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The anti-proliferative effect of metformin in triple-negative MDA-MB-231 breast cancer cells is highly dependent on glucose concentration: Implications for cancer therapy and prevention

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ABSTRACT

Background: Metformin has been shown to have a strong anti-proliferative effect in many breast cancer cell lines, mainly due to the activation of the energy sensing kinase, AMP-activated protein kinase (AMPK). MDA-MB-231 cells are aggressive and invasive breast cancer cells that are known to be resistant to several anti-cancer agents as well as to the anti-proliferative effect of metformin. As metformin is a glucose lowering drug, we hypothesized that normoglycemia will sensitize MDA-MB-231 cells to the anti-proliferative effect of metformin.
Methods: MDA-MB-231 cells were treated with increasing metformin concentrations in hyperglycemic or normoglycemic conditions. The growth inhibitory effect of metformin was assessed by MTT assay. The expression of several proteins involved in cell proliferation was measured by Western blotting.
Results: In agreement with previous studies, treatment with metformin did not inhibit the growth of MDA-MB-231 cells cultured in hyperglycemic conditions. However, metformin significantly inhibited MDA-MB-231 growth when the cells were cultured in normoglycemic conditions. In addition, we show that metformin-treatment of MDA-MB-231 cells cultured in normoglycemic conditions and not in hyperglycemic conditions caused a striking activation of AMPK, and an AMPK-dependent inhibition of multiple molecular signaling pathways known to control protein synthesis and cell proliferation.
Conclusion: Our data show that normoglycemia sensitizes the triple negative MDA-MB-231 breast cancer cells to the anti-proliferative effect of metformin through an AMPK-dependent mechanism.
General significance: These findings suggest that tight normoglycemic control may enhance the anti-proliferative effect of metformin in diabetic cancer patients.

1. Introduction

Breast cancer is the most commonly diagnosed cancer and the leading cause of cancer-related deaths among females worldwide [1]. In the United States, over 200,000 new breast cancer cases are diagnosed every year and approximately 40,000 of these diagnosed patients will die from the disease [1]. Breast cancers are usually classified according to their expression of estrogen receptors (ER), progesterone receptors (PR), or human epidermal growth factor receptor (HER2) [2]. Most of the current successful therapies for breast cancer, which include anti-estrogen therapies, aromatase inhibitors, or Herceptin, target these receptors [3]. Triple-negative breast cancers (TNBCs) which represent about 15% of breast cancer cases [4] do not express any of these receptors and are thus more difficult to treat with existing therapies. Moreover, TNBCs are more likely to metastasize thus resulting in poorer prognosis [5]. MDA-MB-231 cells are aggressive and invasive TNBC cells that are known to be resistant to several anti-cancer agents [6]. In addition to being triple-negative, MDA-MB-231 cells express a mutant p53 and lack the tumor-suppressor kinase LKB1, which make them even more resistant to treatment [7].

Several epidemiological studies have suggested that diabetes is associated with an increased risk of breast cancer [8,9]. Indeed, hyperglycemia and hyperinsulinemia are thought to promote the growth of cancer cells in diabetic patients [10]. Interestingly, metformin, the most commonly prescribed oral anti-diabetic medication, has been shown to have strong anti-proliferative and/or pro-apoptotic properties in several breast cancer cell lines. Indeed, metformin has been shown to inhibit the proliferation of BT474, BT20, MDA-MB-453, T47D, and MCF7 breast cancer cells independent of estrogen receptor, HER2, or p53 status [11]. In addition, metformin has been shown to induce caspase-dependent cell death in several breast cancer cell lines including MCF7, T47D, MDA-MB-453, and BT474 [12]. While the importance of a commonly prescribed and well-tolerated drug that may also function as a cancer therapy and/or in cancer prevention is of extreme clinical significance [13], several investigators have shown that metformin does not inhibit the proliferation or induce cell apoptosis of the aggressive MDA-MB-231 cancer cells [11,12]. Although definitive evidence is not available to explain why the LKB1-deficient MDA-MB-231 cancer cells are

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resistant to metformin, it has been proposed that metformin signals through LKB1 to exert both its anti-diabetic [14] and anti-proliferative [15] effects.

LKB1 is an important upstream kinase of the energy sensing enzyme AMP-activated protein kinase (AMPK) [16] and the activation of AMPK is the most widely accepted mechanism to explain the anti-cancer effects of metformin [17]. While likely not completely delineated, it is thought that metformin activates AMPK via an LKB1-dependent mechanism, which inhibits the mammalian target of rapamycin (mTOR) resulting in a strong inhibition of cell proliferation in several cancer cell lines [18–20]. Consistent with this, it has been suggested that metformin is not able to inhibit the proliferation of MDA-MB-231 cells because these cells do not express LKB1 and thus metformin fails to activate AMPK [18]. However, most of the previous studies have tested the effectiveness of metformin in inhibiting proliferation of cancer cells using culture conditions that contain high concentrations of glucose [21]. Importantly, given the fact that metformin-treated breast cancer diabetic patients have better clinical outcomes than non-metformin-treated patients [22], it is possible that normal serum concentrations of glucose may be involved in the degree of effectiveness of metformin treatment of cancer patients, potentially even in LKB1-deficient cells.

Of particular importance to understanding the role of metformin and serum concentrations of glucose and insulin in cancer patients, high concentrations of glucose and insulin have been shown to enhance the proliferation of MDA-MB-231 cells [23]. On the other hand, the anti-proliferative effect of metformin was dramatically enhanced when pancreatic cancer cells were cultured in media with physiological concentrations of glucose [21]. Based on these previous observations, we hypothesized that normal physiological concentrations of glucose sensitize MDA-MB-231 cells to the anti-proliferative effect of metformin. In the current work, we demonstrate for the first time that metformin significantly inhibits the proliferation of MDA-MB-231 cells when they are cultured in normoglycemic conditions. In addition, we show that the concentrations of metformin that are needed to prevent proliferation of MDA-MB-231 cells are much lower than those used in previous studies using other cell types. Together, our data show that LKB1-deficient MDA-MB-231 cells are not metformin-resistant in normoglycemic conditions and not only highlight the potential importance of good glycemic control as a requirement for the anti-proliferative action of metformin, but also question the involvement of LKB1 in these processes.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM) base, metformin, and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO). OCT-1 primary antibody was obtained from Santa Cruz Biotechnology Inc. (Dallas, TX, USA). BrdU cell proliferation assay kit and other primary and secondary antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Chemiluminescence Western blotting detection reagents were purchased from PerkinElmer. Nitrocellulose membrane was purchased from Bio-Rad Laboratories (Hercules, CA). Fetal bovine serum (FBS) was obtained from Invitrogen (Carlsbad, CA).

2.2. Cell culture

MDA-MB-231 cells were purchased from the ATCC. Wild-type (WT) and AMPKα1/α2 double knock-out (AMPK-DKO) mouse embryonic fibroblasts (MEFs) were generously provided by Dr. B. Viollet (Institut Cochin, Université Paris Descartes, CNRS (UMR 8104); and INSERM, U1016, Paris, France). MDA-MB-231 cells and MEFs were maintained in conventional high glucose (25 mM) DMEM with 10% fetal bovine serum (FBS) supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified 5% CO_2 incubator. Treatments were performed in serum free media supplemented with the specified glucose concentrations.

