Metformin attenuates the stimulatory effect of a high-energy diet on \textit{in vivo} LLC1 carcinoma growth

Carolyn Algire\textsuperscript{1}, Mahvash Zakikhani\textsuperscript{2}, Marie-Jose Blouin\textsuperscript{2}, Jian Hua Shuai\textsuperscript{2} and Michael Pollak\textsuperscript{2}

\textsuperscript{1}Division of Experimental Medicine and \textsuperscript{2}Department of Oncology, Segal Cancer Centre, Jewish General Hospital, McGill University, 3755 Cot Ste. Catherine, Montreal, Quebec, H3T 1E7 Canada

(Correspondence should be addressed to M Pollak; Email: michael.pollak@mcgill.ca)

Abstract

We investigated the effects of metformin on the growth of lewis lung LLC1 carcinoma in C57BL/6J mice provided with either a control diet or a high-energy diet, previously reported to lead to weight gain and systemic insulin resistance with hyperinsulinemia. Forty-eight male mice were randomized into four groups: control diet, control diet + metformin, high-energy diet, or high-energy diet + metformin. Following 8 weeks on the experimental diets, selected groups received metformin in their drinking water. Three weeks following the start of metformin treatment, mice were injected with \(0.5 \times 10^6\) LLC1 cells and tumor growth was measured for 17 days. By day 17, tumors of mice on the high-energy diet were nearly twice the volume of those of mice on the control diet. This effect of diet on tumor growth was significantly attenuated by metformin, but metformin had no effect on tumor growth of the mice on the control diet. Metformin attenuated the increased insulin receptor activation associated with the high-energy diet and also led to increased phosphorylation of AMP kinase, two actions that would be expected to decrease neoplastic proliferation. These experimental results are consistent with prior hypothesis-generating epidemiological studies that suggest that metformin may reduce cancer risk and improve cancer prognosis. Finally, these results contribute to the rationale for evaluation of the anti-neoplastic activity of metformin in hyperinsulinemic cancer patients.

Endocrine-Related Cancer (2008) 15 833–839

Introduction

Population studies provide early circumstantial evidence that patients with type II diabetes treated with metformin have reduced cancer incidence (Evans \textit{et al.} 2005) and mortality (Bowker \textit{et al.} 2006), when compared with type II diabetics receiving alternative therapies. Laboratory studies have shown that metformin, by lowering the ATP/AMP ratio, activates the LKB1/AMP kinase (AMPK) pathway (Hardie 2006) leading to inhibition of gluconeogenesis in hepatocytes (Shaw \textit{et al.} 2005). This leads to decreased hepatic glucose output and a secondary decline in insulin levels, making the drug useful in the treatment of type II diabetes. In tumor cells, the gluconeogenic enzymes found in hepatocytes are not present. However, we recently showed that metformin activates the same LKB1/AMPK pathway in cancer cells \textit{in vitro}, but that in these cells the consequences include reduced signaling at mTOR and growth inhibition (Zakikhani \textit{et al.} 2006).

We wished to extend these \textit{in vitro} results to an \textit{in vivo} model. We chose lewis lung LLC1 carcinoma model because this experimental tumor has recently been shown to exhibit particularly aggressive behavior in mice that are hyperglycemic and obese (Yakar \textit{et al.} 2006). This model therefore provides an opportunity to determine whether metformin has anti-neoplastic activity \textit{in vivo}, particularly in a metabolic context similar to that present in the subjects in the epidemiological studies mentioned above (Evans \textit{et al.} 2005, Bowker \textit{et al.} 2006).
Materials and methods

Animals

All experiments performed were approved by the McGill University Animal Care and Handling Committee. Forty-eight male C57BL/6J-mice aged 5–6 weeks (Charles River, Saint-Constant, QC, Canada) were randomized to four groups of 12 mice: control diet, control diet + metformin, high-energy diet, and high-energy diet + metformin. Food was given ad libitum. Diets were purchased from Harlan Teklad (Madison, WI, USA). The high-energy diet (TD 95217) consisted of 18.8% protein, 41.4% carbohydrate, 39.8% fat, and provided 4.3 kcal/g consumed. The control diet was the standard rodent diet, Harlan Teklad 2016, which consisted of 16% protein, 60% carbohydrate, 3.5% fat, and provided 3.3 kcal/g consumed.

