.

Carbon contribution to tricarboxylic acid cycle and palmitate

Labeled tissue cultures were washed with saline and metabolism was quenched with −20°C cold 70% methanol. After cell scraping in 70% methanol (containing internal standard norvaline), −20°C cold chloroform was added and the samples were vortexed at 4°C to extract metabolites. Phase separation was achieved by centrifugation at 4°C. Methanol phase (polar metabolites) and chloroform phase (fatty acids) were separated and dried by applying constant air flow. Dried metabolite samples were stored at −80°C.

Polar metabolite samples were derivatized with methoxyamine (TS-45950 Thermo Scientific) for 90 minutes at 40°C and subsequently with N-(tert-butyldimethylsilyl)-N-methyltrifluoroacetamid, with 1% tert-butyldimethylchlorosilane (375934 Sigma) for 60 minutes at 60°C. Fatty acids were esterified with 2% sulfuric acid in methanol for 120 minutes at 60°C and subsequently extracted with hexane. Isotopomer distributions of polar metabolites and fatty acids were measured with a 6890N GC system (Agilent Technologies) combined with a 5975B Inert XL MS system (Agilent Technologies). Isotopomer distributions were analyzed using the MATLAB-based software Metran (30–33). Total contribution of carbon was calculated using the following equation (34):

\[ \text{Total contribution of carbon} = \frac{\sum_{i=0}^{n} i \times m_i}{n \times \sum_{i=0}^{n} m_i} \]

where \( n \) gives the number of C atoms in the metabolite, \( i \) is the different mass isotopomers, and \( m \) is the abundance of a certain mass.

Isotopomer distributions of fatty acids were further fitted to an isotopic spectral analysis model, assuming that the measured mass distribution vectors are a function of the rate of fatty acid synthesis and contribution of the labeled carbon source to fatty acids (35, 36).

Net glutamine flux was calculated from glutamine uptake rates and glutamate secretion rates.

Small molecules for in vitro experiments

Metformin (D5035 Sigma) was dissolved in milliQ water and applied at concentrations of 0.5 to 2.5 mM/L. Rotenone (R8875 Sigma) was dissolved in dimethyl sulfoxide (DMSO) and applied at concentrations of 10 to 50 ng/mL. BPTES was a gift from Agios Pharmaceuticals. BPTES and 968 (AG-690 Spees) were dissolved in DMSO and applied at concentrations of 4 or 5 and 10 µM/L, respectively.

TSC2 knockdown and mRNA expression levels

Lentivirus with shRNA against TSC2 (TRCN0000040178, TRCN0000040181; Sigma) was applied. Control cells were infected with empty pLKO.1. For quantitative real-time PCR, RNA was isolated using RNeasy Mini Kit (Qiagen), and cDNA was generated from 1 µg RNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to manufacturer’s protocols. cDNA was analyzed using the Light Cycler 480 II (Roche) with SYBR Green Master Mix from Bio-Rad. Primers sequences were obtained from Primer Bank (37).
Metformin quantification

Tumor tissue samples (20–30 mg) were homogenized in 0.9% NaCl using a mortar and pestle and centrifuged (10 minutes, 13,000 rpm). An aliquot of the supernatant was used for protein determination; in an additional aliquot, metformin was quantified. Metformin was quantified with an Agilent 6460 triple quadrupole mass spectrometer (Agilent) coupled to an Agilent 1200 HPLC system. Ionization mode was electrospray (ESI), polarity positive. Electrospray jetstream conditions were as follows: capillary voltage 3,500 V, nozzle voltage 1,000 V, drying gas flow 11 L/min nitrogen, drying gas temperature 350°C, nebulizer pressure 55 psi, sheath gas temperature 350°C, and sheath gas flow 11 L/min. The mass spectrometer was operated in the multiple reaction monitoring (MRM) mode using m/z 130.1 and the product ion m/z 60.1. Dwell time was 100 ms, the fragmentor was set at 60, and the collision energy at 10. High-performance liquid chromatography (HPLC) separation was achieved on a HILIC plus column (50 x 2.1 mm² I.D., 3.5 µm particle size; Agilent) using 0.1% formic acid in water/acetonitrile 20:80 (v/v) as mobile phase at a flow rate of 0.65 mL/min.

