

Carbon Source and Myc Expression Influence the Antiproliferative Actions of Metformin

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Abstract

Epidemiologic and experimental data have led to increased interest in possible roles of biguanides in cancer prevention and/or treatment. Prior studies suggest that the primary action of metformin is inhibition of oxidative phosphorylation, resulting in reduced mitochondrial ATP production and activation of AMPK. *In vitro*, this may lead to AMPK-dependent growth inhibition if AMPK and its effector pathways are intact or to an energetic crisis if these are defective. We now show that the effect of exposure of several transformed cell lines to metformin varies with carbon source: in the presence of glutamine and absence of glucose, a 75% decrease in cellular ATP and an 80% decrease in cell number is typical; in contrast, when glucose is present, metformin exposure leads to increased glycolysis, with only a modest reduction in ATP level and cell number. Overexpression of *myc* was associated with sensitization to the antiproliferative effects of metformin, consistent with *myc* involvement in "glutamine addiction". Our results reveal previously unrecognized factors that influence metformin sensitivity and suggest that metformin-induced increase in glycolysis attenuates the antiproliferative effects of the compound.

Introduction

Metformin is a partial inhibitor of complex I of the mitochondrial electron transport chain (1, 2). By decreasing mitochondrial ATP production, metformin activates the AMP-activated protein kinase signaling pathway, a key regulator of cellular energy homeostasis (3). When this pathway is activated, energy-consuming processes such as protein synthesis and fatty acid synthesis are downregulated, which tends to lessen energetic stress but limits anabolic metabolism and proliferation (4–9). In cells with defective AMPK signaling or effector pathways, metformin exposure decreases oxidative phosphorylation without a compensatory decrease in energy expenditure, leading to an energy crisis (10, 11).

Metformin is widely used in the treatment of type II diabetes (12). In the special case of hepatocytes, energetic stress induced by the compound leads to downregulation of gluconeogenesis (13, 14), which represents energy export from the liver as glucose. This, in turn, reduces the hyperglycemia of type II diabetes, with secondary reduction in the hyperinsulinemia seen in this condition (15). These systemic actions may contribute to the antineoplastic effects of metformin seen in some

in vivo models (10, 16). Cell autonomous actions, such as those described in this paper, may also play critical roles in antineoplastic actions of biguanides, provided adequate drug concentrations are achieved *in vivo*.

Glucose and glutamine are major metabolic substrates for cancer cells, providing a carbon source for generating ATP. Glutamine also provides precursors for the synthesis of nucleic acids, proteins, and lipids by replenishment of the tricarboxylic acid (TCA) cycle intermediates via anaplerosis (17, 18). Oncogenic levels of *myc* induce a transcriptional program that increases glutamine consumption and reliance on glutamine as a bioenergetic substrate (19, 20).

Some pharmacoepidemiologic studies have suggested that metformin may have antineoplastic activity (21–27) but others have not supported this view (28–31). Similarly, some (10) but not all (32) laboratory studies suggest that metformin has antineoplastic activity. It is possible that these inconsistencies may result from unrecognized genetic and/or metabolic characteristics of the tumor or the host that determine metformin sensitivity. Prior work has identified host insulin levels (10), LKB1 status (10), and expression of transport molecules for metformin uptake (33) as candidate predictors of metformin sensitivity. Here, we examined carbon source and *myc* expression as candidate predictive markers for antineoplastic activity of metformin.

Materials and Methods

Chemicals

Cell culture materials were obtained from Invitrogen. Metformin (1, 1-Dimethylbiguanide hydrochloride), glucose, oligomycin, and myxothiazol were purchased from Sigma-Aldrich. L-glutamine, dialyzed FBS, and Dulbecco's Modified Eagle's

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Medium (DMEM) without D-glucose, sodium pyruvate, and L-glutamine were purchased from Wisent. HEPES was purchased from EMD chemicals. Anti-phospho-mTOR (Ser²⁴⁴⁸), anti-phospho-S6 ribosomal protein (S6RP; Ser^{235/236}) and anti- β -actin were purchased from Cell Signaling Technology. Horseradish peroxidase-conjugated anti-rabbit IgG, anti-mouse IgG, and enhanced chemiluminescence (ECL) reagents were from Pharmacia-Amersham.

Cell lines and culture conditions

MC38 colon carcinoma, a mouse tumor cell line derived from a C57BL/6 mouse was generously donated by Dr. Pnina Brodt (McGill University, Montreal, Quebec, Canada). TGR1 rat fibroblasts, isogenic HO15.19 cells with targeted disruption of both alleles of *c-Myc*, and HOMyc cells in which *c-Myc* expression has been restored in the knockout cells (34) were kindly provided by Dr. John Sedivy (Brown University, Providence, RI) and maintained with DMEM supplemented with 10% FBS and 100 U/mL gentamycin at 37°C and 5% CO₂. The mouse embryonic fibroblasts were generously donated by Dr. Wilson Miller (McGill University) and maintained in DMEM supplemented with 10% FBS and 100 U/mL gentamycin at 37°C and 5% CO₂. The cell line P493-6 (generously donated by Dr. Chi Van Dang (Abraham Cancer Center, University of Pennsylvania, Philadelphia, PA) was established by stable transfection of EREB2-5 cells with the construct *pmyc-tet* (35, 36). Cells were grown in RPMI-1640 medium supplemented with 10% FBS, 100 U/mL gentamycin, 2 mmol/L L-glutamine (Wisent). For repression of *myc*, 0.1 μ g/mL tetracycline was added to the culture medium. To reinduce *myc*, cells were washed 3 times with tetracycline-free, prewarmed phosphate-buffered saline (PBS) containing 10% FBS (37). Uninduced cells (0 hours), which were arrested by tetracycline for 72 hours, served as a control. The control cells were washed similarly to the induced cells, except that tetracycline was present in all washing solutions. All experiments were carried out in the absence of EBNA2 function (without estrogen; ref. 36).

Cell treatments

Cells were plated in 96-well plate (3,500 cells/well), 12-well plate (10⁵ cells/well), 6-well plate (3 \times 10⁵ cells/well), or in 10-cm petri dish (1.5 \times 10⁶ cells) for 24 hours. Medium was changed for fresh medium containing either glucose or glutamine (20 or 5 mmol/L) or both in the absence or presence of metformin (5 mmol/L) for another 12, 48, or 72 hours depending on the experiment. After treatment, supernatant was collected and stored at -80°C until use and cells were trypsinized and counted (Trypan blue exclusion) for extraction or colorimetric assays. Other treatments included increasing concentrations of carbon source with or without metformin. All experiments were carried out at least twice and each condition was in triplicates or 4 replicates.

