Metformin blocks the stimulative effect of a high-energy diet on colon carcinoma growth *in vivo* and is associated with reduced expression of fatty acid synthase

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Abstract

The molecular mechanisms responsible for the association of obesity with adverse colon cancer outcomes are poorly understood. We investigated the effects of a high-energy diet on growth of an *in vivo* colon cancer model. Seventeen days following the injection of 5×10^5 MC38 colon carcinoma cells, tumors from mice on the high-energy diet were approximately twice the volume of those of mice on the control diet. These findings were correlated with the observation that the high-energy diet led to elevated insulin levels, phosphorylated AKT, and increased expression of fatty acid synthase (FASN) by the tumor cells. Metformin, an antidiabetic drug, leads to the activation of AMPK and is currently under investigation for its antineoplastic activity. We observed that metformin blocked the effect of the high-energy diet on tumor growth, reduced insulin levels, and attenuated the effect of diet on phosphorylation of AKT and expression of FASN. Furthermore, the administration of metformin led to the activation of AMPK, the inhibitory phosphorylation of acetyl-CoA carboxylase, the upregulation of BNIP3 and increased apoptosis as estimated by poly (ADP-ribose) polymerase (PARP) cleavage. Prior work showed that activating mutations of PI3K are associated with increased AKT activation and adverse outcome in colon cancer; our results demonstrate that the aggressive tumor behavior associated with a high-energy diet has similar effects on this signaling pathway. Furthermore, metformin is demonstrated to reverse the effects of the high-energy diet, thus suggesting a potential role for this agent in the management of a metabolically defined subset of colon cancers.

Introduction

Many population studies (for example Pietinen *et al.* (1999), Calle *et al.* (2003), Meyerhardt *et al.* (2003, 2007), Dignam *et al.* (2006) and Moon *et al.* (2008)) provide evidence that excess energy intake and/or obesity is associated with increased risk of colorectal cancer and/or with poor prognosis of this disease. Further epidemiological evidence (Ma *et al.* 2004, Wei *et al.* 2005, 2006, Wolpin *et al.* 2009) suggests the hypothesis that one of the underlying mechanisms involves stimulation of malignant proliferation by insulin. This is plausible in the context of the

well-known association of obesity with hyperinsulinemia (Cahill 1971) and with insulin receptor expression by transformed cells (Frasca *et al.* 1999, Becker *et al.* 2009), reviewed in Pollak (2008).

Metformin, an antidiabetic drug, lowers the elevated insulin levels found in type II diabetes by inhibiting gluconeogenesis and hepatic glucose output, which in turn reduces blood glucose levels and results in a secondary decline in insulin levels (Shaw *et al.* 2005). Retrospective studies involving cancer outcomes among diabetic patients receiving metformin therapy have provided preliminary evidence that this compound favorably influences cancer outcomes

(Evans *et al*. 2005, Bowker *et al*. 2006). The drug acts by impairing oxidative phosphorylation, which increases the intracellular AMP/ATP ratio, leading to the activation of the LKB1–AMPK signaling pathway (Leverve *et al*. 2003, Buzzai *et al*. 2007). In hepatocytes, this pathway has a special role in the regulation of gluconeogenesis, whereas in neoplastic cells, AMPK activation changes gene expression patterns in ways that result in decreased proliferation (Wang & Guan 2009). The ability of metformin to activate AMPK depends on the presence of functional LKB1 (Shaw *et al*. 2005) and *in vitro*, in cells where the LKB1–AMPK signaling pathway is intact, metformin-induced AMPK activation is known to decrease protein synthesis and cell proliferation (Zakikhani *et al*. 2006, 2008, Dowling *et al*. 2007), and we have shown this to be the case for colon cancer cells (Zakikhani *et al*. 2008). Thus, metformin may reduce neoplastic proliferation by indirect (insulin lowering) actions and/or direct (activation of AMPK in transformed cells) mechanisms. A prior study (Tomimoto *et al*. 2008) demonstrated that metformin suppresses polyp formation in ApcMin/ + mice, but effects of the drug on colon cancer growth *in vivo* in the context of obesity and/or excess energy intake have not yet been reported.