Transgenic adenocarcinoma of mouse prostate

Transgenic adenocarcinoma of mouse prostate (TRAMP) mice were injected with 3 consecutive intraperitoneal injections (with a 24-hour interval) of vehicle or metformin (300 or 500 mg/kg/d). Metformin was dissolved in PBS, and the vehicle was PBS. Mice were sacrificed 2 hours after the last injection. Prostate tumor tissue was removed and quenched in liquid nitrogen. Pulverized tumor tissue was extracted and analyzed as described above. Metabolite pool sizes were normalized to analyzed tumor tissue weight. Mass isotopomer distributions of citrate were normalized to the glutamine enrichment of the tissue.

All animal experiments were approved by the Committee on Animal Care at MIT and/or Harvard.

Statistics

Error bars depict SD. R and P values for the depicted correlations were calculated with the MATLAB integrated function correlation: [R,P] = corrcoef returns P, a matrix of P values for testing the hypothesis of no correlation. Each P value is the probability of getting a correlation as large as the observed value by random chance, when the true correlation is zero. P values were also calculated for bar graphs using the Student t test (2-tailed, unequal variance) to determine whether 2 samples are likely to have come from the same 2 underlying populations that have the same mean.

Results

Metformin decreases proliferation and promotes glucose fermentation in prostate cancer cell lines

To begin to characterize the effect of metformin on the metabolism of 3 different prostate cancer cell lines, we measured proliferation, glucose uptake rate, and lactate secretion rate in the absence or presence of increasing doses of metformin. For all 3 cell lines, the decrease in proliferation observed with increasing metformin concentration was accompanied by an increase in the rate of glucose uptake and lactate secretion (Fig. 1A). Interestingly, the magnitude of the decrease in proliferation along with the increase in glucose uptake/lactate secretion was different across all 3 cell lines. LNCaP cells exhibited the greatest response, PC3 cells showed an intermediate response, and DU145 hardly responded to metformin. In the presence of 2.5 mmol/L metformin compared with no-treatment, all cell lines displayed an increase in the amount of lactate secretion relative to glucose uptake, suggesting that a larger percentage of the glucose is converted to lactate in these cells. However, the increase in lactate secretion relative to glucose uptake was different in different cell lines (increase in this ratio for DU145: 13% ± 3.9%, PC3: 23% ± 2.2%, and LNCaP: 49% ± 3.0%), suggesting that metformin differently affected carbon metabolism in these different cell lines. Furthermore, the degree to which metformin altered glucose metabolism correlated with the decrease in proliferation rate when cells were treated with the drug (Fig. 1B).

The metabolic response to metformin is consistent with mitochondrial complex I inhibition

To understand how metformin influences glucose metabolism, we first asked whether the known action of metformin as an inhibitor of mitochondrial complex I is also observed in these cells. Complex I is a component of the electron transport chain that transfers electrons derived from nutrient oxidation to oxygen. To determine whether metformin affected this process in prostate cancer cells, we measured oxygen consumption in the absence or presence of increasing concentrations of metformin. Metformin inhibited oxygen consumption in a dose-dependent manner in all 3 cell lines (Supplementary Fig. S1), consistent with complex I inhibition. To determine whether complex I inhibition could account for the differential metformin sensitivity across the 3 cell lines, we tested the effect of increasing doses of the established complex I inhibitor rotenone on cell proliferation (Fig. 2A). Rotenone inhibited cell proliferation in a dose-dependent manner that varied across cell lines. Consistent with complex I being involved in metformin toxicity, the sensitivity of all 3 cell lines to rotenone was the same as that observed with metformin (i.e., DU145 cells were the least sensitive, PC3 cell had an intermediate phenotype, and LNCaP cells were the most sensitive). Moreover, addition of metformin did not have any further effect on cells treated with the highest dose of rotenone, supporting the hypothesis that the antiproliferative effect of metformin is mediated by complex I inhibition in these cells (Fig. 2A). Furthermore, we tested whether the correlation between decreased proliferation and increased glucose uptake was also observed with rotenone treatment. Consistent with a role for complex I inhibition in altering glucose metabolism in response to metformin, proliferation, and glucose uptake rates in the presence of rotenone were highly correlated (Fig. 2B). Finally, we tested whether hypoxia affects metformin sensitivity. Hypoxic cells are more dependent on glycolysis as an adaptation to low oxygen (38), raising the possibility that hypoxic cells could be less sensitive to metformin. Indeed we found...
that PC3 cells in hypoxia are unaffected by metformin and that LNCaP cells were much less affected in hypoxia compared with normoxia (Fig. 2C). Together, these data suggest that complex I inhibition by metformin was responsible for both altered glucose metabolism and the inhibition of cell proliferation in prostate cancer cell lines and that the magnitude of the effect on glucose metabolism is associated with the ability of metformin to suppress cell proliferation.