Cell proliferation assay

We used the MTT (Sigma Chemical) assay to determine the effects of various carbon sources and metformin on cell growth (cell viability). After appropriate treatment time

(48 hours), MTT was added to a final concentration of 1 mg/mL, and the reaction mixture was incubated for 3 hours at 37°C. The resulting crystals were dissolved in 0.04% HCl in isopropanol and the absorbance was read at 562 nm. Triplicates were used for each treatment, and each experiment was repeated twice. Cell proliferation in presence of various concentrations of glucose and glutamine was also evaluated by 0.4% Trypan blue exclusion cell counting. Duplicates were used for each condition and the experiment was repeated 3 times (38). A Bromodeoxyuridine Assay Kit (Calbiochem) was also used to assess proliferation. The kit was used as per the manufacturer's instructions, with 2 \times 10⁵ cells per well.

ATP measurement

Cellular ATP levels were measured using the Invitrogen ATP Determination Kit A22066, (Invitrogen). Cells were treated in 5% FBS DMEM in the absence or presence metformin and under various substrate conditions, for 48 hours. The kit was used as per the manufacturer's instructions with 2 \times 10⁵ cells per well. Measurements were done in triplicate.

Measurements of glucose consumption

Cells were cultured in complete medium with 10% FBS. After 24 hours, the complete medium was replaced with test medium in the absence or presence of metformin. Cells were incubated for 48 hours and the culture medium was then collected and analyzed for measurement of glucose and lactate concentrations using colorimetric kits according to manufacturer's instructions. Glucose levels were determined using a Glucose assay kit (Eton Bioscience, Inc.). Results were indexed to cell-free media and to the number of cells.

Lactate production assay

Lactate levels were determined in 10 μ L culture medium collected from treated cells and results were standardized with the number of cells. Lactate was calculated using a Lactate Kit (BioVision, Inc.).

Cellular respiration

Cells were rinsed, trypsinized, and spun twice at 1,200 rpm for 5 minutes, the final pellet was resuspended in DMEM, 5% dialyzed FBS, 25 mmol/L HEPES with either 20 mmol/L glucose or glutamine. Oxygen consumption was measured by a Clarke type electrode (Rank Brothers) using 4 \times 10⁶ cells/mL at 37°C. Total respiration was assessed without the presence of any inhibitors. Nonmitochondrial respiration was measured using myxothiazol (12 μ g/1 \times 10⁶ cells) and subtracted from the baseline respiration rate.

NAD⁺/NADH quantification

Cellular NAD⁺ and NADH levels were measured using the NAD⁺/NADH Quantification Kit, (BioVision). Cells were treated in 5% FBS DMEM in the absence or presence of metformin and under various substrate conditions for 48 hours. The kit was used as per the manufacturer's instructions with 2 \times 10⁵ cells per well. Measurements were done in triplicate.

Protein extraction and Western blot analysis

Cells were washed 3 times with ice-cold PBS and lysed in 100 to 400 μ L lysis buffer [20 mmol/L Tris HCl (pH 7.5)], 150 mmol/L NaCl, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β -glycerol phosphate, 1 mmol/L Na_3VO_4 , 1 mmol/L EGTA, 1% Triton, and Complete Protease Inhibitor Cocktail Tablet from Roche Diagnostic. Cell debris were removed by centrifugation at $14,000\times$ rpm for 20 minutes at 4°C . Following the assay for total protein (Bio-Rad), clarified protein lysates from each experimental condition (40–50 μ g) were boiled for 5 minutes and subjected to electrophoresis in denaturing 10% SDS-PAGE. Separated proteins were transferred to a nitrocellulose membrane and after blocking, the membranes were probed with antibodies of interest. In some cases, developed blots were stripped in stripping buffer [62 mmol/L Tris HCl (pH 6.8), 100 mmol/L β -mercaptoethanol, 2% SDS] to confirm equal protein loading. Horseradish peroxidase-conjugated anti-rabbit IgG and anti-mouse IgG were used as secondary antibodies. The position of proteins was visualized using the enhanced chemiluminescence reagent ECL.

Statistical analysis

Data are presented as mean \pm SEM. The distribution of variables was tested for normality. The significance of differences between paired or unpaired sets of values was assessed using GLM or Mixed Procedures. Two-way and one-way analysis of variance (ANOVA) was used to determine the effects of different variables. In addition, Least-squares means *post hoc* for multiple unpaired comparisons of means (LSMEANS statement with Bonferroni correction) was applied. All statistical analyses were conducted using Statistical Analysis System software, version 9.2 (SAS Institute), with P values < 0.05 considered significant.

Results and Discussion

Carbon source influences the antiproliferative effect of metformin

We measured proliferation of MC38 colon carcinoma cells grown in increasing concentrations of glucose, glutamine, or both glucose and glutamine. Proliferation was considerably higher when media contained glutamine as compared to growth under the glucose-only condition (proliferation in 20 mmol/L glutamine, 0.71 ± 0.04 arbitrary units (AU) vs. proliferation in 20 mmol/L glucose, 0.22 ± 0.08 AU, $P < 0.0001$; ref. Fig. 1A). Following 48-hour exposure, we observed that metformin (5 mmol/L) had no significant inhibitory activity for cells growing in glucose, but inhibited cells using glutamine as a carbon source (inhibition by metformin in glucose containing media, 7% vs. inhibition by metformin in glutamine-only media, 78%, $P < 0.0001$; Fig. 1B). Furthermore, increasing concentrations of glutamine in the absence of glucose did not diminish the inhibitory effects of metformin, as shown in Fig. 1B. When both glucose and glutamine were present, the inhibitory activity of metformin was intermediate between the glucose-only and glutamine-only conditions, but increasing glucose concentrations did attenuate the metformin effect (inhibition by metformin in glutamine containing media, 78%

vs. inhibition by metformin in glutamine and glucose-containing media, 20%, $P = 0.0007$).

To confirm these results, which were determined by MTT assay, we counted Trypan blue excluding MC38 cells (Fig. 1C), and the same trends were observed (inhibition by metformin in 20 mmol/L glucose, 12% vs. inhibition by metformin in 20 mmol/L glutamine, 89%, $P < 0.0001$). Furthermore, similar effects of metformin were seen with the Trypan blue exclusion assay when glucose and glutamine were provided at physiologic concentrations (5 mmol/L) rather than those conventionally used in tissue culture media (20 mmol/L; results not shown). To further confirm and establish the generality of this observation, mouse embryonic fibroblasts were studied using bromodeoxyuridine (BrdUrd) labeling and physiologic glucose and glutamine concentrations (Fig. 1D). Again, metformin was a better growth inhibitor in media with 5 mmol/L glutamine than media with 5 mmol/L glucose not only in terms of absolute decrease in viable cells, but also in terms of percentage growth inhibition relative to control (inhibition by metformin in glucose containing media, 0% vs. inhibition by metformin in glutamine-only media, 94%, $P < 0.0001$).