Metformin

Metformin (Spectrum Chemical Corp., New Brunswick, NJ, USA) was added to the drinking water 8 weeks after initiation of the experimental diets. Metformin was administered at a dose of 50 mg/kg body weight per day. For example, a 30 g mouse would thus consume 1.5 mg of metformin per day provided as 7 ml of a 0.214 mg/ml solution. A pilot study confirmed that addition of metformin at this concentration did not effect water consumption. The water and metformin were changed daily and the dose adjusted for weight gain every 2 weeks.

C-peptide

C-peptide was measured following 11 weeks of study. Blood was taken from fasting mice and was measured by mouse C-peptide ELISA kit (Gentaur, Aachen, Germany).

Insulin tolerance test

Insulin tolerance tests were performed at noon, 11 weeks after the start of the experimental diets and 3 weeks after mice randomized to metformin had started to receive the drug (Fig. 1). Mice were injected i.p. with 0.75 U/kg insulin (Cell Sciences, Canton, MA, USA). Blood glucose concentration was measured at the indicated time points using the Ultra Smart Touch Glucometer and Ultra Smart glucose strips (Diabetesshop.ca).

Syngeneic tumor model

Mice were injected sc with $0.5 \times 10^6$ lewis lung LLC1 cells (ATCC, Manassas, VA, USA), following 11 weeks on the experimental diets and 3 weeks after start of the metformin (Fig. 1). Tumors were palpable 11 days after injection and were measured daily using electronic calipers (Lee Valley Tools, Ottawa, ON, Canada).

Protein extraction and Western blot analysis

Fat, muscle, liver, and tumor tissue were removed immediately after the mice were killed. Tissues were homogenized in 1 ml ice-cold tissue lysis buffer (20 mM HEPES buffer, 1% Triton X-100, 150 mM NaCl, 0.02% sodium azide, 60 mM β-glycerophosphate, 1 mM sodium vanadate, 1 mM dithiothreitol, 1 Roche complete protease inhibitor tablet, and 5 μl of 5 mM pepstatin A per 50 ml lysis buffer). Lysates were sonicated and cell debris was removed by centrifugation at 18,000 g for 1 h at 4 °C. Following homogenization, protein content was determined by protein assay (Bio-Rad). From each tissue sample, 100 μg of protein were boiled and subjected to electrophoresis in denaturing 10% SDS-PAGE. All primary antibodies were purchased from Cell Signaling Technologies (Danvers, MA, USA), except the insulin receptor antibody that was purchased from Chemicon (Temecula, CA, USA), and visualized using the enhanced chemiluminescence reagent ECL (Amersham).
Statistical analysis

Prior to statistical analysis, data were square-root transformed to satisfy the assumptions of analysis. Statistical significance was evaluated using the GLM Procedure. A one-way ANOVA was used to determine whether there was significant difference among all treatment groups. Additionally, Student–Newman–Keuls test for multiple pairwise comparisons of means and least-squares means multiple unpairwise comparisons of means (LSMEANS statement with Bonferroni correction) were applied. All statistical analyses were performed using Statistical Analysis System software, version 9.1.3 (SAS Institute, Cary, NC, USA), with the $P$ values ≤ 0.05 considered significant.

Results

Diet-induced weight and gain and metabolic changes

Figure 2A shows that mice consuming the high-energy diet gained more weight during the experiment than mice on the control diet (9.63 ± 1.67 g vs 7.2 ± 1.52 g, $P < 0.05$). Although metformin occasionally leads to weight loss in patients due to appetite suppression or gastrointestinal distress (Kirphichnikov et al. 2002), we did not observe a significant difference in body weight between mice randomized to receive metformin and those that did not receive metformin.