2.3. MTT assay

Cell viability was determined by measuring the capacity of reducing enzymes present in viable cells to convert MTT to formazan crystals as described previously [24]. Briefly, after incubating the cells with the increasing concentrations of metformin for 48 or 96 h in a 96-well cell culture plate at 37 °C under a 5% CO_2 humidified incubator, the media were removed and a 100 μl of serum-free media containing 1.2 mM MTT was added to each cell culture well. The plate was then re-incubated at 37 °C for 2 h. The media were then decanted off and 100 μl of isopropyl alcohol was added to each well followed by shaking for 1 h to dissolve the formed formazan crystals. The color intensity in each well was measured at a wavelength of 550 nm. The percentage of cell viability was calculated relative to control wells designated as 100% viable cells.

2.4. BrdU assay

Cell proliferation was assessed by measuring BrdU incorporation into cellular DNA during cell proliferation. Briefly, after incubating the cells with the increasing concentrations of metformin for 96 h in normoglycemic (5 mM glucose) medium in a 96-well cell culture plate at 37 °C under a 5% CO_2 humidified incubator, the BrdU reagent was added to each well and incubated for 16 h. Thereafter, the medium was removed and BrdU incorporation was measured according to manufacturer's instruction. The color intensity in each well was measured at a wavelength of 450 nm. The percentage of cell viability was calculated relative to control wells designated as 100% viable cells.

2.5. Protein extraction and Western blot analysis

Twenty four hours after incubation with increasing metformin concentrations, the cells were harvested in lysis buffer containing 20 mm Tris, 5 mM EDTA, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, and 1% NP-40, and supplemented with protease and phosphatase inhibitor cocktails. The total cell homogenate was prepared and the protein concentration was measured as described previously [25]. The cell homogenates (equivalent to 15 μg protein) were prepared in 3× sample buffer, boiled for 5 min, separated by either 8% or 15% SDS-polyacrylamide gel electrophoresis, and electrophoretically transferred to a nitrocellulose membrane. Protein blots were blocked for 1 h at room temperature in a blocking buffer containing 5% skim milk powder and 0.05% (v/v) Tween 20 in Tris-buffered saline solution (0.15 M sodium chloride, 3 mM potassium chloride, 25 mM Tris-base). After blocking, the blots were incubated with the specified primary antibody overnight at 4 °C in Tris-buffered saline solution containing 0.05% (v/v) Tween 20, 1% skim milk powder, and 0.02% sodium azide. Incubation with a peroxidase-conjugated secondary antibody was performed in a blocking buffer for 1 h at room temperature. The immune complex was visualized using the PerkinElmer chemiluminescence detection kit. The intensity of the phospho-protein bands was quantified, relative to the signals obtained for the total protein, using ImageJ software (National Institutes of Health, Bethesda, MD). Thereafter, the blots were stripped for 60 min in a stripping buffer at 50 °C, washed 3 times in Tris-buffered saline solution containing 0.05% (v/v) Tween 20, then blocked and probed as previously described. In some experiments, the blots were cut at the 100 kDa mark, so that the same blot can be probed with more than one primary anti-body at a time.

2.6. Statistical analysis

Data are presented as mean ± standard error of the mean (SE). Comparisons between control and metformin treatments were

performed using a one-way analysis of variance (ANOVA), with a Bonferroni post hoc test or a *t*-test for unpaired data when appropriate. A probability value of <0.05 is considered significant.

3. Results

3.1. Metformin inhibits MDA-MB-231 cell growth in normoglycemic but not in hyperglycemic conditions

To assess the effect of metformin on the growth of the triple-negative MDA-MB-231 breast cancer cells, the cells were cultured in 96-well plates in conventional high glucose medium containing 10% fetal bovine serum. After 24 h, the medium was changed to serum free medium supplemented with 25 mM glucose (hyperglycemic conditions) or 5 mM glucose (normoglycemic conditions). Thereafter, the cells were treated with increasing concentrations of metformin (from 0.1 mM to 5 mM) for 48 h or 96 h. In hyperglycemic conditions, none of the concentrations of metformin affected MDA-MB-231 cell growth at the 48 h (Fig. 1A) or at the 96 h (Fig. 1C) time-point. On the contrary, in normoglycemic conditions, metformin significantly inhibited MDA-MB-231 cell growth in a concentration-dependent manner at both the 48 h (Fig. 1B) and the 96 h (Fig. 1D) time-points. As expected, the growth inhibitory effect of metformin was greater after the 96 h treatment compared to that after the 48 h treatment. Curves from both time-points indicated an IC_{50} of approximately 2 mM and 1 mM at the 48 h and the 96 h, respectively. To further confirm the anti-proliferative effect of metformin, MDA-MB-231 cells were cultured in normoglycemic conditions for 96 h and cell proliferation was assessed by the BrdU cell proliferation assay. Similar to the MTT assay, metformin caused a significant inhibition of cell growth with an IC_{50} of 1 mM (data not shown).

3.2. Effect of metformin on AMPK signaling in MDA-MB-231 cells

As metformin is thought to inhibit the proliferation of cancer cells through the activation of AMPK [20], it was important to determine the effect of metformin on AMPK signaling. Therefore, the cells were treated with increasing concentrations of metformin (0.1–5 mM) in hyperglycemic and normoglycemic conditions for 24 h. In hyperglycemic conditions, metformin (1–5 mM) caused a modest two-fold increase in AMPK phosphorylation at the threonine 172 (T172) phosphorylation site, which is a surrogate marker for AMPK activation (Fig. 2A). In contrast, metformin (0.5–5 mM) caused a substantial concentration-dependent 4- to 8-fold activation of AMPK in normoglycemic conditions (Fig. 2B). In order to confirm the activation of AMPK by metformin, the phosphorylation status of one of its known downstream targets, acetyl CoA carboxylase (ACC), was measured. In agreement with the modest AMPK activation by metformin in hyperglycemic conditions, metformin caused a two-fold increase in ACC phosphorylation at the AMPK-specific phosphorylation site (Fig. 2C). On the other hand, metformin caused a more marked increase in ACC phosphorylation in normoglycemic conditions (Fig. 2D) that paralleled the substantial AMPK activation also observed, thus confirming AMPK activation by metformin.

