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. (Cancer Res 2006; 66(21): 10269-73)

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).

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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 (Thr^{172}) AMPKα and anti-AMPKα, anti–phospho-p70S6K (Thr^{389}), anti–phospho-mammalian target of rapamycin (anti–phospho-mTOR; Ser^{2448}), anti-mTOR, anti–phospho-specific S6 ribosomal protein ($Ser^{235/236}$), anti–phosphospecific acetyl-CoA carboxylase (Ser^{79}), 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

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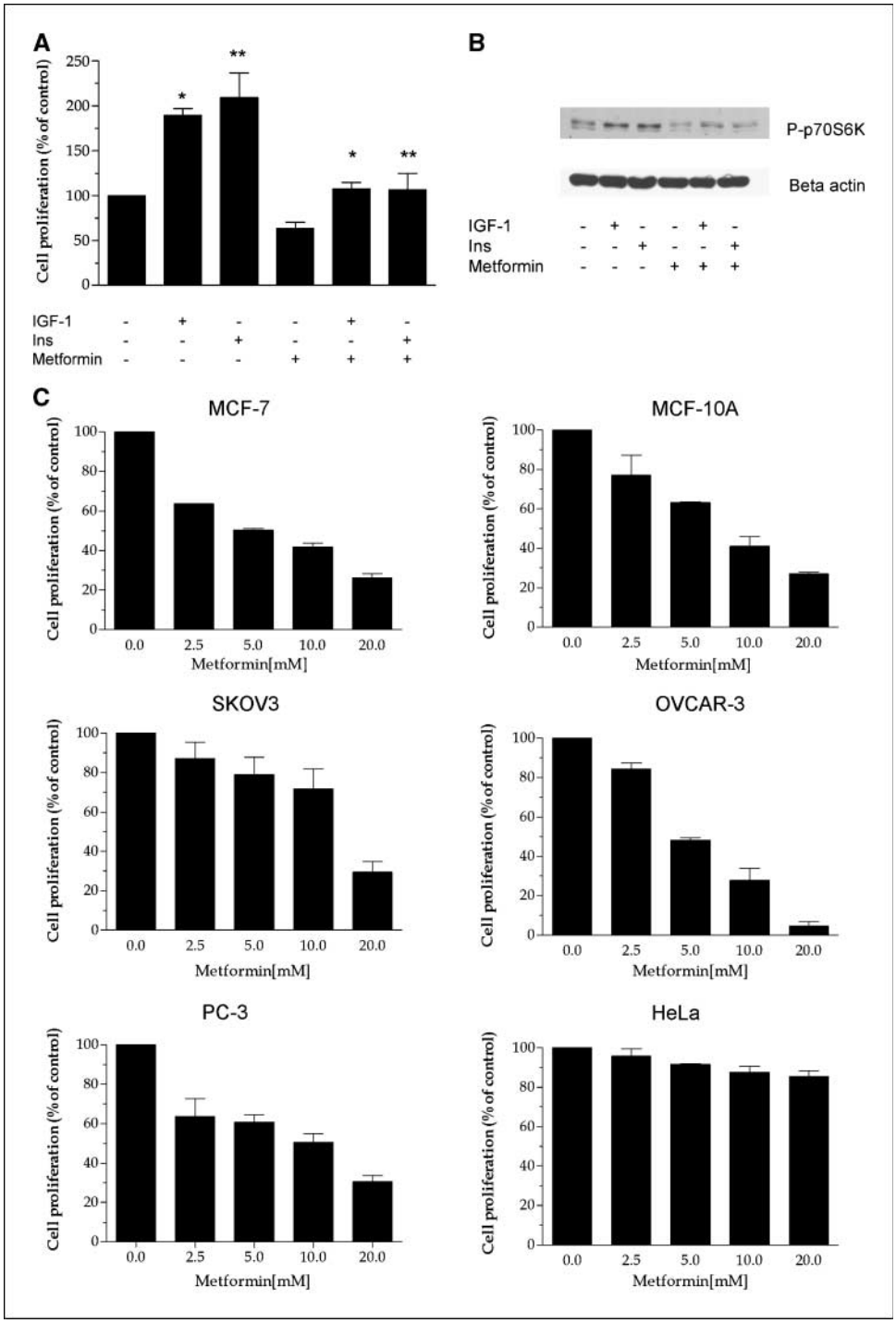

