Metformin Is an AMP Kinase–Dependent Growth Inhibitor for Breast Cancer Cells

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Abstract
Recent population studies provide clues that the use of metformin may be associated with reduced incidence and improved prognosis of certain cancers. This drug is widely used in the treatment of type 2 diabetes, where it is often referred to as an “insulin sensitizer” because it not only lowers blood glucose but also reduces the hyperinsulinemia associated with insulin resistance. As insulin and insulin-like growth factors stimulate proliferation of many normal and transformed cell types, agents that facilitate signaling through these receptors would be expected to enhance proliferation. We show here that metformin acts as a growth inhibitor rather than an insulin sensitizer for epithelial cells. Breast cancer cells can be protected against metformin-induced growth inhibition by small interfering RNA against AMP kinase. This shows that AMP kinase pathway activation by metformin, recently shown to be necessary for metformin inhibition of gluconeogenesis in hepatocytes, is also involved in metformin-induced growth inhibition of epithelial cells. The growth inhibition was associated with decreased mammalian target of rapamycin and S6 kinase activation and a general decrease in mRNA translation. These results provide evidence for a mechanism that may contribute to the antineoplastic effects of metformin suggested by recent population studies and justify further work to explore potential roles for activators of AMP kinase in cancer prevention and treatment.

Introduction
Metformin (1,1-dimethylbiguanide hydrochloride) is a biguanide commonly used in the treatment of type 2 diabetes mellitus (1). It is frequently referred to as an “insulin sensitizer” because in settings of insulin resistance and hyperinsulinemia, it lowers circulating insulin levels. There is some evidence that suggests that the mechanism of action of metformin involves enhancement of signaling through the insulin receptor, leading to improvement of insulin resistance, followed by reduction in insulin levels (2). However, recent work (3) provides evidence that the key action of metformin is the inhibition of hepatic glucose output by inhibition of gluconeogenesis, with a secondary decline in insulin levels, in the absence of a major effect on insulin signaling. There is strong evidence that in the liver, this mechanism involves the activation of AMP kinase via an LKB1-dependent mechanism (3, 4).

Although the molecular mechanisms of metformin have been studied in tissues such as liver, muscle, and fat, in relation to glucose homeostasis and insulin action, relatively little is known about the effects of this compound on epithelial tissues. This represents an important gap in knowledge, given that the recently described control system that links metformin to suppression of gluconeogenesis in hepatocytes involves activation of AMP kinase via LKB1. LKB1 was previously described as a tumor suppressor gene with relevance to epithelial neoplasia (5). Loss of function of LKB1 is associated with Peutz-Jeghers syndrome, which is characterized not only by multiple gastrointestinal polyps but also by a significantly increased lifetime risk (approaching 80%) of various epithelial cancers, including breast cancer (6). The molecular mechanism of action of LKB1, both as a regulator of gluconeogenesis in hepatocytes and more generally as a tumor suppressor gene in epithelial tissues, is thought to involve in large part its action as an activator of AMP kinase (ref. 3; reviewed in ref. 7). In general, physiologic activation of the AMP-activated protein kinase (AMPK) pathway by conditions of nutrient depletion down-regulates processes that consume energy, such as protein translation and cell division, and up-regulates those that generate energy.

Recent pilot studies carried out using population registries raise the possibility that metformin may reduce cancer risk and/or improve cancer prognosis. One showed an unexpectedly lower risk of a cancer diagnosis among diabetics using metformin compared with a control group of diabetics using other treatments (8); another showed lower cancer-specific mortality among subjects with diabetes using metformin compared with diabetics on other treatments (9). There have been occasional reports of antineoplastic activity of metformin in various experimental models. However, the underlying mechanistic aspects have not been explored, and these observations might be related to uncharacterized direct actions of metformin on cancer cells or to indirect actions of the drug, such as reduction of insulin levels. Here, we describe in vitro experiments carried out to investigate the hypothesis that metformin exhibits direct antiproliferative actions on epithelial cells in vitro.