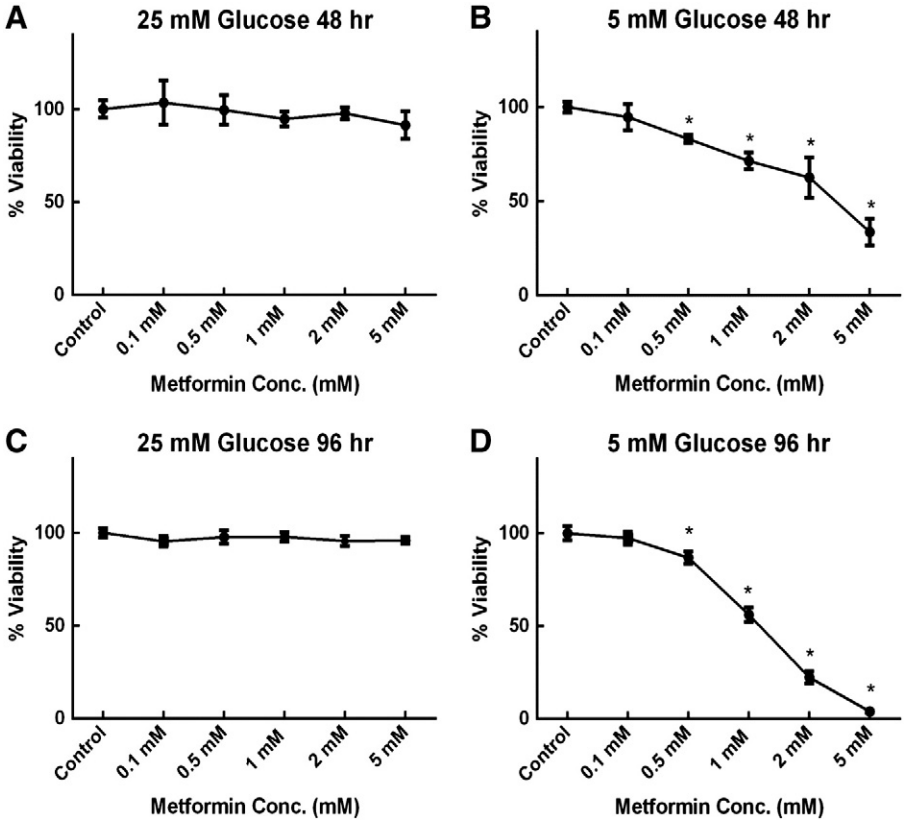


Fig. 1. Metformin inhibits MDA-MB-231 cell growth in normoglycemic but not in hyperglycemic conditions. MDA-MB-231 cells were treated with increasing concentrations of metformin (from 0.1 mM to 5 mM) for 48 h or 96 h in serum free medium supplemented with either 25 mM glucose (hyperglycemic conditions; panels A and C) or 5 mM glucose (normoglycemic conditions; panels B and D). Thereafter, the cell viability was determined by the MTT assay as described under Materials and methods. The percentage of cell viability was calculated relative to control wells designated as 100% viable cells. The values are presented as mean ± standard error of the mean (SE) and analyzed by ANOVA followed by a Bonferroni post-hoc test; *, $p < 0.05$ versus control ($n = 8$ from three independent experiments).

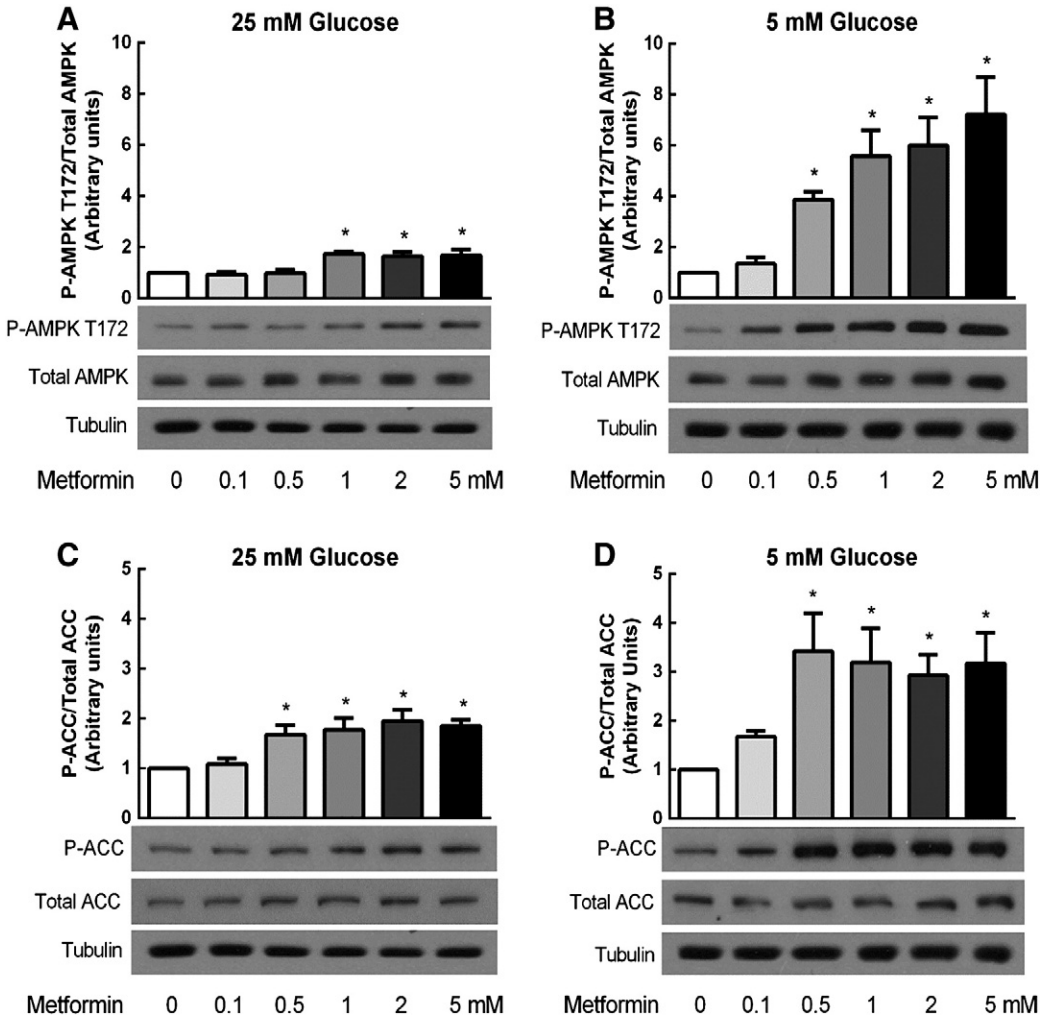


Fig. 2. Effect of metformin on AMPK signaling in MDA-MB-231 cells. MDA-MB-231 cells were harvested 24 h after incubation with increasing concentrations of metformin (0.1–5 mM) under hyperglycemic or normoglycemic conditions. Lysates from cells cultured in hyperglycemic medium (A) or in normoglycemic medium (B) were blotted with anti-phospho-AMPK(T172), anti-total-AMPK, and anti-tubulin antibodies. Results are presented as mean ± SE of densitometry measurements of P-AMPK/total AMPK; *, $p < 0.05$ versus control ($n = 4$; three independent experiments). Similarly, lysates from cells cultured in hyperglycemic medium (C) or in normoglycemic medium (D) were blotted with anti-phospho-ACC(Ser79), anti-total-ACC, and anti-tubulin antibodies. Results are presented as mean ± SE of densitometry measurements of P-ACC/total ACC; *, $p < 0.05$ versus control ($n = 4$; three independent experiments). The blots were stripped and cut as described under Materials and methods, so that the same blot can be probed with several primary antibodies.

3.3. Effect of metformin on the mTOR signaling pathway

In an attempt to determine the mechanism responsible for the anti-proliferative effect of metformin, its effect on mTOR signaling was assessed. As mTOR is activated by phosphorylation at Ser2448 to promote protein synthesis and cell proliferation [26], we used phospho-specific antibodies to this site in order to assess mTOR activity. In hyperglycemic conditions, metformin did not cause any change in mTOR phosphorylation at Ser2448, which parallels metformin's lack of effect on MDA-MB-231 cell proliferation when cultured in hyperglycemic conditions (Fig. 3A). In contrast, metformin caused a significant concentration-dependent 50%–75% inhibition of mTOR phosphorylation at concentrations of 0.5 to 5 mM, respectively (Fig. 3B). In addition to measuring mTOR activation, we also examined the phosphorylation of the regulatory associated protein of mTOR (Raptor) at site Ser792, which is a site of inhibitory phosphorylation [27]. Raptor acts as a scaffold to recruit downstream substrates such as p70S6K to the mTORC1 complex via the TOR signaling motif [15], and its inhibition would result in inability of the mTORC1 to act on its downstream targets. While metformin did not cause any significant changes of Raptor in hyperglycemic conditions (Fig. 3C), metformin caused a striking inhibition of Raptor by increasing its phosphorylation at the Ser792 site in MDA-MB-231 cells cultured in normoglycemic conditions (Fig. 3D).