Metformin decreases entry of glucose carbon into the tricarboxylic acid cycle and increases glutamine anaplerosis

To investigate the metabolic effects of metformin beyond the macroscopic parameters of glucose uptake and lactate secretion, we cultured all 3 cell lines in the presence of uniformly labeled $^{13}$C-glucose or uniformly labeled $^{13}$C-glutamine and measured the incorporation of labeled carbon from either glucose or glutamine into the tricarboxylic acid cycle. The results showed that metformin induced a significant decrease in glucose incorporation into the tricarboxylic acid cycle and an increase in glutamine anaplerosis, consistent with the hypothesis that metformin inhibits complex I of the electron transport chain, thereby reducing the availability of reducing equivalents and promoting the use of glutamine as a metabolic substrate.

Figure 1. Metformin decreases proliferation and promotes glucose fermentation in prostate cancer cell lines. A, proliferation, glucose uptake rate, and lactate secretion rate of cell lines treated with metformin. B, correlation between absolute alteration in proliferation and glucose uptake or lactate secretion rate for different metformin concentrations compared with the condition with no metformin added. All error bars indicate SD. SD, correlation coefficient $R$, and $P$ values were calculated from 3 biologic replicates.
nutrient into the tricarboxylic acid cycle metabolite α-ketoglutarate by gas chromatography/mass spectrometry. In all cell lines tested, metformin decreased glucose contribution to α-ketoglutarate in a dose-dependent manner, whereas glutamine contribution to α-ketoglutarate increased in a reciprocal pattern (Fig. 3A). As in most cultured cells, glutamine accounted for the majority of tricarboxylic acid cycle carbon in all 3 lines tested (39). However, variability in the degree of glucose contribution was observed. Interestingly, the magnitude of glucose carbon contribution to the tricarboxylic acid cycle was also correlated with the sensitivity of the cell lines to metformin; DU145 was the least sensitive to metformin and showed the least amount of glucose oxidation, PC3 cells had an intermediate phenotype, and the LNCaP cells displayed the most glucose oxidation and were most sensitive to metformin (Fig. 3A).

Inhibition of electron transport has been reported to switch glutamine metabolism from an oxidative to a reductive pathway (40). Consistent with the inhibition of complex I by metformin in these cells, metformin treatment led to an increase in reductive glutamine metabolism. This was shown by an increase in the M + 5 mass isotopomer of citrate (Fig. 3B) and further confirmed by measuring the contribution of glutamine labeled with 13C only at the C5 position, to palmitate, which is lost if glutamine contributes oxidatively to fatty acids. Metformin increased the reductive contribution of glutamine to palmitate in a dose-dependent manner (Supplementary Fig. S2), as well as the overall contribution of carbon to the tricarboxylic acid cycle. These data suggest that complex I inhibition by metformin decreased glucose entry into the tricarboxylic acid cycle and subsequently increased glutamine anaplerosis via reductive carboxylation to citrate. Furthermore, these phenomena track with sensitivity to metformin.