Insulin secretion was estimated by C-peptide ELISA following 11 weeks on the experimental diets (Fig. 2B). Consistent with previous studies (Venkateswaran et al. 2007), we found the high-energy diet was sufficient to significantly raise the C-peptide levels in those mice that received the high-energy diet compared with those on the control diet (high-energy diet at $1.147 ± 0.134$ ng/ml versus control diet at $0.728 ± 0.028$ ng/ml; $P < 0.05$). Interestingly, we observed that metformin treatment lowered the C-peptide levels associated with high-energy diet to a degree that approached statistical significance despite a small sample size (high-energy diet at $1.147 ± 0.134$ ng/ml versus high-energy diet + metformin at $0.999 ± 0.063$ ng/ml; $P = 0.11$). The influence of metformin on C-peptide levels was anticipated, as metformin is known to reduce gluconeogenesis and serum glucose levels that would have an indirect effect on insulin secretion. Insulin tolerance tests (Fig. 2C) showed that following the initial drop in blood glucose, those mice on the high-energy diet had significantly reduced ability to maintain a lowered blood glucose concentration 45 min after the insulin injection.
compared with those mice on the control diet (control diet blood glucose at \( t = 45 \): 3.9 ± 0.10 mmol/l versus high-energy diet blood glucose at \( t = 45 \): 7.7 ± 0.71 mmol/l; \( P < 0.0001 \); Fig. 2C). The mice on the high-energy diet with metformin had significantly improved insulin tolerance compared with those on the same high-energy diet without metformin. These animals were able to maintain a lowered blood glucose concentration at 45 min following insulin injection (high-energy diet + metformin blood glucose at \( t = 45 \): 5.47 ± 0.43 mmol/l versus high-energy diet blood glucose at \( t = 45 \): 7.7 ± 0.71 mmol/l; \( P < 0.05 \)). However, metformin did not completely reverse the effects of the high-energy diet. Metformin had no effect on the insulin tolerance of those mice on the control diet (control diet blood glucose at 45 min: 4.45 ± 0.34 mmol/l versus control diet blood glucose, 3.9 ± 0.10 mmol/l; \( P > 0.05 \)).

**Effect of diet and metformin on tumor growth**

Tumor growth curves are shown in Fig. 3. In keeping with a prior report (Yakar et al. 2006), the high-energy diet was associated with increased tumor growth (tumor size of 2056 ± 240 mm\(^3\) for high-energy diet versus 1313 ± 204 mm\(^3\) for control diet at day 17; \( P < 0.0001 \)). Metformin treatment was observed to significantly decrease tumor growth rate and tumor size in mice on the high-energy diet (tumor size of 1231 ± 207 mm\(^3\) for high-energy diet + metformin versus 2056 ± 240 mm\(^3\) for high-energy diet at day 17; \( P < 0.0001 \)). There was no statistically significant effect of metformin on tumor growth in those mice on the control diet (tumor size of 1537 ± 114 mm\(^3\) for control diet + metformin versus 1313 ± 204 mm\(^3\) for control diet at day 17; \( P > 0.05 \)).

**Metformin alters signaling in tumors**

Our prior work demonstrated that *in vitro* growth inhibition of cancer cells by metformin involves the activation of the AMPK pathway (Zakikhani et al. 2006). In order to determine whether this mechanism occurs in the *in vivo* models, we measured AMPK activation in the normal and neoplastic tissue. Metformin activated AMPK in the neoplastic tissue, as shown by western blot using a phospho-specific antibody against AMPK Thr\(^{172} \) (Fig. 4A). However, because we observed no significant effect of metformin on tumor growth for mice on the control diet, we hypothesized that metformin attenuated tumor growth through lowering of insulin levels, in addition to any effect on AMPK.