3.4. Effect of metformin on p70S6K and ribosomal S6 protein

As p70S6K and its downstream target, ribosomal S6 protein, are crucial players in protein synthesis and cell proliferation [28], the effect of metformin on these proteins was assessed. In agreement with the lack of a metformin effect on cell proliferation in hyperglycemic conditions, metformin had no effect on p70S6K T389 phosphorylation in MDA-MB-231 cells cultured in hyperglycemic conditions (Fig. 4A), suggesting no change in p70S6K activity. On the contrary, metformin caused a significant 95% inhibition of p70S6K T389 phosphorylation under normoglycemic conditions, suggesting that this may be involved in the strong anti-proliferative effect of metformin in such conditions (Fig. 4B). Indeed, phosphorylation at Thr389 is critical for the kinase function and closely correlates with p70S6K activity in vivo [28]. Similarly, metformin did not cause any significant change of p70S6K T421 phosphorylation in hyperglycemic conditions; whereas it caused a marked inhibition of p70S6K T421 phosphorylation in normoglycemic conditions (Fig. 4C and D). Phosphorylation at the T421 site is thought

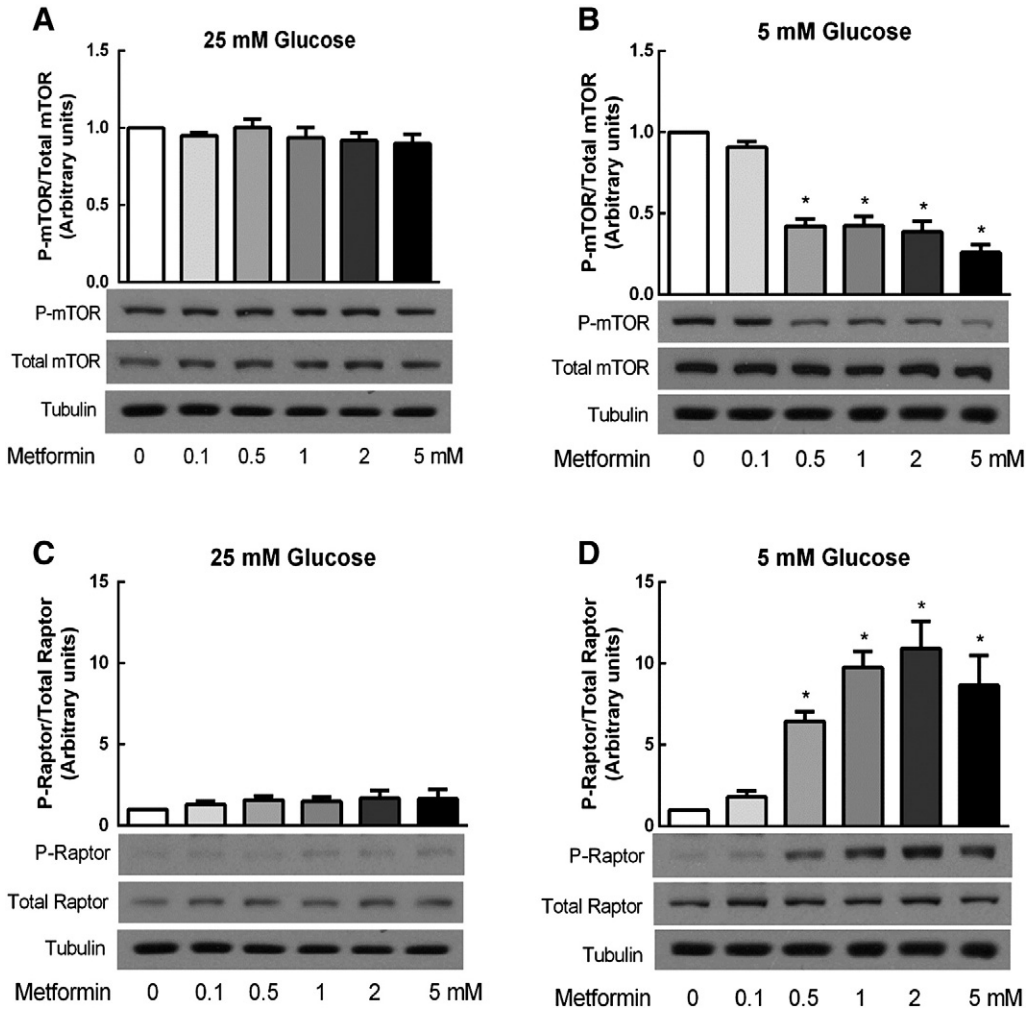


Fig. 3. Effect of metformin on mTOR signaling in MDA-MB-231 cells. MDA-MB-231 cells were harvested 24 h after incubation with increasing concentrations of metformin (0.1–5 mM) under hyperglycemic or normoglycemic conditions. Lysates from cells cultured in hyperglycemic medium (A) or in normoglycemic medium (B) were blotted with anti-phospho-mTOR(Ser2448), anti-total-mTOR, and anti-tubulin antibodies. Results are presented as mean ± SE of densitometry measurements of P-mTOR/total mTOR; *, $p < 0.05$ versus control ($n = 4$; three independent experiments). Similarly, lysates from cells cultured in hyperglycemic medium (C) or in normoglycemic medium (D) were blotted with anti-phospho-Raptor(Ser792), anti-total-Raptor, and anti-tubulin antibodies. Results are presented as mean ± SE of densitometry measurements of P-Raptor/total Raptor; *, $p < 0.05$ versus control ($n = 4$; three independent experiments). The blots were stripped and cut as described under Materials and methods, so that the same blot can be probed with several primary antibodies.

to activate p70S6K by relieving pseudosubstrate inhibition effects [28]. As expected with unaltered p70S6K activity, metformin did not cause any change in ribosomal S6 phosphorylation under hyperglycemic conditions (Fig. 4E). However, metformin did cause a substantial inhibition of ribosomal S6 phosphorylation when cells were cultured in normoglycemic conditions (Fig. 4F), which is in agreement with the significant drop in p70S6K activation under these conditions.

3.5. Effect of metformin on the MAPK pathways

The MAPK pathways, which include ERK1/2, p38, and JNK, are very important regulators of cancer cell proliferation, survival, motility, and invasiveness [29,30]. Therefore, to examine whether or not metformin exerted its anti-proliferative effect via these pathways, we investigated the effect of metformin effect on these kinases. While metformin had no significant effect on ERK1/2 phosphorylation in MDA-MB-231 cells cultured in hyperglycemic conditions (Fig. 5A), it caused a dramatic inhibition of ERK1/2 phosphorylation when the cells were treated in normoglycemic conditions (Fig. 5B). In contrast to ERK1/2, metformin caused a modest activation of the p38 MAPK in hyperglycemic conditions (Fig. 5C), but a substantial concentration-dependent p38 activation in normoglycemic conditions (Fig. 5D). There was no change in JNK phosphorylation by metformin either in hyperglycemic or in normoglycemic conditions(data not shown).

3.6. Effect of metformin on apoptotic markers

In order to investigate whether metformin also has pro-apoptotic effects in MDA-MB-231 cells cultured in normoglycemic conditions, MDA-MB-231 cells were cultured in 5 mM glucose concentration with increasing metformin concentrations (0.1–5 mM) for 24 h; thereafter the expression of the apoptotic markers cleaved-caspase 3, cleaved-caspase 6, cleaved-caspase 7, cleaved-caspase 9, BAX, and cleaved PARP was measured by Western blotting. Metformin did not cause any significant change in these apoptotic markers under the aforementioned experimental conditions (data not shown).