To further confirm that complex I inhibition by metformin results in the switch from glucose toward additional glutamine for fueling the tricarboxylic acid cycle, we measured glutamine contribution to α-ketoglutarate and the reductive glutamine contribution to palmitate in the presence of rotenone (Supplementary Fig. S3). Similar to metformin, rotenone increased the contribution of glutamine carbon to the tricarboxylic acid cycle and reductive glutamine contribution to palmitate, providing further support for the role of complex I inhibition in the antiproliferative effect for metformin in prostate cancer cells.

**Decreased glutamine flux increases sensitivity to metformin**

We next questioned whether increased glutamine metabolism is an adaptive response to complex I inhibition that might attenuate the antiproliferative effect of metformin. To test this hypothesis, we took advantage of the small-molecule inhibitors 968 and BPTES, both of which target glutaminase (refs. 15, 16; Fig. 4A). Glutaminase catalyzes the conversion of glutamine to glutamate, and the increased activity of this enzyme is at least partially responsible for elevated glutamine metabolism.
in cancer (16–41). Consistent with increased glutamine metabolism being an adaptive response following metformin treatment, we detected larger decrease in cell number with the simultaneous administration of metformin and a glutaminase inhibitor (968 or BPTES) than observed with either metformin or glutaminase inhibitor acting alone for all 3 cell lines (Fig. 4B; Supplementary Fig. S4A). The combination of rotenone and 968 produced a similar effect (Fig. 4C). Finally, we tested whether withdrawal of glutamine from the medium yields increased metformin sensitivity. As none of the prostate cancer cell lines proliferate in the absence of glutamine, we used Huh7 liver cancer cells, which have been previously reported to grow without glutamine (42). Indeed, Huh7 cells cultured without glutamine displayed a significantly increased metformin sensitivity (Supplementary Fig. S4B).

**Increased glutamine flux decreases metformin sensitivity**

To test whether increased glutamine flux is sufficient to limit the antiproliferative effect of metformin, we took advantage of the recently reported ability of mTOR to positively regulate net glutamine flux into the tricarboxylic acid cycle (43). mTOR was activated in these cells by a knockdown of its negative regulator TSC2 (Supplementary Fig. S5A). Net glutamine flux into the tricarboxylic acid cycle increased in the TSC2-knockdown cell lines by approximately 2-fold (Fig. 5A). In line with the hypothesis that increased glutamine metabolism mitigated metformin toxicity, cells with activated mTOR were approximately 3-fold less sensitive to metformin when compared with control cells (Fig. 5B). To further test the importance of glutamine conversion to α-ketoglutarate as an adaptive response to metformin, we used a cell-permeable form of α-ketoglutarate, dimethylketoglutarate, in LNCaP cells following drug treatment. In line with our previous findings, dimethyl-ketoglutarate supplementation in the presence of metformin increased cell number by 30% (Supplementary Fig. S5B). These results suggest that augmenting glutamine metabolism limited the antiproliferative effect of metformin.

**Metabolic alterations triggered by metformin in vivo match the in vitro response**

To test whether our findings are relevant to tumors in vivo, we first established that metformin reaches prostate tumors in mice. Using the TRAMP prostate cancer mouse model, we determined that tumors of mice dosed 3 times with 500 mg/kg
(body weight) metformin had taken up between 23 and 103 ng/mg (tissue protein) metformin into the prostate tumors. Metabolite analysis of both prostate cancer cell lines and prostate tumor tissue derived from TRAMP mice treated with vehicle or metformin showed that the most pronounced alteration in metabolite concentration as a result of metformin treatment was a fall in the citrate pool, which is associated with an activation of reductive glutamine metabolism (Fendt and colleagues, submitted; Fig. 6A). This effect of metformin was observed both in cells and in tissues (Fig. 6A and B). This

![Diagram of glutamine metabolism pathway](image1)

Figure 4. Decreased glutamine flux increases metformin sensitivity. A, enzymatic conversion steps from glutamine to α-ketoglutarate including known inhibitors of the pathway. Relative cell count of cell lines treated with a combination of metformin (B) or rotenone (C) with the glutaminase inhibitor 968. Cell counts were normalized to the condition with no metformin and no 968 added. SD and P values were calculated from 3 biologic replicates.