Figure 1. Effects of metformin on insulin or IGF-stimulated proliferation of MCF-7 breast cancer cells under serum-free conditions and more generally on epithelial cell proliferation. *A,* MCF-7 cells were plated onto 96-well plates in culture medium containing 10% FBS. After 24 hours, serum-free medium 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. *B,* MCF-7 cells were plated in culture medium containing 10% FBS. After 24 hours, serum-free medium was replaced and incubated for 16 hours. Starved cells were pretreated with vehicle or 40 ng/mL IGF-1 or 5 μg/mL insulin (*Ins*) for 30 minutes followed by adding 10 mmol/L metformin. Cells were incubated for 48 hours. Cell lysates were analyzed by Western blot for phosphorylation of p70S6K (Thr^{389}). β-Actin is shown as a loading control. *C,* growth inhibition of cancer cells MCF-7 (breast), PC-3 (prostate), and SKOV3 and OVCAR3 (ovary) was observed. Untransformed MCF-10A human breast epithelial cells were also growth inhibited. HeLa cells, which have no functional *LKB1* allele, were not inhibited. Cells were seeded into 96-well plates in the presence of 1% FBS and after 24 hours treated with different concentrations of metformin. Cell proliferation in each well was measured by Alamar Blue dye reduction. *Columns,* mean from three independent experiments done in triplicate; *bars,* SE.

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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 RPM1 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% CO_2 in 75-cm^2 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 5×10^3 per well in triplicate in 96-well plates and incubated in medium containing 10% FBS. After 24 hours, the complete medium was replaced with test medium containing vehicle control or various doses of metformin for 72 hours at 37°C. Alamar Blue was then added, and all plates were incubated at 37°C, and a colorimetric change was measured according to the methods provided by the supplier.

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,

1 mmol/L β-glycerol phosphate, 1 mmol/L Na_3VO_4, 1 mmol/L EGTA, 1% Triton, and complete protease inhibitor mixture inhibitors (Roche, Mannheim, Germany). Cells debris was removed by centrifugation at 14,000 × *g* for 20 minutes at 4°C. Following assay for total protein (Bio-Rad, Mississauga, Ontario, Canada), clarified protein lysates from each experimental condition (40-50 μg) were boiled for 5 minutes and subjected to electrophoresis in denaturing 8% SDS-polyacrylamide gel for mTOR, or 10% SDS-PAGE for other proteins. After stripping the membranes with stripping buffer [62 mmol/L Tris-HCl (pH 6.8), 100 mmol/L β-mercaptoethanol, 2% SDS], the membranes were probed with antibodies of interest. Horseradish peroxidase–conjugated anti-rabbit IgG and anti-mouse IgG were used as secondary antibodies. The position of proteins was visualized using the ECL reagent.

Cell transfection. MCF-7 cells were transfected with siRNA targeting the AMPKα1 or a negative control siRNA using Oligofectamine (Invitrogen) as described by the manufacturer's instructions. Cells cultures were incubated for 24 hours with various concentrations of siRNA before metformin treatment.

[^{35}S]methionine metabolic labeling. MCF-7 cells (seeded in 24-well plates) were incubated with increasing doses of metformin for 24 hours in DMEM supplemented with 10% FBS. After 24 hours, cells were washed once with PBS and incubated with metformin in methionine-free DMEM without FBS for 2 hours. The medium was then replaced with methionine-free DMEM containing ^{35}S-protein labeling mix (20 μCi/mL). After 45 minutes, the cells were washed with cold PBS and lysed in buffer (described in Protein Extraction and Western Blot Analysis), and radioactivity incorporated into the TCA precipitable material was measured.

Statistical analysis. Before statistical analysis, data were square root transformed to satisfy the assumptions of analysis. Statistical significance was evaluated using GLM Procedure, Student Newman-Keuls test, with Statistical Analysis System software, version 8e (SAS Institute, Cary, NC), with Ps ≤ 0.05 considered significant.

Results

Metformin acts as a growth inhibitor rather than an insulin sensitizer for MCF-7 human breast cancer cells. MCF-7 cells are known to be responsive to insulin and insulin-like growth factors (IGF), and this is confirmed by the data in Fig. 1*A*. However, metformin, rather than enhancing insulin or IGF-I-stimulated growth, acted as a growth inhibitor, an action correlated with a suppression of phosphorylation of p70S6K at Thr^{389} (Fig. 1*B*). To examine the effect of metformin on proliferation more generally, dose-response studies were carried out on several cell lines (Fig. 1*C*). Interestingly, several cancer cell lines, but not the LKB1$^{-/-}$ HeLa cell line (10), were growth inhibited. Untransformed MCF-10A human breast epithelial cells were also growth inhibited.