3.7. Effect of glucose deprivation on OCT-1 expression and AMPK activation

As the organic cation transporter 1 (OCT-1) is the transporter required for the uptake of metformin [31], it was important to investigate whether the increased sensitivity of MDA-MB-231 cells to metformin in normoglycemic conditions is due to altered expression of this transporter. Therefore, the cells were cultured in a range of decreasing glucose concentrations (from 25 mM to 0 mM) for 24 h; thereafter the

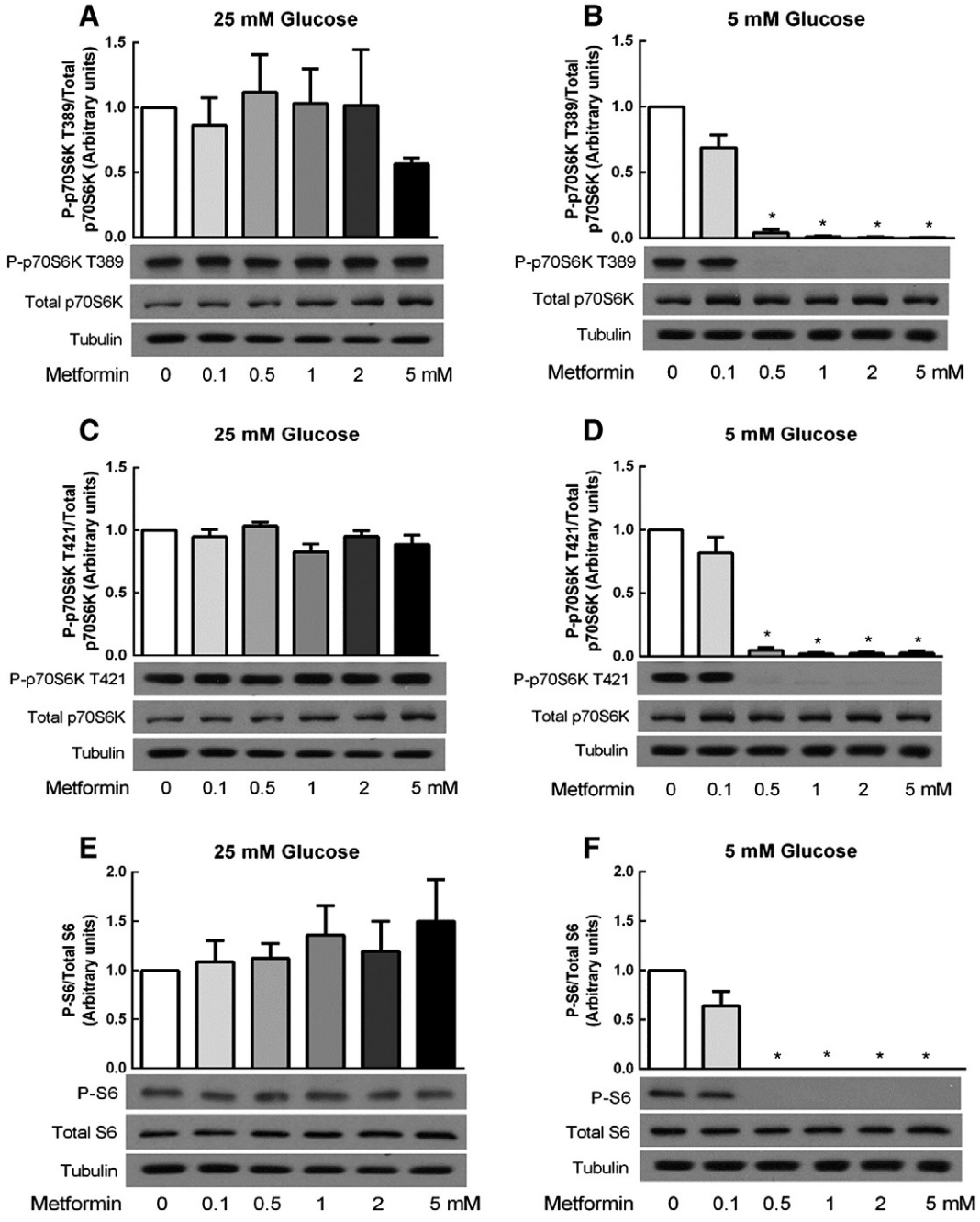


Fig. 4. Effect of metformin on p70S6K and ribosomal S6 protein. MDA-MB-231 cells were harvested 24 h after incubation with increasing concentrations of metformin (0.1–5 mM) under hyperglycemic or normoglycemic conditions. Lysates from cells cultured in hyperglycemic medium (A and C) or in normoglycemic medium (B and D) were blotted with anti-phospho-p70S6K (T389 and T421, respectively), anti-total-p70S6K, and anti-tubulin antibodies. Results are presented as mean ± SE of densitometry measurements of P-p70S6K/total p70S6K; *, $p < 0.05$ versus control ($n = 4$; three independent experiments). Similarly, lysates from cells cultured in hyperglycemic medium (E) or in normoglycemic medium (F) were blotted with anti-phospho-S6(Ser240/244), anti-total-S6, and anti-tubulin antibodies. Results are presented as mean ± SE of densitometry measurements of P-S6/total S6; *, $p < 0.05$ versus control ($n = 4$; three independent experiments). The blots were stripped and cut as described under Materials and methods, so that the same blot can be probed with several primary antibodies.

expression of OCT-1 was measured by Western blotting. There was no significant change in OCT-1 expression in MDA-MB-231 cells with glucose deprivation (Fig. 6A) which excludes the possibility that the increased metformin sensitivity is due to a change in its uptake through the OCT-1 transporter. In addition, we assessed the effect of glucose deprivation on AMPK activation to investigate whether the cells cultured in normoglycemic conditions exhibit a moderate increase in baseline AMPK activation, which makes it more sensitive to metformin. After 24 h incubation with a range of decreasing glucose concentrations (25 mM to 5 mM), there was no significant change in AMPK phosphorylation at the activation site T172. However, complete glucose deprivation (0 mM glucose) caused a significant 2 fold increase in AMPK activation (Fig. 6B).

3.8. The effects of metformin are not altered in the presence of insulin

In type 2 diabetic patients, hyperglycemia is usually associated with significant hyperinsulinemia. Taking into account that insulin also stimulates breast cancer growth [32], it was important to investigate whether insulin could modulate the metformin effects on MDA-MB-231 cells. Therefore, the effect of metformin on MDA-MB-231 cells was assessed in hyperglycemic and normoglycemic