Effect of metformin on mTOR signaling as a function of carbon source

Our previous findings showed that *in vitro*, metformin, like rapamycin, decreases mTOR phosphorylation and prevents phosphorylation of S6K1 (8, 10, 39), consistent with the previously described role of AMPK activation as an inhibitor of mTOR (7). In our MC38 cell experimental system, glutamine availability was associated with increased mTOR activation, as expected (40). While metformin attenuated mTOR activity when cells were grown in glucose alone or both nutrients to a slight degree, it drastically repressed mTOR activity when cells were grown in glutamine. Similarly, metformin inhibited phosphorylation of S6RP, a downstream effector of mTOR, in cells provided with glutamine, as shown in Fig. 2.

Effect of metformin on ATP level, glucose consumption, lactate production, and NAD⁺/NADH ratio as a function of carbon source

Having established that metformin is a more effective growth inhibitor when MC38 cells use glutamine rather than glucose as a fuel, we examined additional metabolic endpoints. Figure 3A shows a significant drop ($\sim 75\%$) in ATP level when cells in glutamine-only media were exposed to metformin, whereas a metformin-induced decrease in ATP level was not observed when glucose was available. As metformin inhibits mitochondrial ATP production (2), this is in keeping with the fact that the use of glutamine to generate ATP is more dependent on functional mitochondria than the use of glucose, because glucose can provide ATP via glycolysis or oxidative phosphorylation, but generation of ATP from glutamine requires the TCA cycle and oxidative phosphorylation.

Glucose consumption ($P < 0.0001$) and lactate production increased ($P < 0.0001$; Fig. 3B and C) when MC38 cells with access to glucose were exposed to metformin in the presence or absence of glutamine. This is consistent with a decrease in