In this study, we investigated the effects of metformin on the *in vivo* growth of MC38 colon carcinoma. Bearing in mind that the epidemiological evidence for the antineoplastic activity of metformin was obtained from type II diabetic populations who tend to be hyperinsulinemic and obese, we chose to carry out *in vivo* studies of metformin in mice provided with either a high-energy diet that induced weight gain and elevated insulin levels, or a control diet. We also explored the relationship between expression of fatty acid synthase (FASN) and diet and/or metformin administration. FASN catalyzes the biosynthesis of long-chain fatty acids from acetyl-CoA and malonyl-CoA. The fatty acids generated by FASN are essential constituents of all biological membrane lipids and are important substrates for energy metabolism. In well-nourished mammals, FASN activity and expression in untransformed cells are low due to ample supply of fatty acids derived from diet. In contrast, neoplastic cells continue to utilize FASN and display high levels of constitutive endogenous fatty acid synthesis, even in the presence of dietary fatty acids. Overexpression and hyperactivity of FASN are a common characteristic of aggressive malignancies, and inhibition of FASN by siRNA has been shown to induce apoptosis in breast cancer cells (Bandyopadhyay *et al*. 2006). Furthermore, FASN expression by colorectal cancers has been described as an adverse prognostic factor in obese patients (Ogino *et al*. 2008), and thus, it is possible that metabolic hormones that are influenced by excess energy intake may be involved in regulating the expression of FASN in neoplastic cells.

Materials and methods

Animal work

All experiments performed were approved by the McGill University Animal Care and Handling Committee. Forty-eight male C57BL/6 mice purchased from Charles River (Saint-Constant, QC, Canada) at 5–6 weeks of age were housed 4–5 per cage and put on either a control or high-energy diet *ad libitum* for 17 weeks. Diets were purchased from Harlan Teklad (Madison, WI, USA). The control diet was TD 2016 and consisted of 16% protein, 3.5% fat, and 60% carbohydrate, and this diet provided 3.3 kcal/g consumed. The high-energy diet was TD 95 217 and consisted of 18.8% protein, 39.8% fat (lard), and 41.4% carbohydrate, and this diet provided 4.3 kcal/g consumed. During the first 12 weeks on the diets, there were 24 mice on the control diet and 24 mice on the high-energy diet.

Metformin

Following 12 weeks on the experimental diets, a subgroup of mice from each dietary group ($n = 12$ mice) was given metformin in the drinking water. Metformin was administered at a dose of 50 mg/kg body weight per day. It was assumed that the mice drank 7.0 ml of water per day, and pilot experiments have shown that mice do not consume less water when metformin is added. Metformin was dissolved in 200 ml making for a concentration of 0.28 mg/ml for mice on the high-energy diet and 0.23 mg/ml for mice on the control diet. The dose of metformin was adjusted biweekly for changes in body weight.

Hormone measurement

Blood was collected in heparinized capillary tubes following 17 weeks on the diet (5 weeks with or without metformin). Plasma was used for insulin (Millipore, Billerica, MA, USA), insulin-like growth factor 1 (IGF1; R and D Systems, Minneapolis, MN USA), and leptin (Millipore) ELISAs.

Tumor growth

MC38 cells were generously donated by Dr Pnina Brodt. Cells were cultured in flasks and in 10% FBS–DMEM without antibiotic. Cells were

trypsinized, counted, and re-suspended in PBS prior to injection. Mice were injected s.c. with 5×10^5 MC38 colon carcinoma cells, a syngeneic tumor cell line. Tumors were palpable in the majority of the mice 8 days following tumor inoculation. Tumor growth was measured every other day with electronic calipers (Lee Valley Tools, Ottawa, ON, Canada).