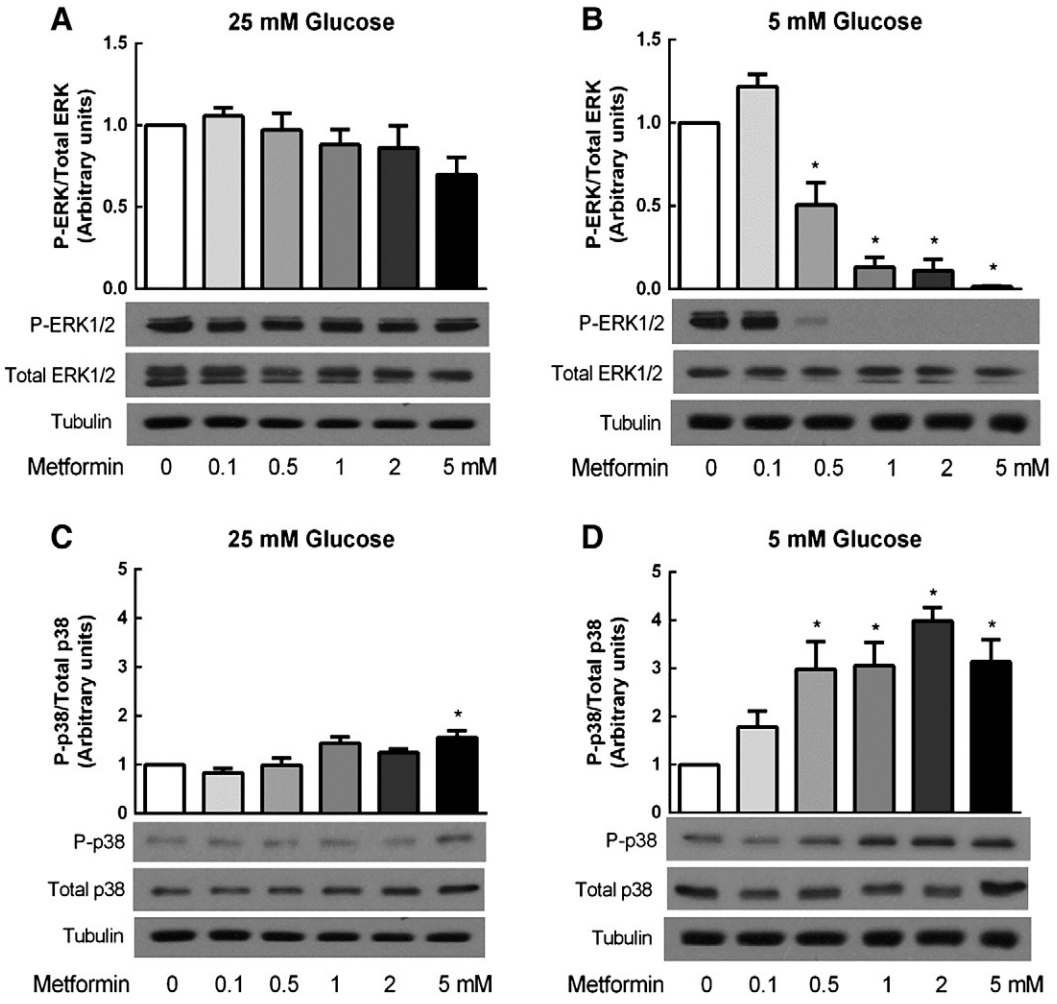


Fig. 5. Effect of metformin on the MAPK pathway. MDA-MB-231 cells were harvested 24 h after incubation with increasing concentrations of metformin (0.1–5 mM) under hyperglycemic or normoglycemic conditions. Lysates from cells cultured in hyperglycemic medium (A) or in normoglycemic medium (B) were blotted with anti-phospho-ERK1/2(Thr202/Tyr204), anti-total-ERK1/2, and anti-tubulin antibodies. Results are presented as mean ± SE of densitometry measurements of P-ERK1/2/total ERK1/2; *, $p < 0.05$ versus control ($n = 4$; three independent experiments). Similarly, lysates from cells cultured in hyperglycemic medium (C) or in normoglycemic medium (D) were blotted with anti-phospho-p38(Thr180/Tyr182), anti-total-p38, and anti-tubulin antibodies. Results are presented as mean ± SE of densitometry measurements of P-p38/Total p38; *, $p < 0.05$ versus control ($n = 4$; three independent experiments). The blots were stripped and cut as described under Materials and methods, so that the same blot can be probed with several primary antibodies.

conditions in the presence of a range of insulin concentrations: 10 μg/ml which represents an insulin concentration used in some culture media, 100 ng/ml which represents plasma insulin concentration in severe hyperinsulinemic patients, and 1 ng/ml which represents the physiological plasma insulin concentration. In hyperglycemic conditions, there was no significant change in ERK or p70S6K phosphorylation by metformin in all the examined insulin concentrations (Fig. 7A, C, E). In contrast, in normoglycemic conditions, metformin caused a significant inhibition of ERK phosphorylation (Fig. 7B), as well as p70S6K phosphorylation at both sites T389 and T421 (Fig. 7D and F) in the presence of insulin at the three examined concentrations. Of interest, higher insulin concentrations caused a significant increase in p70S6K phosphorylation at both sites T389 and T421 as compared to the physiological insulin concentration when the cells are cultured under normoglycemic conditions (Fig. 7D and F).

3.9. Compound C reverses the effect of metformin in MDA-MB-231 cells cultured in normoglycemic conditions

In order to investigate whether the inhibition of mTOR by metformin was due to AMPK activation, cells were treated with metformin in the absence and presence of the AMPK inhibitor, compound C. As expected, compound C caused a significant inhibition of metformin-induced AMPK activation (Fig. 8A) as well as the inhibition of ACC phosphorylation (Fig. 8B). Of interest, treatment with compound C prevented the inhibitory effect of the mTOR (Fig. 8C) and Raptor (Fig. 8D) by metformin demonstrating that the inhibition of mTOR and Raptor was due to metformin-induced AMPK activation. Similar to the mTOR/Raptor signaling, treatment of cells with compound C also restored metformin-inhibited ERK1/2 phosphorylation (Fig. 9A) as well as metformin-induced p38 activation (Fig. 9B), confirming that the changes in MAPK signaling were also due to metformin-induced AMPK activation. As a downstream target of both mTOR and ERK1/2, p70S6K phosphorylation at both T389 and T421 was also completely normalized by treatment with compound C (Fig. 9C and D) as was ribosomal S6 (Fig. 9E). To demonstrate that metformin effects on proliferation are specifically caused by AMPK activation, MDA-MB-231 cells were cultured in normoglycemic conditions in the presence of the AMPK inhibitor, compound C (5 μM). After 2 h, the cells were treated with increasing metformin concentrations (0.1–5 mM), and cell growth was assessed by the MTT assay after 48 h as described under the Materials and methods. Interestingly, metformin had no effect on MDA-MB-231 cell growth in the presence of

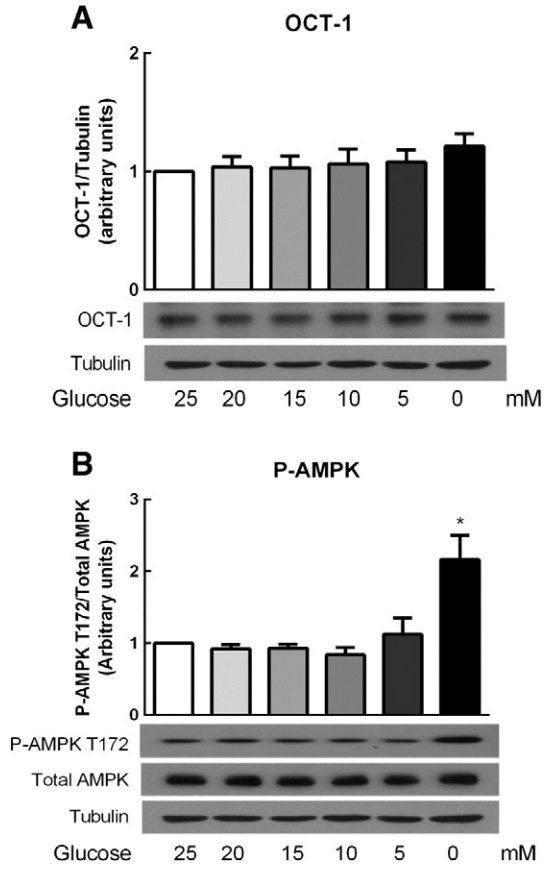


Fig. 6. Effect of glucose deprivation on OCT-1 expression and AMPK activation. MDA-MB-231 cells were harvested 24 h after incubation with decreasing concentrations of glucose (25–0 mM). Total cell lysates were blotted with anti-OCT-1 and anti-tubulin antibodies. Results are presented as mean ± SE of densitometry measurements of OCT-1/tubulin; *, $p < 0.05$ versus control ($n = 4$; four independent experiments) (A). Similarly, total cell lysates were blotted with anti-phospho-AMPK(T172), anti-total AMPK, and anti-tubulin antibodies. Results are presented as mean ± SE of densitometry measurements of P-AMPK/total AMPK; *, $p < 0.05$ versus control ($n = 4$; four independent experiments). The blots were stripped as described under Materials and methods, so that the same blot can be probed with several primary antibodies.

compound C, further supporting that AMPK activation is the main mechanism of the anti-proliferative effect of metformin (Fig. 9F).