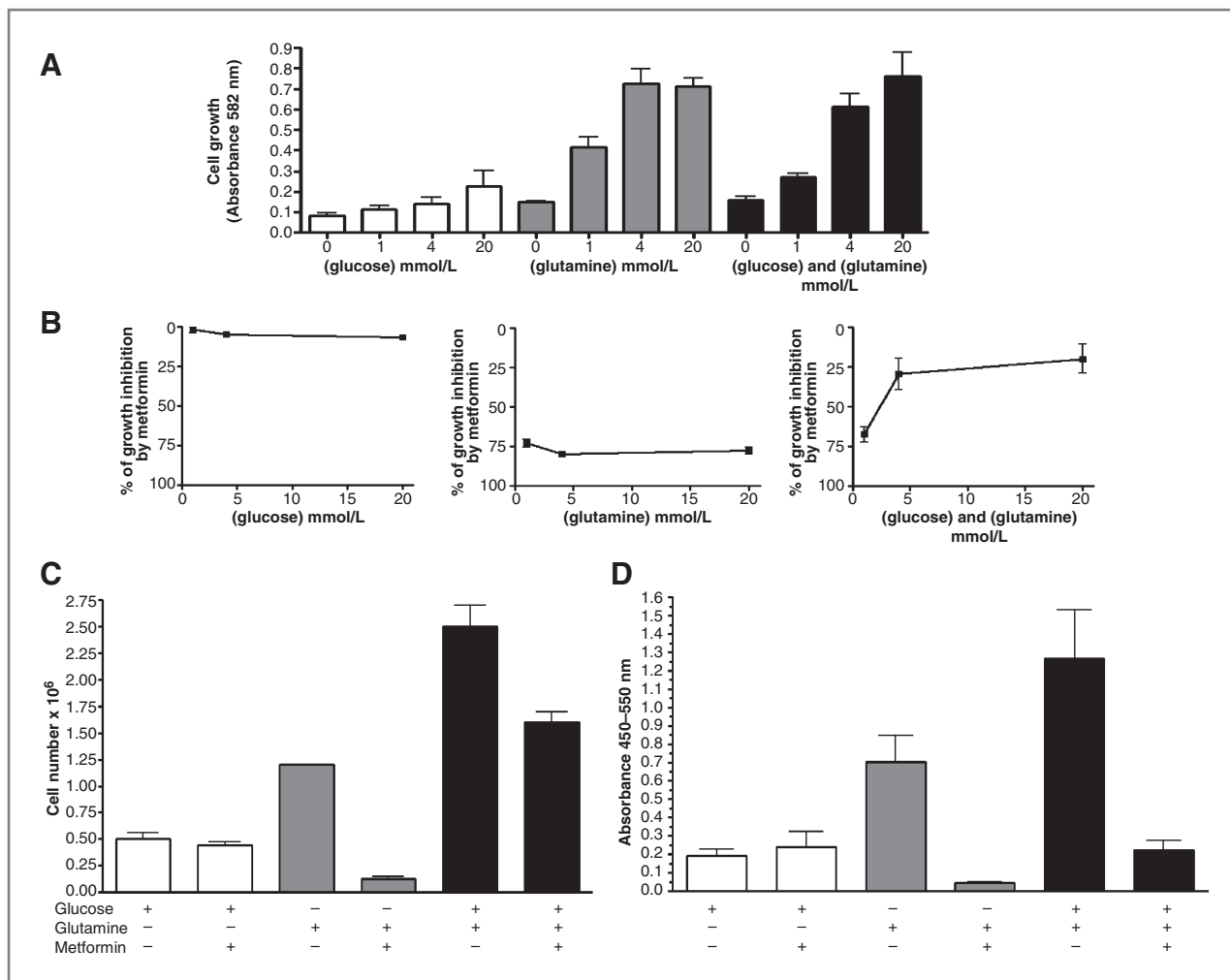


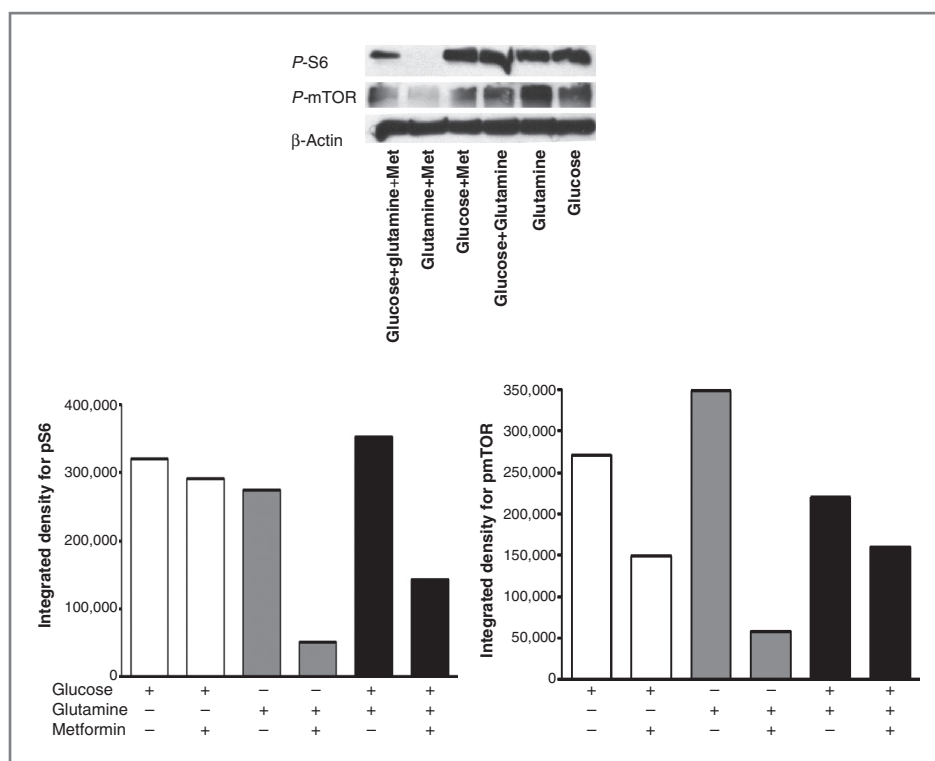
Figure 1. Carbon source influences the antiproliferative effect of metformin. **A**, effect of carbon source on proliferation. MC38 colon cancer cells were cultured in increasing concentrations of glucose, glutamine, or both for 48 hours. Cell growth in each well was measured by MTT. Proliferation was higher when media contained either glutamine or both nutrients as compared with growth under the glucose-only condition (proliferation in glutamine 20 mmol/L, 0.71 ± 0.04 AU vs. proliferation in glucose 20 mmol/L, 0.22 ± 0.08 AU, $P < 0.0001$). Data are presented as mean \pm SE from 3 independent experiments carried out in triplicate. **B**, growth inhibition induced by metformin (5 mmol/L) as a function of concentration of carbon source. MC38 colon cancer cells were cultured in increasing concentrations of glucose, glutamine, or both in the presence or absence of metformin for 48 hours. Growth inhibition varied with carbon source (inhibition by metformin in glucose 20 mmol/L, 7% vs. inhibition by metformin in glutamine 20 mmol/L, 78%, $P < 0.0001$). Increasing concentration of glutamine in the absence of glucose did not diminish the inhibitory effect of metformin. **C**, effect of metformin on cell number. MC38 colon cancer cells were cultured in 20 mmol/L glucose, 20 mmol/L glutamine or both in the presence or absence of metformin (5 mmol/L) for 48 hours. Cell growth in each condition was measured by counting Trypan blue, excluding cells. **D**, mouse embryonic fibroblasts were exposed to 5 mmol/L glucose, 5 mmol/L glutamine or both in the presence or absence of metformin for 48 hours. Cell growth in each condition was measured by BrdUrd labeling. Metformin was a better growth inhibitor in glutamine-only media than glucose-only media (inhibition by metformin in glucose containing media, 0% vs. inhibition by metformin in glutamine containing media, 94%, $P < 0.0001$).

oxidative phosphorylation and an increase in glycolysis. Interestingly, when both glutamine and glucose are available, in either the presence or absence of metformin, cells consume less glucose than in the glucose-only condition (glucose consumption with both glutamine and glucose, 8.59 ± 0.02 mmol/L/ 10^6 cells/48 hours vs. the glucose alone condition, 18.55 ± 0.11 mmol/L/ 10^6 cells/48 hours, $P = 0.0002$; both glutamine and glucose in the presence of metformin, 22.14 ± 0.05 mmol/L/ 10^6 cells/48 hours vs. glucose alone in the presence of metformin, 31.83 ± 0.25 mmol/L/ 10^6 cells/48 hours, $P < 0.0001$), consistent with

a contribution of glutamine to ATP generation via anapleurosis.

Figure 3C shows higher lactate production in the presence or absence of metformin when glutamine is excluded from media, consistent with increased glycolysis under such conditions. Metformin exposure was associated with increased lactate production in both glucose-only and glucose plus glutamine media. Maximal lactate production was seen in the absence of glutamine and the presence of metformin. Lactate was not detected when cells were provided with glutamine in either the presence or absence of

Figure 2. Effect of metformin on mTOR signaling as a function of carbon source. Western blot analysis of MC38 colon cancer cells exposed to 20 mmol/L glucose, 20 mmol/L glutamine, or both in the presence or absence of metformin (5 mmol/L) for 48 hours using antibodies against P-S6RP (Ser^{235/236}) and P-mTOR (Ser²⁴⁴⁸) is shown. Results were quantified by densitometry.



metformin, indicating that in these cells glutamine is not converted into pyruvate and then to malate to produce lactate.

When both glutamine and glucose are present, lactate production is substantially less than in the glucose-only condition. This is observed in both the presence or absence of metformin (lactate production in media with both glucose and glutamine, 10.08 ± 0.08 nmol/ 10^6 cells/48 hours vs. glucose-only media, 39.24 ± 2.78 nmol/ 10^6 cells/48 hours, $P = 0.0003$; media with both glucose and glutamine in the presence of metformin, 24.34 ± 0.42 nmol/ 10^6 cells/48 hours vs. glucose-only media in the presence of metformin, 56.96 ± 0.61 nmol/ 10^6 cells/48 hours, $P = 0.0005$). This provides further evidence that glutamine availability leads to reduced glycolysis.

A metformin-induced decline in the NAD⁺/NADH ratio was only observed in the absence of glucose (41.06% decline in NAD⁺/NADH ratio in the glutamine-only condition, $P = 0.0045$ vs. a 0% decline in conditions where glucose was available; Fig. 3D). This is in keeping with the inhibition of complex I by metformin, with accumulation of NADH. This effect of metformin is attenuated in the presence of glucose; under these conditions, increased lactic acid production by lactate dehydrogenase (LDH) consumes NADH but further analysis will be of interest as the increased glycolysis induced by metformin would also be expected to increase NADH generation by glyceraldehyde-3-phosphate dehydrogenase. In the absence of glucose, no lactic acid is produced, indicating minimal reductive metabolism of pyruvate to lactate by LDH, with reduced generation of NAD⁺.

***myc* overexpression leads to sensitization to metformin**

In the context of prior evidence that glutamine uptake and metabolism are increased by *myc* (41) and our observation that glutamine use sensitizes cells to metformin, we examined the influence of *myc* on metformin action. We used TGR-1 Rat1 fibroblasts expressing physiologic levels of *myc* (designated Myc +/+), the isogenic *myc* null HO15.19 cell line (designated Myc -/-), and the isogenic HOmyc cell line, which overexpressed *myc* (designated Myc +++). The highest proliferation was seen in the Myc +++ cell line using glutamine as a carbon source, consistent with evidence that oncogenic levels of *myc* lead to glutamine addiction (ref. 40; Fig. 4A).