In support of this hypothesis, there was increased tyrosine phosphorylation of the insulin receptor, an indicator of insulin receptor activation, in tumor tissue from mice on the high-energy diet compared with tumor tissue from mice on the control diet (Fig. 4B). In addition, among mice on the high-energy diet, metformin administration decreased insulin receptor tyrosine phosphorylation intensity by 38%, compared with mice on the same high-energy diet without metformin. The inhibitory effect of metformin on insulin receptor phosphorylation was smaller in magnitude than the stimulatory effect of the high-energy diet (Fig. 4B). We also examined downstream signaling through insulin receptor substrate 1 (IRS-1). Under conditions of insulin resistance, serine phosphorylation of IRS-1 is increased through negative feedback regulation (Giraud et al. 2004). However, not all serine phosphorylation sites are inhibitory; the phosphorylation site 302 is known to be associated with increased insulin receptor signaling and increased nutrient availability (Giraud et al. 2004). We found elevated serine 302 phosphorylation of IRS-1 in tumors from mice on the high-energy diet compared to tumors from mice on the same high energy with metformin, as well as tumors taken from both groups on the control diet.
In the context of the finding that metformin acted as an inhibitor of tumor growth under conditions where the high-energy diet stimulated tumor growth, these results suggest that in vivo the mechanism by which metformin reduces tumor proliferation involves suppression of insulin levels and subsequent reduction of insulin signaling, rather than being entirely attributable to AMPK activation.

**Discussion**

There is increasing interest in potential roles of the biguanide metformin or other AMPK activators (Cool et al. 2006) in cancer treatment and prevention. We confirmed the results of a prior report (Yakar et al. 2006) showing that a high-energy diet is associated with more aggressive tumor behavior in the lewis lung LLC1 model. We also observed that metformin almost completely attenuated the effect of high-energy diet on tumor growth, while having no significant impact on tumor growth of animals on the control diet.

Prior experiments provide evidence that AMPK activation by metformin leads to growth inhibition of cancer cells in vitro (Zakikhani et al. 2006). We studied the in vivo effects of metformin on tumor growth and employed a model that allowed us to make observations under standard metabolic conditions and also under conditions of dietary-induced hyperinsulinemia and systemic insulin resistance. This model allowed us to study tumor growth in the context of the metabolic characteristics of metformin-treated patients with type II diabetes, where there is early epidemiologic evidence of possible anti-neoplastic activity of the drug (Evans et al. 2005, Bowker et al. 2006).

The finding that metformin attenuates the stimulatory effect of a high-energy diet on tumor growth in this model, but has no effect on baseline tumor growth is intriguing. The signaling data provide evidence that metformin at the dose and route we employed activates AMPK in neoplastic tissue in animals on both a control and a high-energy diet. However, metformin-induced growth inhibition of tumors was only observed in animals on the high-energy diet, thus suggesting that mechanisms other than APMK activation may be involved. We observed by western blotting, using a figure showing western blots for AMPK and insulin receptor signaling.

**Figure 4** Effect of diet and metformin on signaling in C57 neoplastic tissues. (A) Tumor tissue was analyzed by western blot and immunoprecipitation. We observed increased phosphorylation of AMPK at Thr172 in tumor tissue from those mice on the control diet and the high-energy diet that received metformin (relative intensity of control diet versus control diet + metformin; \( P < 0.005 \); high-energy diet versus high-energy diet + metformin; \( P < 0.05 \)). (B) For insulin receptor phosphorylation, tumor tissue was analyzed by immunoprecipitation of the insulin receptor and western blotting with an anti-phospho-tyrosine antibody. The high-energy diet (HED) was associated with increased insulin receptor activation as shown by elevated tyrosine phosphorylation of the insulin receptor in tumor tissues of mice in this group (relative intensity high-energy diet versus control diet \( P < 0.001 \)). Metformin attenuated the increased insulin receptor activation in tumors from mice on the high-energy diet (relative intensity of high-energy diet versus control diet \( P < 0.0001 \)). Metformin attenuated the increased insulin receptor activation in tumors from mice on the high-energy diet (relative intensity of high-energy diet versus high-energy diet + metformin, \( P < 0.05 \)), but not in the tissues from mice on the control diet. IRS-1 Ser 302 activity was measured using a site-specific antibody. We found elevated signaling through IRS-1 in those mice on the high-energy diet compared with that on the control diet (relative intensity high-energy diet versus control diet \( P < 0.001 \)), indicating increased insulin receptor signaling and increased nutrient availability. The increased IRS-1 signaling was attenuated in the high-energy diet group that received metformin (relative intensity high-energy diet versus high-energy diet + metformin; \( P < 0.001 \)). \( \beta \)-Actin was measured as a loading control.
specific anti-insulin receptor β-chain antibody, that insulin receptors are expressed by Lewis lung LLC1 cells. Furthermore, the tumor tissue from mice on the high-energy diet was found to have elevated insulin receptor activation as shown by increased tyrosine phosphorylation of insulin receptor, compared with tumor tissue from mice on the control diet. This occurs in the context of the known association of elevated insulin levels with obesity and excess caloric intake (Shuldiner et al. 2001) and the elevated insulin levels and weight associated with the high-energy diet in the model we employed. It is possible that the anti-neoplastic action of metformin in this model involves its well-known action as an insulin-lowering agent, leading to a reduction in insulin signaling in tumor cells, rather than the in vitro mechanism of AMPK activation. Thus, our observations are compatible with the increasingly accepted view (Shaw et al. 2005) that metformin reduces insulin levels in hyperinsulinemic states by a mechanism involving reduced hepatic glucose output and do not support the possibility that metformin lowers insulin levels by enhancing insulin receptor signaling (Holland et al. 2004). Our results therefore imply that in hosts with diet-induced insulin resistance, neoplastic tissue is not necessarily also insulin resistant to the same degree, if at all.