Materials and Methods

Chemicals. Cell culture materials were obtained from Invitrogen (Burlington, Ontario, Canada). Anti–phospho-specific (Thr172) AMPKα and anti-AMPKα, anti–phospho-p70S6K (Thr389), anti–phospho-mammalian target of rapamycin (anti–phospho-mTOR; Ser2481), anti-mTOR, anti–phospho-specific S6 ribosomal protein (Ser235/236), anti–phospho-specific acetyl-CoA carboxylase (Ser79), and anti–β-actin were purchased from Cell Signaling Technology (Beverly, MA). Anti-AMPKα1 and anti-AMPKα2 were purchased from Upstate (Charlottesville, VA). Horseradish peroxidase–conjugated anti-rabbit IgG, anti-mouse IgG, and enhanced chemiluminescence (ECL) reagents were from Pharmacia-Amersham (Baie d’Urfé, Quebec, Canada). Metformin was obtained from Sigma-Aldrich (St. Louis, MO). Small
interfering RNA (siRNA) SMARTpool/AMPKα1 was obtained from Upstate (Charlottesville, VA). Negative control siRNA (Alexa Fluor 488) was purchased from Qiagen (Mississauga, Ontario, Canada).

Cell lines and culture conditions. Cell lines were purchased from the American Type Culture Collection (Manassas, VA). Cells were cultured in RPMI 1640 (MCF-7, PC-3, SKOV3, and OVCAR3), DMEM (HeLa), or D-MEM/F12 with MEGM Single Quots (Cambrex, Walkersville MD; MCF10A) and supplemented with 10% fetal bovine serum (FBS) and 100 units/mL gentamicin at 37°C and 5% CO2 in 75-cm² flasks. Cells were passaged by 0.25% Trypsin-EDTA when they reached ~80% confluence.

Cell proliferation assay. The effect of metformin on cell lines was evaluated by the indicator dye Alamar Blue (Biosource International, Camarillo, CA). Cells were plated at 5 × 10³ per well in triplicate in 96-well plates and incubated in medium containing 10% FBS. After 24 hours, serum-free medium containing metformin (10 mmol/L) alone or with IGF-1 (40 ng/mL) or insulin (5 μg/mL) were provided, and cells were incubated for 72 hours. Cell proliferation in each well was measured by Alamar Blue dye reduction. Columns, mean of two independent experiments carried out in triplicate (n = 6); bars, SE. *, P = 0.0359, difference between the IGF-I condition and IGF-I plus metformin condition; **, P = 0.0346, difference between the insulin condition and insulin plus metformin condition.

Protein extraction and Western blot analysis. Cells were washed thrice with ice-cold PBS and lysed in 100 to 400 μL lysis buffer [20 mmol/L Tris-HCl (pH 7.5)], 150 mmol/L NaCl, 2.5 mmol/L sodium pyrophosphate,
require AMP kinase (3), we hypothesized that the antiproliferative effects of metformin involve the same pathway. We examined the phosphorylation of AMPK in MCF-7 cells. Western blot analysis indicated that metformin stimulates AMPK phosphorylation in a dose-dependent manner (Fig. 2A). AMPK activation is associated with decreased phosphorylation of mTOR and S6 kinase. The effect of AICAR, an AMP analogue, on proliferation was similar to that of metformin (Fig. 2B). Similar effects were also observed for other cell lines (data not shown).

**siRNA against AMP kinase (α1 subunit) rescues cells from metformin-induced growth inhibition.** To determine if activation of AMP kinase by metformin is required for the antiproliferative effect of the drug, we carried out experiments with siRNA against AMP kinase. The siRNA rescued cells from the inhibitory effect of metformin (Fig. 3A). As shown in Fig. 3B, AMPKα1 siRNA reduced the stimulatory effect of metformin on AMPK phosphorylation. This was correlated with reduction of levels of AMPKα1 by siRNA, as detected by Western blot. Levels of total AMPKα showed a pattern similar to that seen for AMPKα1. It has previously been reported (11) that AMPKα2 is mainly expressed in muscle and liver, and we observed only low levels of expression of the α2 isoform, which did not change with the siRNA targeting and/or metformin treatments.

We hypothesized that metformin, by up-regulating AMP kinase activity, would inhibit mTOR activation and downstream events. Consistent with this prediction, metformin inhibited levels of phospho-p70S6K and phospho-S6, whereas AMP kinase siRNA had opposite effects. Acetyl-CoA carboxylase (ACC) is also regulated by AMP kinase, and we observed an increase in P-ACC with metformin.

Similar results were obtained with MCF-10A cells (data not shown).

**Effect of metformin on mRNA translation.** In view of the effect of metformin on mTOR and S6 kinase activation, we hypothesized that protein translation would be decreased by this drug in epithelial cells. Data in Fig. 4 support this hypothesis by showing a general decline in protein synthesis on exposure to growth inhibitory concentrations of metformin.