Protein extraction and immunoblotting

Tumor tissue was removed immediately after killing and was snap frozen in liquid nitrogen. Tissue was homogenized in lysis buffer (20 mM HEPES buffer, 1% Triton X-100, 150 mM NaCl, 0.02% sodium azide, 60 mM b-glycerophosphate, 1 mM sodium vanadate, 1 mM dithiothreitol, 1 Roche complete protease inhibitor tablet, and 5 ml of 5 mM pepstatin A per 50 ml lysis buffer), and 100 μg of tissue was subjected to electrophoresis in denaturing 8–10% SDS-PAGE. Primary antibodies for p-AKT, AKT total, p-AMPK, AMPK total, β-actin, BNIP3, poly (ADP-ribose) polymerase (PARP), FASN, and acetyl-CoA carboxylase total were purchased from Cell Signaling Technologies (Danvers, MA, USA). The antibody for p-acetyl-CoA carboxylase was purchased from Millipore (Concord, MA, USA), and the antibody for SREBP-1 as well the secondary antibodies was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All primary antibodies were diluted 1:500, and the secondary antibody was diluted 1:5000. Westerns were developed using Western Lightning Plus-ECL (Perkin-Elmer, Waltham, MA, USA), and density of the western blots bands was determined by Scion Image.
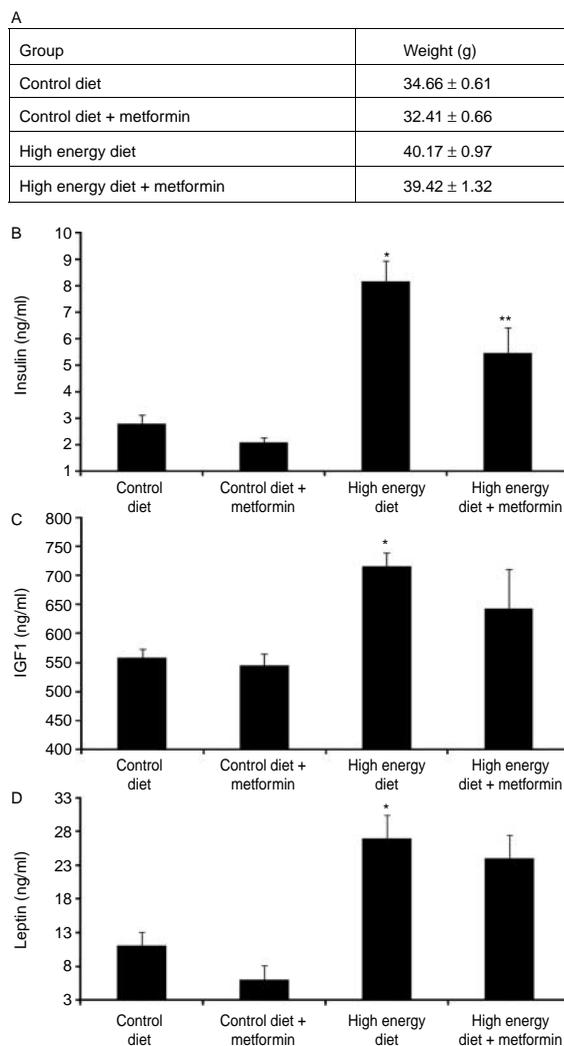
Statistical analyses

For tumor data, 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.

For direct comparison between two groups (such as comparing dietary effect on insulin levels), Student's t-test was used in the Microsoft Excel Program, and error bars represent the s.e.m.

Results

Influence of diet and metformin on body weight and circulating insulin, IGF1, and leptin levels

Figure 1A shows the effect of diet, and metformin, on weight gain in our model. The weights shown were measured following 17 weeks on the diets, including the final 5 weeks with or without metformin.

A

Group	Weight (g)
Control diet	34.66 ± 0.61
Control diet + metformin	32.41 ± 0.66
High energy diet	40.17 ± 0.97
High energy diet + metformin	39.42 ± 1.32

Figure 1 Effects of diet and/or metformin on weight and serum insulin, IGF1, and leptin levels. Mice were separated into four groups: control diet±metformin or high-energy diet±metformin. The diets were maintained for 17 weeks. Those groups with metformin received metformin in the drinking water at a concentration of 50 mg/kg per day for the last 5 weeks on the respective diets. The mice on the high-energy diet weighed significantly more than those mice on the control diet. Metformin did not have a significant effect on weight for either dietary group. CD, control diet; CD + M, control diet + metformin, HED, high-energy diet and HED + M, high-energy diet + metformin. (A) Weight prior to tumor injection; (B) insulin, *$P < 0.001$, **$P < 0.05$; (C) IGF1, *$P < 0.001$; (D) leptin; $P = 0.0008$.

The high-energy diet was sufficient to induce significant weight gain compared to the control diet (control diet: 34.66 ± 2.02 g versus high-energy diet: 40.17 ± 3.22 g; $P < 0.0001$, $n = 12$/group). Metformin was given in the drinking water at 50 mg/kg per day for 5 weeks. Although metformin may cause minor gastro-intestinal distress or appetite suppression (Kirpichnikov et al. 2002), in our model, there were no adverse effects of metformin at the dose employed. The mice that were administered metformin in the drinking water did not have body weight that differed significantly from the mice in the same dietary group that did not receive the drug.