We observed that as *myc* levels decrease, proliferation when glutamine was the carbon source also declined (Myc +++, 1.50×10^6 vs. Myc +/+, 0.97×10^6 , $P = 0.0049$), whereas *myc* level had no effect on proliferation when glucose was the carbon source. While *myc* has been reported to increase glycolysis and LDH expression (42) as well as glutamine use (40), this observation suggests that functionally, the effect of *myc* on glutamine use is dominant.

To better understand the relation between *myc* expression and metformin inhibition, a more physiologic replete media consisting of 5 mmol/L glucose and 5 mmol/L glutamine was used and *myc* expression was varied (Fig. 4B). BrdUrd incorporation measurements revealed that even in media where both glucose and glutamine are available, metformin reduced proliferation to the greatest degree when *myc* was overexpressed (% inhibition for Myc +++ cells, 62%, $P < 0.0001$, %

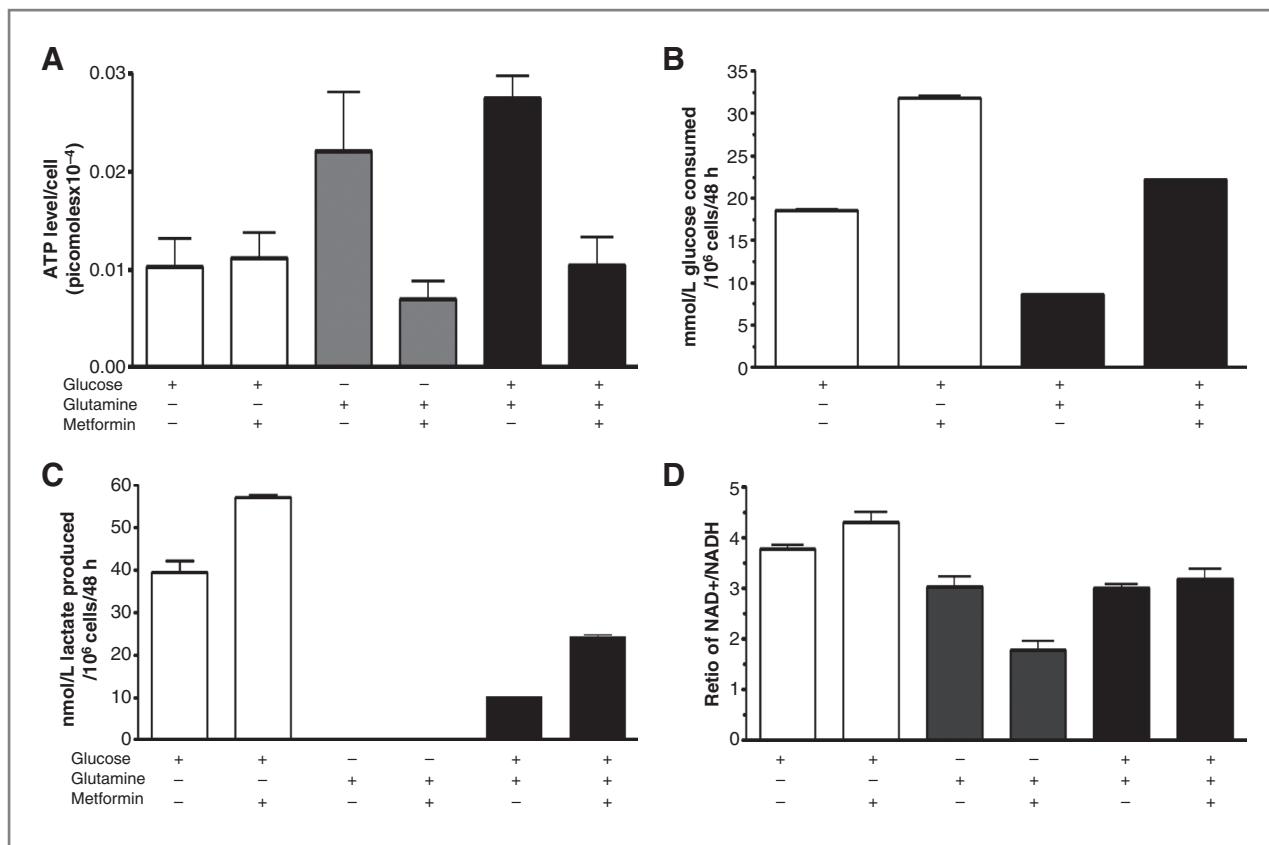


Figure 3. Effect of metformin on ATP level, glucose consumption, lactate production, and NAD⁺/NADH ratio as a function of carbon source. MC38 colon cancer cells were cultured in 20 mmol/L glucose, 20 mmol/L glutamine, or both in the presence or absence of metformin (5 mmol/L) for 48 hours. A, ATP levels. Exposure to metformin (5 mmol/L) resulted in a decrease (75%) in ATP level in the glutamine-only condition, whereas this decline in ATP level was not observed in the glucose-only condition. B, glucose consumption. Metformin exposure also led to an increase in glucose consumption consistent with an increase in glycolysis. Less glucose was consumed when cells were exposed to both nutrients in the presence or absence of metformin ($P < 0.0001$). C, lactate production. Metformin exposure resulted in an increase in lactate production when cells were either grown on glucose or both nutrients ($P < 0.0001$). Lactate was not detected when cells were exposed to glutamine either in the presence or absence of metformin. D, NAD⁺/NADH ratios. Metformin exposure resulted in a 41.06% decrease ($P = 0.0045$) in NAD⁺/NADH ratio. This decline was not observed in glucose-only media or media containing both nutrients.

inhibition for Myc +/+ cells, 14% $P = NS$). The fact that metformin decreased proliferation in *myc*-overexpressing cells growing in replete media and glutamine-only media, but not in the glucose-only media, suggests that when *myc* is overexpressed, the inhibitory effects of metformin are related to glutamine metabolism.