Metformin up-regulates AMP kinase activity in MCF-7 breast cancer cells. As the metabolic actions of metformin 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. 2*A*). 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. 2*B*). 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. 3*A*). As shown in Fig. 3*B*, 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

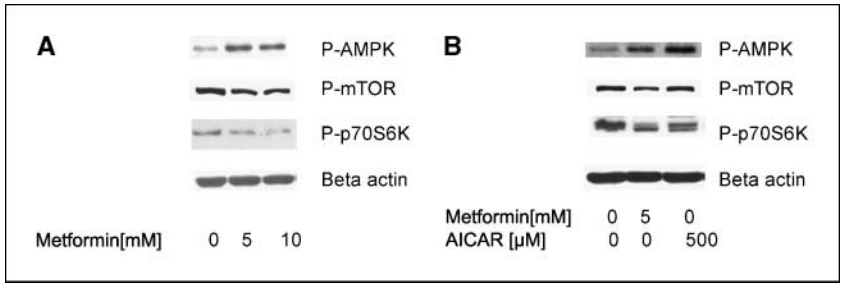

Figure 2. *A,* metformin activates AMPK and reduces p70S6K activation in MCF-7 cells. MCF-7 cells were treated with various doses of metformin for 72 hours. After harvesting, cells were lysed and prepared for immunoblot analysis using antibodies against phospho-AMPK (Thr^{172}), phosphorylated (Ser^{2448}) and non-phosphorylated mTOR, and phosphorylated (Thr^{389}) p70S6K. β-Actin is shown as a loading control. *B,* to determine if the AMP analogue AICAR has a similar effect to metformin, MCF-7 cells were treated with metformin or AICAR in the presence of 1% FBS for 72 hours. After harvesting, cells were lysed and prepared for immunoblot analysis using antibodies against phospho-AMPK (Thr^{172}), phospho-mTOR (Ser^{2448}), and phospho-p70S6K. β-Actin is shown as a loading control.

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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