Elevated levels of insulin and IGF1 often are seen in the context of excess energy intake, obesity, and associated metabolic diseases, and are also known to negatively influence cancer outcomes (Pollak 2009, Wolpin et al. 2009). Insulin, IGF1, and leptin levels were measured by ELISA using blood taken from the mice following 17 weeks on the diets, with or without metformin treatment for the final 5 weeks. As expected, mice on the high-energy diet had significantly higher insulin levels compared to mice on the control diet (Fig. 1B), consistent with diet-induced hyperinsulinemia (control diet: 2.80 ± 0.34 ng/ml versus high-energy diet: 8.14 ± 0.77 ng/ml; $*P < 0.0001$, $n = 12$/group). Metformin reduced elevated insulin in hyperinsulinemic mice, but had little effect on the insulin levels of mice on the control diet (high-energy diet: 8.14 ± 0.77 ng/ml versus high-energy diet + metformin: 5.45 ± 0.97 ng/ml; $**P < 0.05$, $n = 12$/group). Figure 1C shows the effect of the high-energy diet on IGF1 levels (control diet: 559.10 ± 14.25 ng/ml versus high-energy diet: 716.80 ± 22.45 ng/ml; $*P < 0.0001$). Clinically, metformin is not considered to have important effects on IGF1 level, but there was evidence for an IGF1-lowering effect of metformin in an experimental model that used high doses of the drug (Phoenix et al. 2009). In our model, we observed no significant effect of metformin on IGF1 in either dietary group. We observed a greater than twofold increase in leptin levels (Fig. 1D) in mice on the high-energy diet compared to mice on the control diet (control diet: 11.00 ± 2.04 ng/ml versus high-energy diet: 27.00 ± 3.40 ng/ml; $*P = 0.0008$), but metformin had no significant effect on the leptin levels. Recent reports (Cirillo et al. 2008) have suggested that leptin signaling may play a role in tumorigenesis of tumor cells that express the leptin receptor; however, the MC38 cell line is null for the leptin receptor, and treatment of this cell line with leptin did not affect cell growth as measured by MTT assay (data not shown).

Influence of diet and metformin on *in vivo* tumor growth

In keeping with a prior report (Yakar et al. 2006), we observed a potent stimulative effect of the high-energy diet on MC38 tumor growth (final tumor volume: control diet: 1186 ± 99 mm^3 versus high-energy diet: 2073 ± 319 mm^3; $*P = 0.003$) (Fig. 2). Metformin significantly attenuated the effect of the high-energy diet on tumor growth, but interestingly had no significant effect on tumor growth of mice on the control diet (tumor volume at day 17 following injection: high-energy diet: 2073 ± 319 mm^3 versus high-energy diet + metformin: 1275 ± 223 mm^3; $**P = 0.005$). These results indicate that the growth inhibitory activity of metformin for MC38 colon cancer is strongly influenced by the metabolic status of the host.

Effect of diet and metformin on intracellular signaling pathways

AMPK
Activation of AMPK was measured by western blot of AMPK phosphorylated at Thr^{172}. We observed (Fig. 3) increased phosphorylated AMPK in the tumor tissue from mice administered metformin, indicating that the dose and route of administration were sufficient to allow the drug to act as an indirect AMPK activator in transformed cells, as described *in vitro* (Zakikhani et al. 2008), and *in vivo* in liver (Shaw et al. 2005). AMPK activation stimulates many downstream pathways that alter cell metabolism in ways that
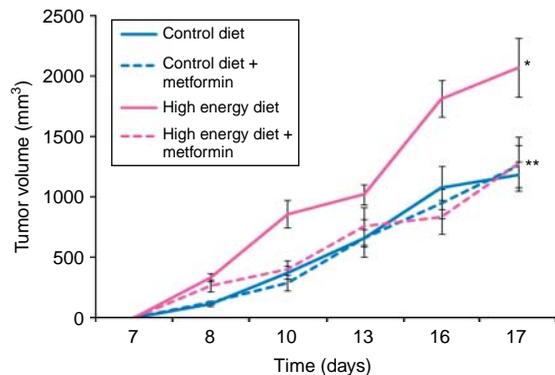
Figure 2 Effect of diet and metformin on MC38 colon cancer growth *in vivo*. Following 17 weeks on the experimental diets (with or without 5 weeks of metformin), 5×10^5 MC38 colon carcinoma cells were injected s.c., and tumor growth was observed for 17 days. This shows that mice on the high-energy diet had significantly larger tumor volume compared to mice on the control diet ($*P = 0.003$). Metformin significantly attenuated the stimulative effect of the high-energy diet on tumor growth in the high-energy diet + metformin group ($**P = 0.005$), while having no significant effect on the tumor growth of mice in the control diet + metformin group.
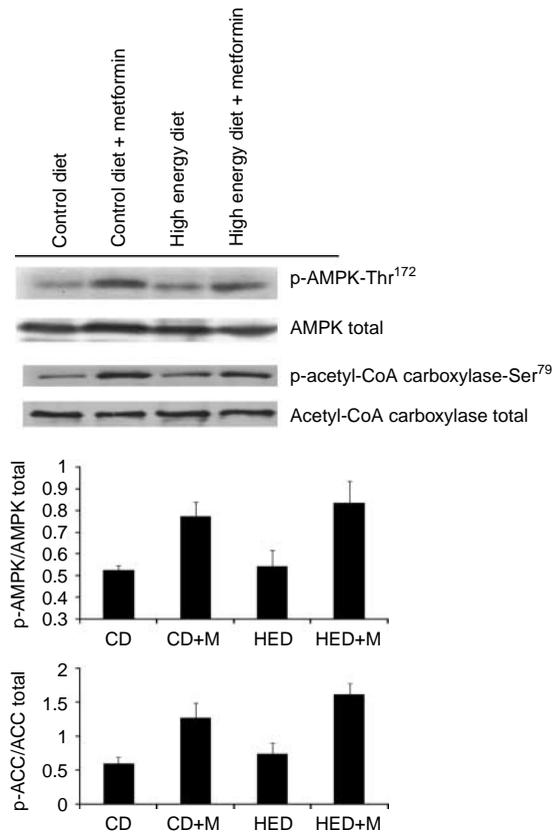
Figure 3 Metformin leads to activation of AMPK and phosphorylation of acetyl-CoA carboxylase. Western blot analyses show that the addition of metformin was sufficient to activate AMPK in the tumor tissue, as shown by increased phosphorylation at Thr^{172}. Activation of AMPK results in the inhibitory phosphorylation of acetyl-CoA carboxylase at Ser^{79}. We also observed this downstream effect of AMPK activation in the tumor tissue taken from mice in the metformin groups: phosphorylation at Ser^{79} was significantly higher in the metformin group compared to the parental dietary group indicating inhibition of the rate-limiting step in *de novo* fatty acid synthesis. CD, control diet; CD + M, control diet + metformin; HED, high-energy diet; HED + M, high-energy diet + metformin.