![Graphs of cell counts](image2)

Figure 5. Increased glutamine flux decreases metformin sensitivity. A, glutamine flux of TSC2-knockdown cell lines relative to control cell line. Control cells for knockdown were infected with an empty plasmid. B, metformin sensitivity of TSC2-knockdown cell lines, which exhibit increased glutamine flux based on cell counts. All error bars indicate SD. SD and P values were calculated from at least 3 biologic replicates.
uniformly labeled $^{13}$C-glutamine were administered to TRAMP mice treated with metformin or vehicle. Following tumor harvest the enrichment of M + 5 citrate was measured in prostate tumors as a surrogate for reductive glutamine metabolism. We found a significant increased enrichment of M + 5 citrate in different parts of the prostate from metformin treated animals (Fig. 6C). This increase in M + 5 citrate enrichment was accompanied by a decrease in the relative citrate pool size (Fig. 6D). In conclusion, these data show that the metabolic effects of metformin in tumor cells in vitro extend to similar responses in tumors in vivo.

### Discussion

The oral anti-diabetic drug metformin is beneficial for at least a subset of patients with cancer, but whether metformin can directly affect metabolism in tumor cells was not known. We systematically dissected the metabolic alterations metformin triggers in prostate cancer cell lines through complex I inhibition and found that this results in an adaptive increase in glutamine metabolism (Fig. 7). It is possible that in addition to the adaptive response in glutamine metabolism described here in response to metformin, energy homeostasis regulation via AMPK following metformin treatment further diminishes the effect of the drug as shown recently for non–small lung carcinoma (44). Our finding of an adaptive response in glutamine metabolism also complements a recent study showing that cell lines grown on glutamine as the major carbon source are sensitive to metformin (45). The finding of this study can be explained by our data, as we show that metformin leads to reductive glutamine metabolism and consequently leads to a dramatic decrease in ATP production by the tricarboxylic acid cycle. Importantly, we additionally show that inhibition of glutamine metabolism can promote metformin toxicity, suggesting that therapy with an agent that limits glutamine metabolism may potentiate the effect of metformin.

Our in vivo model showed that mice tolerate metformin at a dose of 500 mg/kg/d, considerably higher that than the conventional anti-diabetic dose in humans of ~20 mg/kg/d, which led to accumulation of the drug to ~60 ng/mg (tissue protein) in cancer tissue. This exposure level was observed to have in vivo metabolic effects on prostate tissues that matched our in vitro data. We show for the first time that this is correlated with effects on cellular carbon metabolism, specifically a dependency on reductive glutamine metabolism. This finding suggests that high-dose biguanide exposure might be a method to therapeutically induce metabolic stress in a subset of tumors, either alone or in rational combinations. It is important to recognize, however, that the effect of conventional anti-diabetic metformin doses currently being examined for antineoplastic activity in clinical trials may have a smaller effect on metabolism. Our work suggests that a dose escalation study of biguanides may be warranted to determine whether the dose range with more profound direct effects on cancer metabolism can safely be administered to humans.

Because the antiproliferative effect of metformin tracks with its ability to inhibit complex I, and this effect is mitigated by increased glutamine metabolism, it is possible that cells with a high dependence on oxidative glucose metabolism and low
Metformin Increases Dependency on Glutamine Metabolism

**Figure 7.** Metformin attenuates proliferation in prostate cancer cells by inhibiting complex I of the respiratory chain. This effect of metformin increases the dependency of the prostate cancer cell lines on reductive glutamine anaplerosis of the tricarboxylic acid cycle. Consequently, a combination therapy between metformin and inhibitors of the glutamine pathway might have a therapeutic benefit.

**Disclosure of Potential Conflicts of Interest**
M.G. Vander Heiden and L.C. Cantley have ownership interest (including patents) from and are consultants/advisory board members of Agios Pharmaceuticals. No potential conflicts of interest were disclosed by the other authors.

**Authors' Contributions**
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**References**
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