3.10. Metformin has no effect on ERK1/2 or p70S6K signaling in AMPK-DKO MEFs

To further confirm that metformin effects are AMPK-dependent, we treated WT and AMPK-DKO MEFs with 2 mM metformin for 24 h in normoglycemic medium; thereafter we assessed the effect of metformin on AMPK, ERK, and p70S6K signaling. Fig. 10A demonstrates that metformin caused a significant AMPK activation in the WT MEFs, whereas the AMPK-DKO MEFs do not express the AMPK protein (Fig. 10A). In agreement with the previous chemical inhibition results, metformin caused a significant inhibition of ERK phosphorylation in the WT but not in the AMPK-DKO MEFs (Fig. 10B). Similarly, metformin caused a significant inhibition of p70S6K phosphorylation at both phosphorylation sites T389 and T421 in the WT MEFs but not in the AMPK-DKO MEFs (Fig. 10C and D). Taken together, these data confirm that the aforementioned metformin effects are AMPK-dependent.

4. Discussion

Several lines of evidence drawn from in vitro, in vivo, and epidemiological studies have suggested that metformin may be of benefit to diabetic cancer patients [33,34]. Of interest, breast cancer risk has been shown to be lower in diabetic patients treated with metformin than in those treated with other anti-diabetic medications [35]. In addition, metformin-treated diabetic breast cancer patients have better clinical outcomes than non metformin-treated patients [22]. On the other hand, some studies have also shown that metformin use did not affect the risk of breast cancer, although it was associated with lower risk of colon and pancreatic cancer in type 2 diabetic patients [36]. Mechanistically, it is believed that metformin inhibits complex 1 of the mitochondrial respiratory chain, reduces ATP synthesis and increases the AMP/ATP ratio, thus activating the energy sensing enzyme AMPK [37]. AMPK activation is thought to inhibit the growth of cancer cells by switching off protein synthesis and cell proliferation [38]. However, recent studies have shown that metformin may also have anti-cancer effects independent of AMPK activation [39]. Therefore, further research is warranted to confirm the clinical benefit of metformin in breast cancer patients as well as its exact mechanism of action.

The aggressive TNBC MDA-MB-231 cells have been shown by several investigators to be "metformin resistant" [11,18] and it has been proposed that this metformin-resistance is due to the fact that MDA-MB-231 cells lack LKB1 and thus cannot activate AMPK [18]. Interestingly, Zhuang and Miskimins have shown that metformin (8 mM) treatment of MDA-MB-231 cells for 2 days caused a modest activation of AMPK [11], suggesting that other kinases may be responsible for metformin-induced AMPK activation in the absence of LKB1. Indeed, the authors postulated that MDA-MB-231 cells are "metformin-resistant" because they express very low levels of $p27^{Kip1}$ and $p21^{Cip1}$ [11]. Regardless of the pathways involved, it was shown that a modest increase in AMPK activity was not sufficient to inhibit MDA-MB-231 cell proliferation [11]. In contrast to these previous studies, the current work demonstrates for the first time that metformin can activate AMPK when MDA-MB-231 cells are cultured in normoglycemic conditions and that this activation has a strong anti-proliferative effect. As such, our findings may help explain why MDA-MB-231 cells were first thought to be "metformin-resistant" and indicate that AMPK can be activated in these cells by metformin even in the absence of LKB1 and very low levels of $p27^{Kip1}$ and $p21^{Cip1}$.

In agreement with Zhuang and Miskimins [11], we demonstrate that metformin causes a modest activation of AMPK in MDA-MB-231 cells cultured in hyperglycemic conditions. Similar to their work, we also show that the modest activation of AMPK was not sufficient to inhibit MDA-MB-231 cell proliferation. However, in contrast to that observed in cells cultured in hyperglycemic conditions, metformin caused a significant and robust activation of AMPK in MDA-MB-231 cells when they are cultured in normoglycemic conditions. Although MDA-MB-231 cells do not express the major AMPK kinase, LKB1 [16], metformin was still able to activate AMPK suggesting that other kinases may be involved. Indeed, calmodulin-dependent protein kinase kinase, ATM, and other unidentified kinases may be responsible for AMPK activation in cells lacking LKB1 [40,41]. In agreement with our results, a novel AMPK activator, OSU-53, and the natural compound, demethoxycurcumin, were able to activate AMPK in MDA-MB-231 cells despite the lack of LKB1 expression [42,43]. Interestingly, recent research has suggested that metformin can even be more effective against cancer cells with an inactive LKB1 [44]. While we currently do not provide evidence to explain why metformin only activates AMPK in MDA-MB-231 cells when they are cultured in normoglycemic conditions, we speculate that in hyperglycemic conditions, as the glucose uptake rate is very high, cancer cells generate most of their ATP through aerobic glycolysis to meet the demands of rapidly proliferating cells [45]. In contrast, in normoglycemic conditions, the glucose uptake rate becomes lower and the cells depend more on mitochondrial respiration for their ATP needs [46]. Therefore, cancer cells cultured in normoglycemic conditions may become much more sensitive to metformin as metformin inhibits mitochondrial oxidative phosphorylation [21]. Taken together, metformin treatment coupled with limited glucose supply may cause a striking increase in the cellular AMP/ATP ratio and the activation of

AMPK by increasing its phosphorylation by upstream kinases as well as by making it a worse substrate for the competing phosphatases [47]. Similar to other cancer cell lines [48], we have shown that complete glucose deprivation for 24 h has caused a modest AMPK activation in MDA-MB-231 cells. However, the physiological glucose concentration (5 mM) used in the rest of our study did not cause any significant AMPK activation, which confirms that AMPK activation in normoglycemic conditions is indeed due to metformin not due to the physiological glucose concentration used.

To further elucidate the molecular mechanism(s) responsible for controlling the anti-proliferative effect of metformin, we investigated the effect of metformin on mTOR signaling which is a central regulator of