conserve ATP. To confirm that metformin-induced activation of AMPK was associated with downstream pathway activation, we measured phosphorylation status of the AMPK substrate acetyl-CoA carboxylase at its inhibitory Ser^{79} site, and observed increased phosphorylation in the presence of metformin. When active, acetyl-CoA carboxylase catalyzes the ATP-dependent carboxylation of acetyl-CoA to malonyl-CoA. This step is the rate-limiting step in the *de novo* fatty acid synthesis pathway, indicating that metformin can act to suppress the formation of malonyl-CoA and *de novo* fatty acid synthesis. Suppression of anabolic pathways is in keeping with the expected consequences of AMPK activation.

AKT

We observed a 60% increase in p-AKT-Ser^{473} in tumor tissue taken from mice on the high-energy diet compared to tumor tissue taken from mice on the control diet (Fig. 4A), which we attribute to increased signaling through insulin receptors present on the MC38 colon cancer cells (Fig. 4B). Metformin attenuated the effect of diet on AKT signaling in tumors of mice on the high-energy diet, consistent with the observed effects of the drug on insulin levels.

We observed the same trend (Fig. 4A) in levels of cleaved SREBP-1, a transcription factor downstream of AKT. SREBP-1 is a transcription factor that mediates the expression of enzymes involved in lipid and cholesterol homeostasis (Yokoyama *et al.* 1993). Our observation indicates that diet-induced changes in AKT phosphorylation are of sufficient magnitude to have an impact on AKT targets. We also observed a significant reduction in cleaved SREBP-1 in tumors of mice that were administered metformin, independent of dietary group.

There is considerable evidence that FASN plays a key role in the lipogenic phenotype of many human cancers (Menendez & Lupu 2007). Our observation that a high-energy diet increases FASN levels twofold in MC38 colon cancers compared to that seen in tumors of animals on the control diet (Fig. 4C) represents the first evidence that macronutrient intake by the host can influence FASN expression in neoplastic tissue. *In vitro* results (Fig. 4D) show that insulin, more than IGF1, upregulates FASN expression by MC38 cells, suggesting that insulin-stimulated AKT activation may upregulate FASN *in vivo*.

Metformin, shown in Fig. 2 to block the stimulative effect of a high-energy diet on tumor growth, was also observed to completely reverse the stimulative effect of the high-energy diet on FASN expression in tumor tissue (Fig. 4C). This novel action of metformin on neoplastic cells is plausible given the observed decrease in mature SREBP-1 and that inhibition of energy-consuming synthesis of macromolecules is a well-known consequence of AMPK activation (Wang & Guan 2009). Given that there is a rationale for FASN inhibition for cancer treatment (Menendez & Lupu 2007), it is possible that this consequence of metformin action contributes to its antineoplastic activity, particularly where baseline FASN levels are high.