Our study is not the first report of in vivo anti-neoplastic activity of metformin. A recent paper (Buzzai et al. 2007) demonstrated in vivo inhibition of growth of HCT-116 colon cancer cells, but used doses approximately five times larger than those we employed. In that study, anti-neoplastic activity was observed to be restricted to a p53−/− HCT-116 variant, but diet was not treated as a variable and insulin receptor activation was not evaluated.

There is convincing epidemiological evidence that obesity is associated with increased cancer mortality (Calle et al. 2003, Ogden et al. 2006). However, while there is clear laboratory evidence for an inhibitory effect of caloric restriction on tumor growth (Carroll 1975), there are few in vivo models of adverse effects of excess dietary intake or obesity on cancer behavior. The H59 syngeneic tumor model we employed appears to be useful for studies of excess energy intake on tumor behavior (Yakar et al. 2006). The correlation of excess energy consumption with increased insulin receptor phosphorylation in neoplastic tissue suggests that insulin is a mediator of the dietary effect in our model and also a mediator of the association of obesity with adverse outcome seen in population studies (Calle et al. 2003). However, there are many other candidate mediators of the effect of excess energy intake or obesity on tumor growth, including leptin and/or other adipokines, elevated free fatty acids, or altered levels of circulating carbohydrates. In particular, further work investigating the effects of diet and metformin on insulin-like growth factor-I signaling is necessary. Recent population studies have linked serum C-peptide levels (a marker for insulin secretion) to adverse cancer prognosis (Ma et al. 2006). These data, together with evidence for the expression of insulin receptors on tumor cells (Papa & Belfiore 1996, Belfiore 2007), represent further circumstantial evidence consistent with insulin as a mediator of the adverse effect of excess energy intake on cancer prognosis. While it would be interesting to investigate the effects of other pharmaceuticals for treating insulin resistance, such as thiazolidinediones; these PPARγ activators are known to have anti-neo-plastic effects secondary to the effect on insulin signaling (Grommes et al. 2004).

The finding that the inhibitory effect of metformin on tumor growth was restricted to animals on the high-energy diet suggests that any benefits of this drug in reducing cancer aggressiveness may be restricted to a metabolically defined subset of cancer patients. The definition of this subset is an important topic for further translational research. Serum markers of hyperinsulinemia may be more useful than simple body morphometry, due to the existence of the so-called ‘normal weight, metabolically obese’ individuals (St-Onge et al. 2004, Conus et al. 2007). Clinical trials of metformin in cancer treatment have been proposed, and it will be important that these trials are evaluated in a way that is stratified for baseline metabolic profile.

Declaration of interest

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This research did not receive any specific grant from any funding agency in the public, commercial or not-for-profit sector.

Acknowledgements

We wish to thank Dr George Fantus for his help with the insulin tolerance tests, as well as Dr Ian Copeland, Dr Lilian Amrein, and Terrence Kucic for their technical assistance.

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