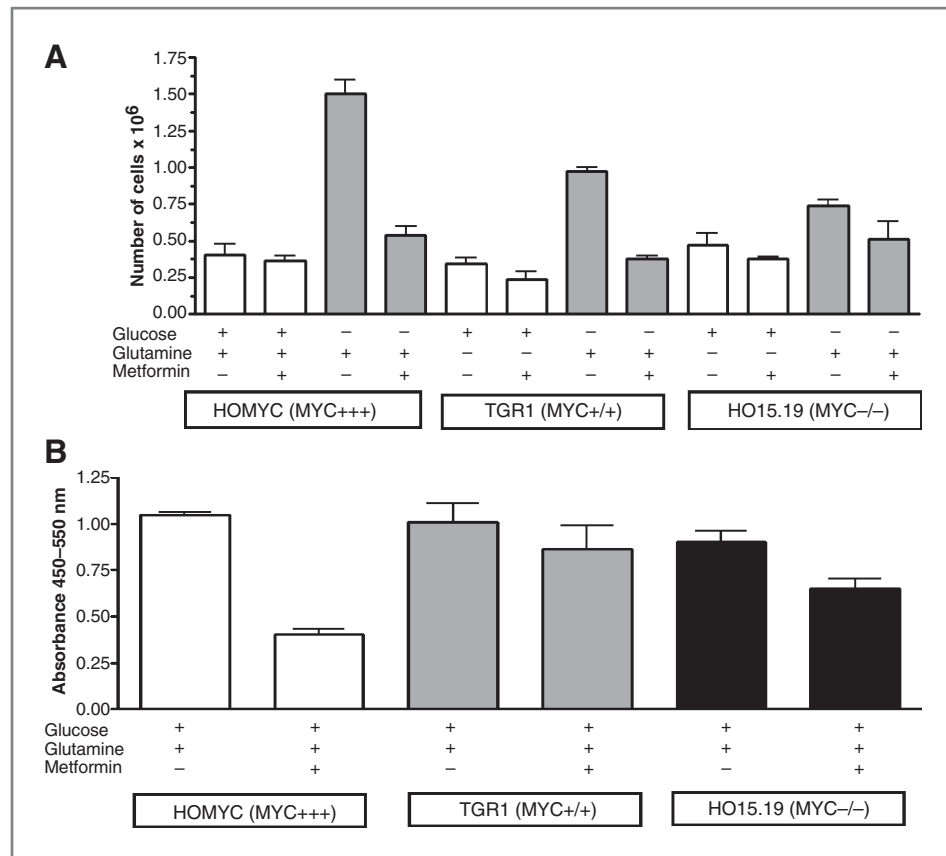
Effect of metformin on glucose consumption and lactate production as a function of carbon source and *myc* expression

Previous reports have shown that *c-myc* upregulates the expression of glucose transporter 1 (GLUT1), phosphofructokinase, and enolase A as well as directly regulating LDH-A (35, 37, 43, 44). We therefore carried out experiments to determine if varying *myc* expression influences the effects of metformin on glucose consumption and lactate production. Figure 5A shows that metformin increases glucose consumption when cells were grown in media without glutamine, and that the amount of glucose consumed on metformin exposure varied significantly with *myc* expression level (glu-

ose consumed in glucose-only media on metformin exposure for Myc +/+ cells, 27.43 mmol/L/10⁶ cells/48 hours vs. for Myc +/+ cells, 10.54 mmol/L/10⁶ cells/48 hours vs. for Myc -/- cells, 2.67 mmol/L/10⁶ cells/48 hours; $P < 0.0001$ for all comparisons). *myc* expression was associated with both higher fold increase in glucose consumption (~3-fold) and higher absolute glucose consumption levels on metformin exposure (~1.5-fold). Similar trends were seen in the amount of lactate produced (Fig. 5B). The high amount of lactate released in conditions where glucose was the only carbon source available indicates that a considerable amount of pyruvate generated from glucose through glycolysis does not support oxidative phosphorylation but is instead reduced to lactate.

In cell lines expressing *myc*, in the presence of both glucose and glutamine, the increase in glucose consumption on metformin exposure was not as marked as in the absence of glutamine (% increase in glucose consumed on metformin exposure in glucose and glutamine, 69% vs. glucose-only, 300%) in keeping with some residual glutamine use for ATP production despite the presence of metformin.

Figure 4. *myc* overexpression leads to sensitization to metformin. A, isogenic HOmyc Rat1 fibroblast cells expressing high levels of *myc* (Myc +++ cells), TGR1 cells expressing physiologic levels of *myc* (Myc +/- cells), and HO15.19 cells in which *myc* was knocked (Myc -/- cells) out were grown with glucose and/or glutamine (20 mmol/L) in the presence or absence of metformin for 48 hours. Cell number was determined by Trypan Blue exclusion. As *myc* levels decrease, proliferation when glutamine was the carbon source also declined (Myc +++, 1.50×10^6 cells vs. Myc +/-, 9.7×10^5 cells, $P = 0.0049$), whereas *myc* had no effect on proliferation when glucose was the carbon source. B, Rat1 fibroblasts exposed to replete media consisting of 5 mmol/L glucose and 5 mmol/L glutamine in the presence or absence of metformin (5 mmol/L) for 48 hours. Cell proliferation measured by BrdUrd labeling. Metformin reduced growth to the greatest degree when *myc* was overexpressed (% inhibition of Myc +++ cells, 62%, $P < 0.0001$, % inhibition of Myc +/- cells, 14%, $P =$ not significant).



Effect of metformin on ATP level and oxygen consumption as a function of carbon source and *myc* expression

We observed higher baseline ATP concentrations when MC38 cells were grown on glutamine as compared with glucose, independent of *myc* status (~3-fold higher in glutamine compared with glucose in Myc +++ and 2.25-fold higher in Myc -/-; Fig. 6A). As discussed previously, a substantial amount of lactate is released from cells when glucose is the only carbon source (Fig. 5B). Thus, much of the pyruvate generated from glucose by glycolysis does not contribute to energy generated by oxidative phosphorylation. However, when cells are provided with only glutamine, no lactate is produced and glutamine carbon is all available for oxidative phosphorylation, explaining the high amounts of ATP and oxygen consumption (see below) generated in this condition.

Previous reports have showed that biguanides reduce mitochondrial ATP production (1, 2). Following 48 hours metformin exposure (5 mmol/L), we observed a substantial decrease (~95%) in cellular ATP levels when cells were grown in glutamine-only media (20 mmol/L), regardless of *myc* expression, as shown in Fig. 6A. In contrast, little decrease in ATP level was detected when cells were grown on glucose-only media (20 mmol/L), ~25% decrease in Myc +++ cells and no decrease in Myc -/- cells. These results suggest that increased ATP production via glycolysis represents a mechanism that partially compensates for the metformin-associated decrease

in ATP production by oxidative phosphorylation, and that this mechanism operates when cells have access to glucose.