Metformin induces apoptotic proteins *in vivo*

It has been shown (Bandyopadhyay *et al.* 2006) that BNIP3, a Bcl-2 family member and pro-apoptotic protein, is upregulated in cells when FASN expression
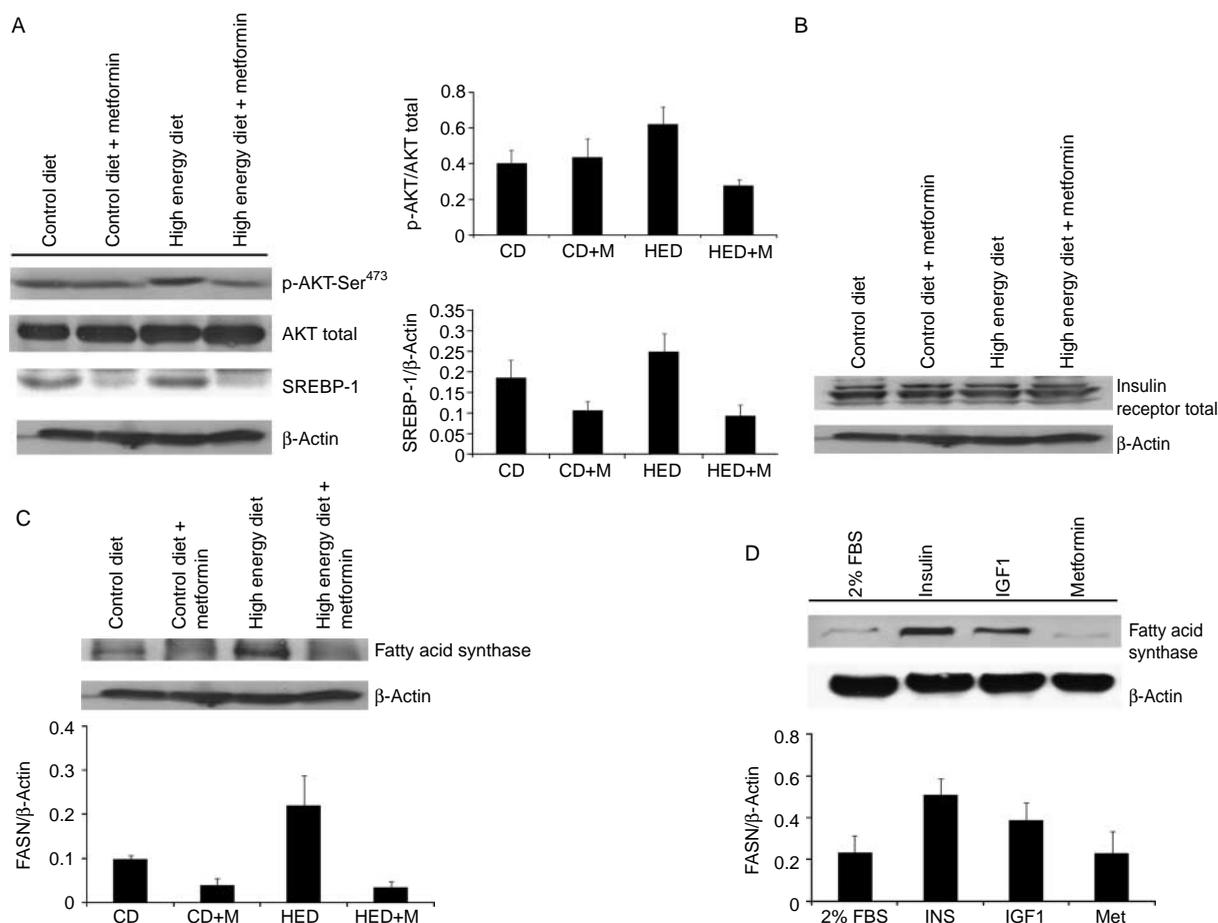
Figure 4 High-energy diet leads to increased phosphorylation of AKT and increased expression of fatty acid synthase *in vivo*, and insulin increased fatty acid synthase expression *in vitro*. (A) We observed increased phosphorylated AKT at Ser[473] in the tumor tissue taken from mice on the high-energy diet (HED) compared to tumor tissue from mice on the control diet (CD) consistent with increased growth factor signaling. The administration of metformin significantly reduced the amount of phosphorylated AKT in tumors of mice on the high energy diet group (HED + M), but we observed no significant difference in the amount of phosphorylated AKT in tumors of mice on the control diet + metformin group (CD + M) compared to the control diet alone. Downstream of AKT, we found elevated expression of the mature form of SREBP-1 in the tumors of mice in the high energy diet group compared to the control diet group. Tumor tissue from mice in the metformin groups had significantly reduced expression of the mature form of SREBP-1 compared to the parental dietary groups. (B) Expression of insulin receptors on MC38 colon carcinoma cells. (C) FASN expression was shown to be significantly higher in tumor tissue from mice on the high-energy diet, while the addition of metformin attenuated the expression of FASN in both dietary groups. (D) MC38 cells were treated in DMEM + 2% FBS with either 10 nmol insulin, 10 nmol IGF1, or 5 mM metformin for 48 h and analyzed for expression of FASN.