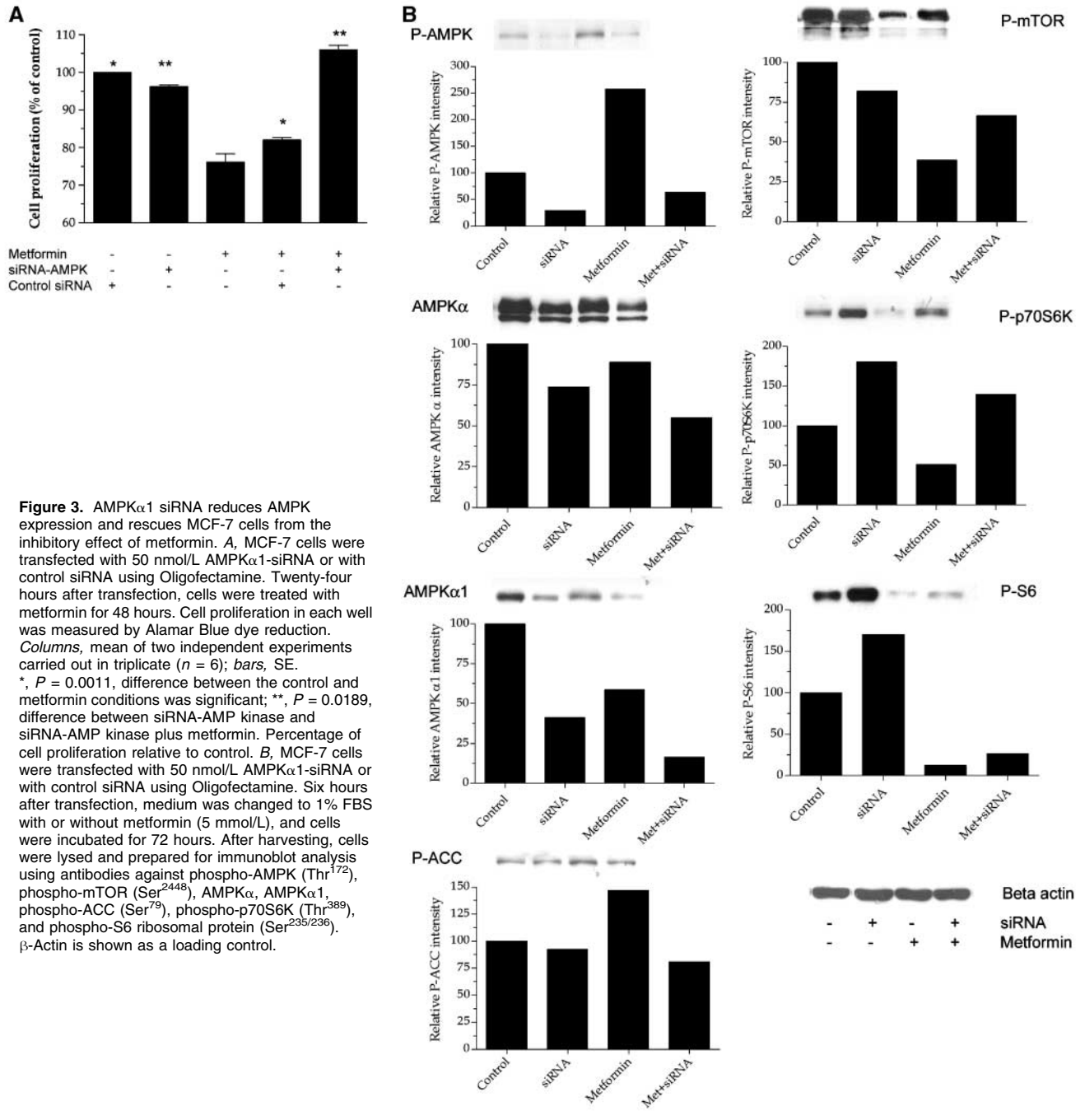

Figure 3. AMPKα1 siRNA reduces AMPK expression and rescues MCF-7 cells from the inhibitory effect of metformin. *A,* MCF-7 cells were transfected with 50 nmol/L AMPKα1-siRNA or with control siRNA using Oligofectamine. Twenty-four hours after transfection, cells were treated with metformin for 48 hours. 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 (Thr^{172}), phospho-mTOR (Ser^{2448}), AMPKα, AMPKα1, phospho-ACC (Ser^{79}), phospho-p70S6K (Thr^{389}), and phospho-S6 ribosomal protein ($Ser^{235/236}$). β-Actin is shown as a loading control.

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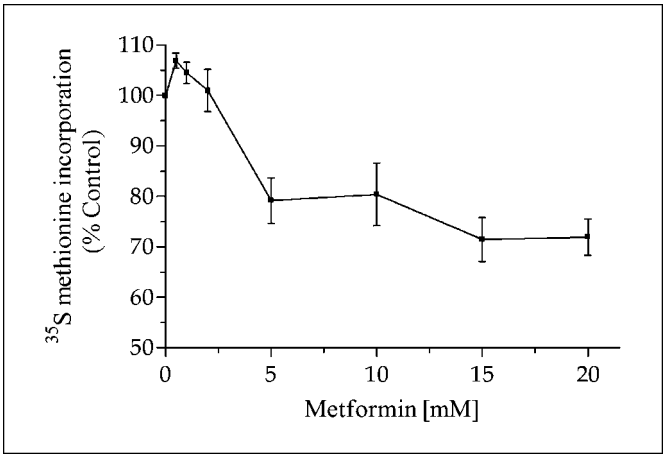

Figure 4. Inhibition of protein synthesis by metformin. MCF-7 cells were incubated with the indicated doses of metformin for 24 hours, and ^{35}S methionine-protein labeling mix (20 μCi/mL) was added to the cells for 45 minutes. Cells were harvested after labeling, and radioactivity incorporated into the TCA precipitable material was measured. Protein synthesis levels are displayed as a percentage of that exhibited in the absence of metformin. The experiment was carried out three separate times. *Points,* mean; *bars,* SE. $P = 0.0019$, difference between protein synthesis in the absence of metformin and in the presence of 15 mmol/L metformin was significant.

muscle and liver, the α1 isoform is physiologically more important than α2 isoform.

Antiproliferative actions of metformin on untransformed or transformed epithelial cells via AMP kinase–dependent pathways are in keeping with the role of AMP kinase as an energy sensor that down-regulates processes, such as protein synthesis, when energy is in short supply. Thus, by pharmacologically activating some of the intracellular control systems physiologically activated by nutrient deprivation, metformin acts as an inhibitor of proliferation. It is a classic experimental observation that severe dietary restriction protects rodents from a variety of carcinogenic influences (12). There is evidence (reviewed in ref. 13) that, at the 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 halploinsufficent 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.

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