To better understand the relevance of carbon source to oxidative phosphorylation as a function of *myc* status and metformin exposure, oxygen consumption was measured as *myc* status and carbon source were varied in the presence or absence of metformin. Figure 6B shows that the basal level of oxygen consumption was consistently higher when cells were using glutamine rather than glucose as a carbon source provided *myc* was expressed (Myc -/- cells, 1.3-fold $P =$ NS; Myc +/- cells, 1.8-fold $P = 0.0097$; Myc +++ cells, 1.8-fold $P = 0.0011$). The observation that lactate was not released when cells were grown on glutamine, yet oxygen consumption and ATP levels remained high, suggests that when glutamine is the only carbon source provided, cells rely on oxidative phosphorylation to supply their energy needs. When cells were grown in glucose-only media, metformin decreased oxygen consumption ($P < 0.0001$) but this had minimal effect on ATP levels, in keeping with a high contribution of glycolysis to ATP generation. This is also in keeping with the metformin induced increase in glucose consumption and lactate production under these conditions. Cells in glutamine-only media also displayed decreased oxygen consumption on metformin exposure, in keeping with an important decrease in ATP production by oxidative phosphorylation, but in this case an increase in glycolysis is not possible and a major decline in ATP is observed. This supports the hypothesis that ATP

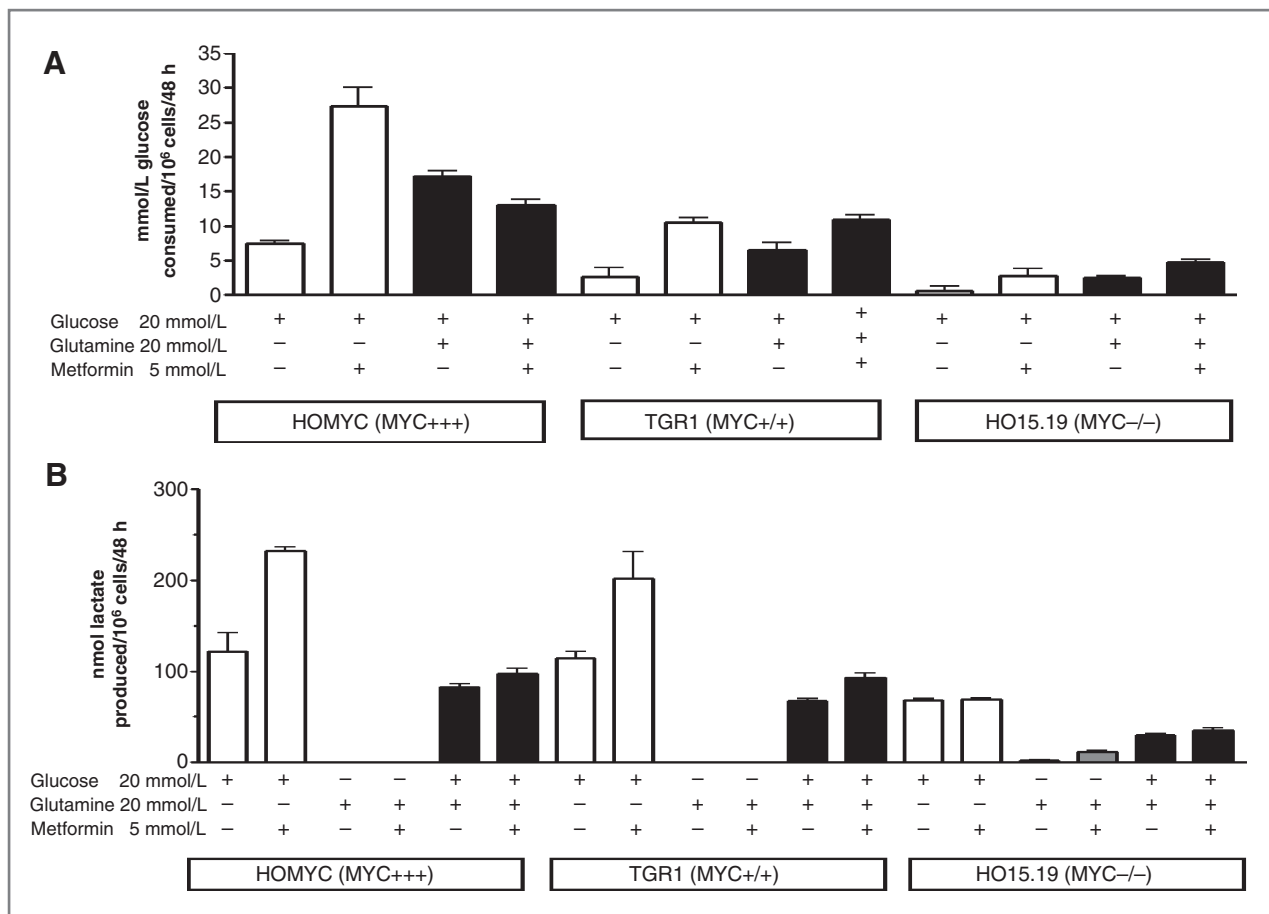


Figure 5. Effect of metformin on glucose consumption and lactate production as a function of carbon source and *myc* expression. Isogenic HOMyc Rat1 fibroblast cells expressing high levels of *myc* (Myc +++ cells), TGR1 cells expressing physiologic levels of *myc* (Myc +/+ cells), and HO15.19 cells in which *myc* was knocked out (Myc -/- cells) were grown in glucose (20 mmol/L) and/or glutamine (20 mmol/L) in the presence or absence of metformin for 48 hours. A, metformin increased glucose consumption regardless of *myc* level when cells were grown in media without glutamine. The amount of glucose consumed on metformin exposure varied significantly according to *myc* expression (glucose consumed in the glucose-only condition with metformin exposure by Myc +++ cells, 27.43 mmol/L/10⁶ cells/48 hours vs. Myc +/+ cells, 10.54 mmol/L/10⁶ cells/48 hours vs. Myc -/- cells, 2.67 mmol/L/10⁶ cells/48 hours; $P < 0.0001$ for all differences according to *myc* state). B, similar trends were observed in the amount of lactate produced regardless of carbon source (lactate produced in the glucose-only condition with metformin exposure by Myc +++ cells, 66.32 nmol/10⁶ cells/48 hours vs. Myc +/+ cells, 57.64 nmol/10⁶ cells/48 hours vs. Myc -/- cells, 19.67 nmol/10⁶ cells/48 hours).

provided by glycolysis protects against metformin-induced energy stress, consistent with a prior report showing enhanced ATP depletion when metformin was combined with 2-deoxyglucose, a glycolysis inhibitor (45). The lowest oxygen consumption was observed in cells that had glucose available for glycolysis, and overexpressed *myc*, with metformin exposure. This is plausible as these conditions favor maximal glycolysis.

Metformin sensitization in a tetracycline repressible *myc* expression model

Finally, to further investigate the relationship between *myc* expression and the antiproliferative effect of metformin, we used the cell line P493-6 carrying a conditional, tetracycline-regulated *myc* (36). Figure 7 shows that in the presence of both glucose and glutamine at physiologic concentrations (5 mmol/L), the cells overexpressing the *myc* oncogene (tetra-

cycline absent) were significantly inhibited by metformin (37% inhibition, $P = 0.02$), whereas cells with low levels of *myc* (tetracycline present) were not affected, consistent with our prior observation that high levels of *myc* expression sensitize cells to metformin inhibition.