is repressed. We found that the low-dose metformin administered in our model significantly upregulated the expression of this pro-apoptotic protein in tumors independent of diet (Fig. 5). As PARP is a substrate of apoptosis-inducing caspases, the amount of cleaved PARP is used to assess the propensity of cells to apoptosis. We used this endpoint to assess the effects of metformin on apoptosis (Fig. 5). Metformin increased the quantity of cleaved PARP regardless of diet. Interestingly, the level of apoptosis assessed by the cleaved PARP was lower in mice on the high-energy diet than those on the control diet, and the fold increase in cleaved PARP with the administration of

metformin was greater for mice on the high-energy diet (10.7-fold increase) as compared to that for mice on the control diet (3.87-fold increase). The significance of our incidental observation of an increase in total PARP level with the high-energy diet is under investigation.

Discussion

There are limited prior experimental data concerning the relationship of excess energy intake to colorectal cancer biology, despite the fact that population studies suggest that diet has an important influence on tumor
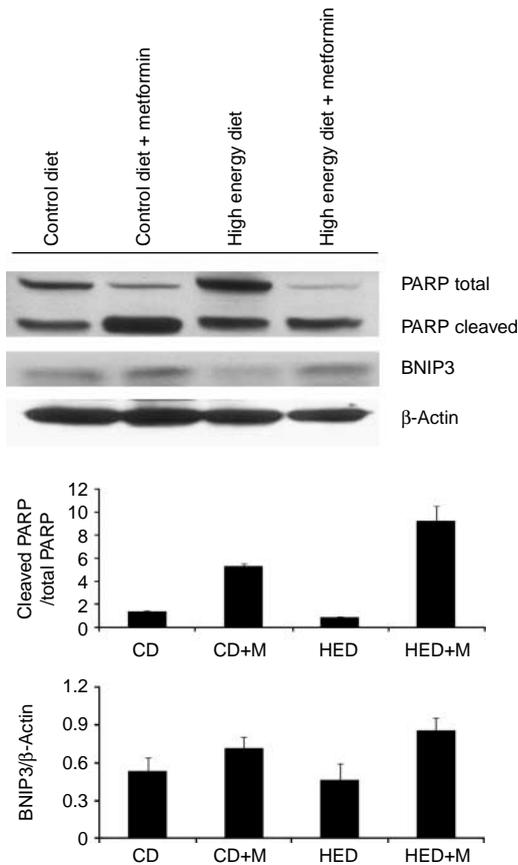
Figure 5 Induction of apoptotic proteins. Tumor tissue was analyzed for expression of BNIP3 and PARP cleavage. For PARP, the 116 and the 89 kDa bands are shown, and cleavage was calculated as the ratio of cleaved PARP to total PARP. BNIP3 expression was normalized to β-actin as loading control. The data indicate that metformin administration leads to increased BNIP3 and cleaved PARP in MC38 colon cancer *in vivo*, while the high diet was associated with reduced BNIP3 and reduced cleaved PARP. CD, control diet; CD+M, control diet+metformin; HED, high-energy diet; HED+M, high-energy diet+metformin.

behavior (Yakar *et al.* 2006). We observed, in a murine model, that increased macronutrient intake leads to an increase in insulin levels, an increase in AKT activation, increased FASN and increased tumor growth of MC38 colon carcinoma.

Furthermore, we observed that metformin attenuates the increase in tumor growth associated with excess energy intake, and also reversed each of the biomarker changes observed in mice of the high energy dietary group. Our findings concerning the effects of metformin on signaling pathways suggest that in our model, the drug may act on neoplastic cells by both direct, AMPK mediated, and indirect insulin-mediated mechanisms. As expected, metformin reduced the high levels of insulin seen in mice consuming a

high-energy diet. This action of metformin has been shown to be a consequence of activation of the LKB1–AMPK pathway in liver, which results in decreased gluconeogenesis, decreased hepatic glucose output, reduced circulating glucose, and a secondary decline in insulin (Shaw *et al.* 2005). This correlated with reduced AKT activation in the colon cancers in our model, an effect which would be expected to reduce proliferation.

Separately, we detected increased activation of AMPK in neoplastic tissue, which is the expected consequence of metformin acting directly on cancer cells, as well as evidence of downstream consequences of AMPK activation, including phosphorylation of acetyl-CoA carboxylase. AMPK activation is known to reduce energy consuming processes such as proliferation and protein translation (Zakikhani *et al.* 2006, Dowling *et al.* 2007, Wang & Guan 2009), and this could contribute to the observed reduction in tumor growth. In order to determine if the antineoplastic activity of metformin is attributable to direct or indirect mechanisms, or if both are necessary, it will be necessary to use genetic approaches involving inactivation of the LKB1–AMPK pathway in neoplastic cells, or using mice in which the indirect mechanism of action has been rendered inoperational by LKB1/AMPK inaction in the liver (Shaw *et al.* 2005). Nonetheless, the fact that we observed maximal antineoplastic activity of metformin under conditions of diet-induced hyperinsulinemia leads us to speculate that the indirect mechanism, in which insulin levels are lowered by metformin, plays an important role.