**Discussion**

Most studies of the effects of metformin on cell signaling networks have been carried out in the context of diabetes research, using tissues classically sensitive to insulin, such as liver, muscle, and fat. Recent work (3) supports the view that the insulin-lowering and glucose-lowering actions of metformin are related to the
suppression of gluconeogenesis, which is a consequence of metformin-stimulated activation of LKB1 and AMP kinase in hepatocytes. In cells other than hepatocytes, LKB1/AMP kinase activation is of course unrelated to gluconeogenesis regulation but rather involves regulation of other downstream pathways, including many relevant to the control of cellular proliferation (7). However, there have been few experimental studies of the effects of metformin on epithelial cells.

Data presented here show that activation of the AMPK pathway by metformin is not confined to hepatocytes but can be observed in epithelial cells as well. In epithelial cells, sequellae of pathway activation include reduced proliferation, an expected consequence of the observed reduction in mTOR activation, S6 kinase inactivation, and general reduction of mRNA translation and protein synthesis. Thus, the view that epithelial cells of organs such as breast, prostate, colon, and lung are “bystanders” unaffected by metformin treatment may be inaccurate. Indeed, the effects of metformin on these pathways in epithelial cells may be relevant to the recent preliminary observations from population studies suggesting that metformin administration reduces cancer risk (8) and mortality (9). Our data are consistent with prior observations (11) emphasizing that in tissue other than

Figure 3. AMPKα1 siRNA reduces AMPK expression and rescues MCF-7 cells from the inhibitory effect of metformin. A, MCF-7 cells were transfected with 50 nmol/L AMPKα1-siRNA or with control siRNA using Oligofectamine. Twenty-four hours after transfection, cells were treated with metformin for 48 hours. Cell proliferation in each well was measured by Alamar Blue dye reduction. Columns, mean of two independent experiments carried out in triplicate (n = 6); bars, SE. *, P = 0.0011, difference between the control and metformin conditions was significant; **, P = 0.0189, difference between siRNA-AMP kinase and siRNA-AMP kinase plus metformin. Percentage of cell proliferation relative to control. B, MCF-7 cells were transfected with 50 nmol/L AMPKα1-siRNA or with control siRNA using Oligofectamine. Six hours after transfection, medium was changed to 1% FBS with or without metformin (5 mmol/L), and cells were incubated for 72 hours. After harvesting, cells were lysed and prepared for immunoblot analysis using antibodies against phospho-AMPK (Thr172), phospho-mTOR (Ser2448), AMPKα, AMPKα1, phospho-ACC (Ser79), phospho-p70S6K (Thr389), and phospho-S6 ribosomal protein (Ser235/236). β-Actin is shown as a loading control.
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level of whole-organism physiology, this protection is mediated at least in part by the suppressive effect of dietary restriction on the circulating level of IGF-I, which influences cancer risk (14). At the cellular level, physiologic or pharmacological activation of AMP kinase would serve to further attenuate signaling in networks downstream of insulin and/or IGF-I receptors, particularly at the level of mTOR (15).

Metformin is unlikely to directly affect those cancers that exhibit biallelic loss of function of LKB1 or other critical downstream signaling molecules, in keeping with our observation of lack of inhibition of LKB1 null HeLa cells shown in Fig. 1. Germ line homozygous loss of function of LKB1 is embryonic lethal (16); subjects with Peutz-Jeghers syndrome have a functional allele that may even be retained in some Peutz-Jeghers polyps and cancers. The possibility that metformin, by up-regulating activation of the retained functional LKB1 allele in halplosufficient epithelial tissues, might attenuate manifestations of neoplasia in Peutz-Jeghers syndrome deserves investigation.

Further work is needed to determine the relative importance of direct (AMP kinase pathway activation) and indirect (reduction of insulin levels) mechanisms by which metformin may act as an antiproliferative agent for normal and/or transformed epithelial cells in vivo. The indirect mechanism may be of considerable importance in subjects with high insulin levels and/or cancers with high levels of insulin and/or hybrid insulin/IGF-I receptors (17, 18), where there is emerging evidence that ligand levels influence risk and prognosis (19, 20). However, the direct action of metformin as an activator of the LKB1/AMP kinase tumor suppressor pathway in epithelial cells reported here suggests the possibility of broader clinical relevance.

References