There is increasing interest in developing cancer therapies that target energy metabolism as compared with current drugs that target signal transduction or function as cytotoxic agents. Many strategies have been proposed (46) and metformin, widely used in treatment of diabetes, as well as other biguanides, deserve investigation in this context (9). There may be specific situations where metformin-induced decline in circulating insulin level leads to antineoplastic effects, but this may be restricted to patients who are hyperinsulinemic at baseline, as small variations of insulin levels within the normal range are of uncertain biologic significance. The present study provides novel information concerning separate

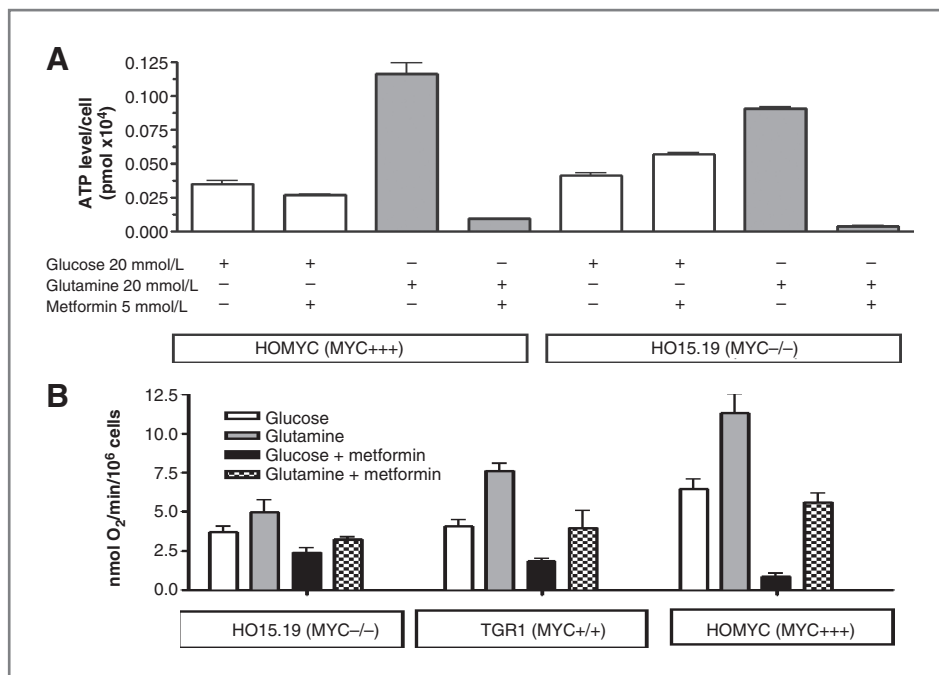


Figure 6. Effect of metformin on ATP level and oxygen consumption as a function of carbon source and *myc* expression. Isogenic HOmyc Rat1 fibroblast cells expressing high levels of *myc* (Myc +/+), TGR1 cells expressing physiologic levels of *myc* (Myc +/-), and HO15.19 cells in which *myc* was knocked out (Myc -/-) were grown with glucose and/or glutamine (20 mmol/L) in the presence or absence of metformin (5 mmol/L) for 48 hours. **A**, ATP level. Higher baseline ATP concentrations were observed when cells were grown on glutamine as compared with glucose, independent of *myc* status (~3-fold higher in glutamine compared with glucose for Myc +/+ cells and 2.25-fold higher for Myc -/- cells). Metformin exposure resulted in a decrease (95%) in cellular ATP levels when cells were grown on glutamine-only media. In contrast, little or no decrease in ATP level was detected when cells were grown in glucose-only media (~25% decrease in Myc +/+ cells and no decrease in Myc -/- cells). **B**, oxygen consumption. The basal level of oxygen consumed was consistently higher when glutamine, as compared with glucose, was the carbon source, provided *myc* was expressed (Myc -/- cells, 1.3-fold $P = \text{NS}$; Myc +/- cells, 1.8-fold $P = 0.0097$; Myc +/+ cells, 1.8-fold $P = 0.0011$). Metformin significantly inhibited oxygen consumption in either nutrient condition regardless of *myc* expression level.

cell-autonomous effects of biguanides that may contribute to antineoplastic activity.

Certain proposed metabolic therapies for cancer seek to downregulate the Warburg phenomenon (46), which is characterized by rapid proliferation in the setting of increased glycolysis and lactate production, regardless of oxygen availability. Metformin and similar compounds paradoxically fur-

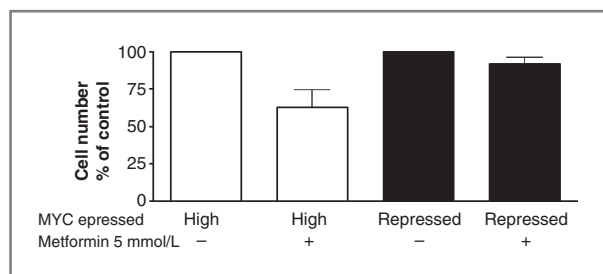


Figure 7. Metformin sensitization in a tetracycline repressible *myc* expression model. P493-6 Burkitt lymphoma cells carrying a conditional, tetracycline-regulated *myc* were exposed to replete media consisting of 5 mmol/L glucose and 5 mmol/L glutamine in the presence or absence of metformin (5 mmol/L). Cells overexpressing *myc* (tetracycline absent) were inhibited by metformin (37% inhibition, $P = 0.02$), whereas cells with low levels of *myc* (tetracycline present) were not affected.

ther increase the substantial rates of glycolysis and lactate production in cancer cells, but inhibit their proliferation. Our data suggest that the increase in glycolysis represents compensation to the metformin-induced decrease in oxidative phosphorylation. This attenuates but does not eliminate metformin-induced energy stress. It is of interest to contrast the metabolic effects of metformin to those recently described with PTEN overexpression. PTEN elevation decreased glycolysis and increased oxidative phosphorylation, resulting in reduced cell proliferation (47), whereas metformin increases glycolysis and reduces oxidative phosphorylation but also leads to a decline in proliferation.

Oxidation of glutamine carbon in the mitochondria is a major source of energy for proliferating cells (48, 49). Our findings, using various experimental systems, show that overexpression of *myc*, previously shown to be associated with increased glutamine use (40), increases sensitivity to metformin. This finding identifies *myc* overexpressing tumors as particularly attractive targets for biguanides such as metformin, either alone or in combination with pharmacologic strategies to inhibit glycolysis.

The use of drugs that induce energy stress in the treatment of those cancers that have genetic alterations that decrease tolerance to such stress is an attractive therapeutic concept. However, careful attention needs to be given to

pharmacokinetic considerations, as conventional metformin doses used in diabetes treatment may not lead to adequate tumor concentrations of metformin, particularly for those cancers that do not express the cell surface transport proteins required for drug influx (50).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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