A prior model (Buzzai *et al.* 2007) of antineoplastic activity of metformin *in vivo* provided convincing evidence that its growth inhibitory effect is influenced by the p53 status of the tumor, with p53-null cells being selectively growth inhibited. The data in that study supported the hypothesis that at the doses employed (approximately fivefold higher than that used in our model), the drug inhibited oxidative phosphorylation to an extent that maintaining cellular energy supply required an AMPK-dependent and p53-dependent switch to autophagy *in vivo* and glycolysis *in vitro*. In the absence of functional p53, this switch is impaired and leads to an energy deficit and growth inhibition. In the presence of intact p53 signaling, the neoplastic cells were not growth inhibited by metformin because of successful metabolic compensation. That model involved a cell line with a mutant PI3K, a molecular lesion which constitutively activates signaling pathways downstream of the insulin receptor. Constitutive activation of the PI3K/AKT pathway has recently been shown to

confer resistance to caloric restriction-induced growth inhibition (which is related to caloric restriction-induced decline in insulin level (Kalaany & Sabatini 2009). It is likely that activating PI3K mutations also confer resistance to any antiproliferative actions of metformin which are attributable to its systemic insulin-lowering effect (Pollak 2009). Thus, in the setting of high-dose metformin and an activating PI3K mutation, dominant consequences of the drug include metformin-induced energy stress and AMPK–p53-dependent metabolic compensation for survival, or in the case of defective compensation, growth inhibition, or death. In other contexts, such as diet induced hyperinsulinemia *in vivo*, metformin-induced antiproliferative effects may be attributable to the declines in insulin levels, reduced AKT phosphorylation and/or AMPK activation, and the induction of energy-conserving pathways.

Prior work (Samuels *et al*. 2004, Guo *et al*. 2007, Ogino *et al*. 2008) demonstrated that AKT activation is an important determinant of colon cancer behavior, but emphasized mutational activation of PI3K as a mechanism resulting constitutive AKT signaling. This mechanism has been estimated to operate in approximately one-third of colon cancers (Samuels *et al*. 2004). Our model demonstrates that high dietary energy intake can activate the AKT pathway in colon cancer cells, increase FASN expression, and accelerate tumor growth. This shows that energy intake and/or systemic metabolic factors may influence a subset of colon cancers by regulating the same signaling pathway that mediates the effects of activating somatic mutations of PI3K. Furthermore, we present evidence that for cancers in which tumor growth and AKT activation are stimulated by a high-energy diet, metformin has growth inhibitory activity, and also reduces AKT activation and FASN expression. AKT-induced cleavage of SREBP-1 leading to increased production of FASN mRNA has been shown (Porstmann *et al*. 2005) and depends on the activity of mTORC1. As the cleavage of SREBP-1 is dependent on mTORC1, our finding is consistent with previous reports that show metformin can inhibit signaling through mTORC1 via activation of AMPK in neoplastic cells (Zakikhani *et al*. 2006). SREBP-1 is involved in the regulation of FASN, which is responsible for the final catalytic step in fatty acid synthesis, and which is commonly upregulated in malignancies.

Despite the well-known association between obesity and poor cancer prognosis (Calle *et al*. 2003), the ultimate molecular mechanisms involved are yet to be described. Cancer cells require *de novo* fatty acid synthesis in order to maintain a constant supply of lipids in a highly proliferative environment. Although the mechanisms responsible for overexpression of FASN in neoplastic cells are not fully understood, in this study we show, for the first time, that the expression of FASN can be manipulated by diet and that insulin may play a role in both the increased tumor growth and increased FASN expression. Elevated serum insulin levels, a result of diet induced hyperinsulinemia, were associated with increased signaling through AKT, SREBP-1 and ultimately increased expression of FASN *in vivo*. Furthermore, the administration of metformin reversed the stimulatory effects of diet on tumor growth, including tumor volume, AKT activation, and FASN expression. These results are consistent with epidemiological and experimental results that showed that metformin may reduce cancer risk and improve cancer prognosis in hyperinsulinemic cancer patients, and suggest that there may be roles for metformin or related compounds in the treatment of a subset of colorectal cancer defined by criteria involving both host metabolic status and molecular pathology of the tumor.

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