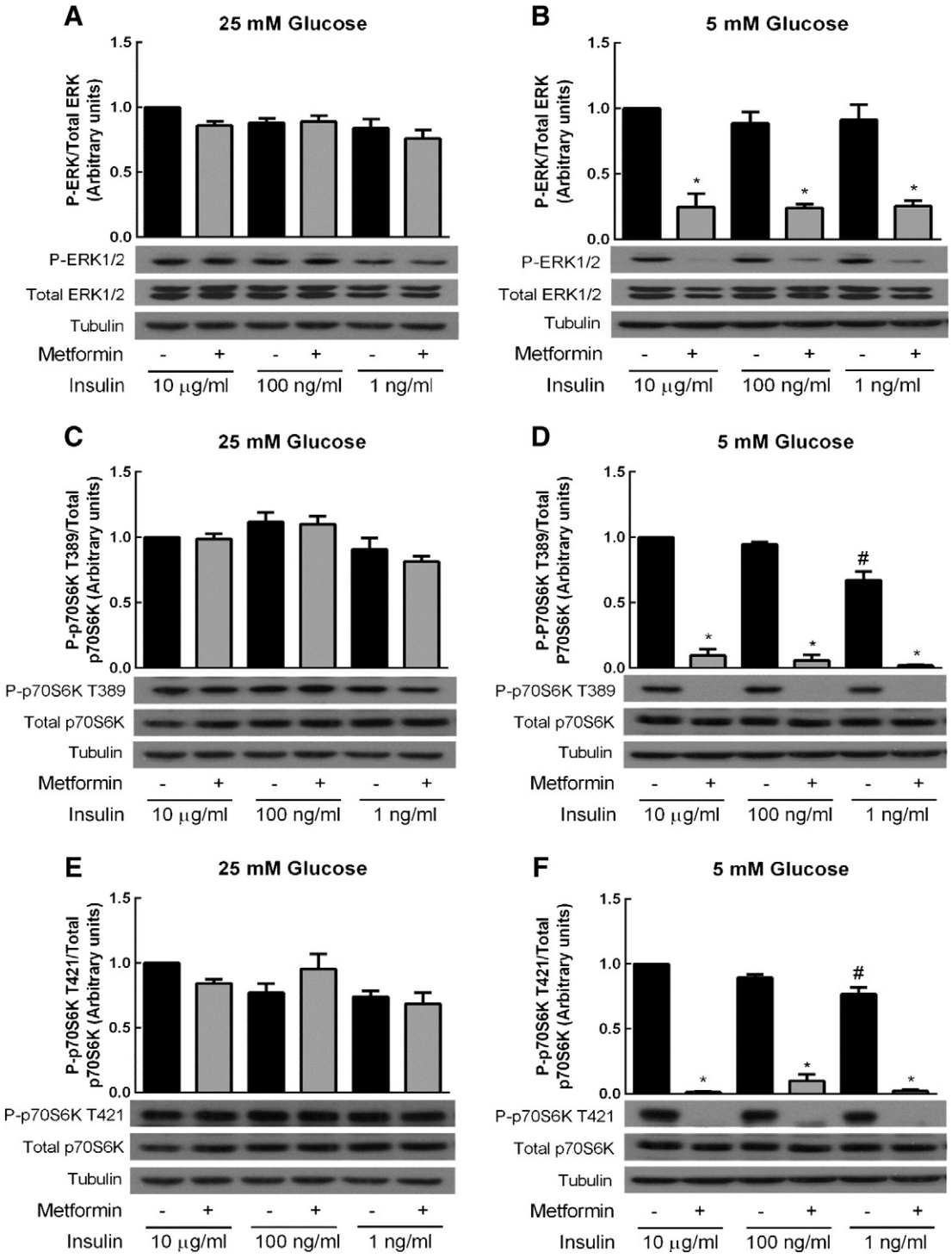


Fig. 7. Effect of metformin on ERK1/2 and p70S6K signaling in the presence of insulin. MDA-MB-231 cells were harvested after 24 h incubation with metformin 1 mM in the presence of a range of insulin concentrations in normoglycemic and in hyperglycemic conditions. Lysates from cells cultured in hyperglycemic medium (A) or in normoglycemic medium (B) were blotted with anti-phospho-ERK1/2(Thr202/Tyr204), anti-total-ERK1/2, and anti-tubulin antibodies. Results are presented as mean ± SE of densitometry measurements of P-ERK1/2/Total ERK1/2; *, $p < 0.05$ versus control ($n = 3$; three independent experiments). Similarly, lysates from cells cultured in hyperglycemic medium (C and E) or in normoglycemic medium (D and F) were blotted with anti-phospho-p70S6K (T389 and T421, respectively), anti-total-p70S6K, and anti-tubulin antibodies. Results are presented as mean ± SE of densitometry measurements of P-p70S6K/total p70S6K; *, $p < 0.05$ versus non-metformin treated cells; # $p < 0.05$ versus higher insulin concentrations ($n = 3$; three independent experiments). The blots were stripped as described under Materials and methods, so that the same blot can be probed with several primary antibodies.

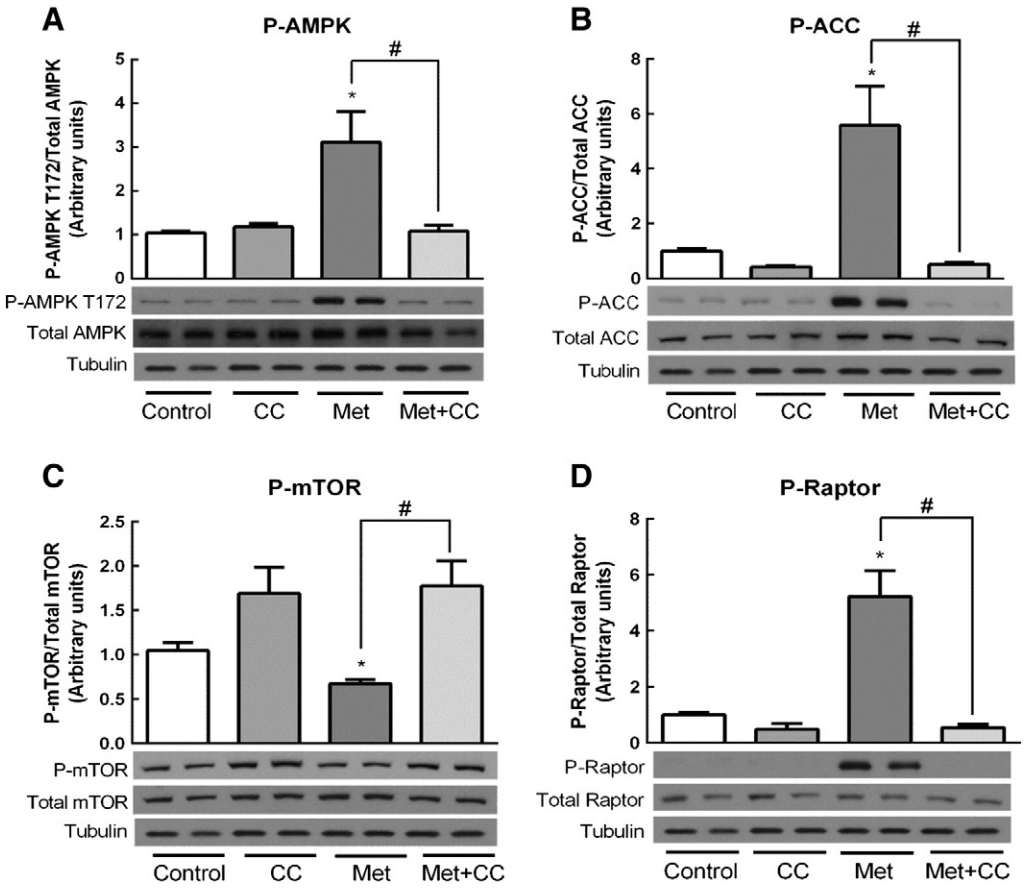


Fig. 8. Compound C reverses the effect of metformin on AMPK and mTOR signaling. MDA-MB-231 cells were harvested 24 h after incubation with 1 mM metformin (Met) in the absence or presence of 5 μM of the AMPK inhibitor compound C (CC) in normoglycemic conditions. Cell lysates were blotted with anti-phospho-AMPK(T172), anti-total-AMPK, and anti-tubulin antibodies (A); with anti-phospho-ACC(Ser79), anti-total-ACC, and anti-tubulin antibodies (B); with anti-phospho-mTOR(Ser2448), anti-total-mTOR, and anti-tubulin antibodies (C); or with anti-phospho-Raptor(Ser792), anti-total-Raptor, and anti-tubulin (D). Results are presented as mean ± SE of densitometry measurements of the phospho/total proteins; *, $p < 0.05$ versus control; # $p < 0.05$ versus metformin-treated cells ($n = 4$; three independent experiments). The blots were stripped and cut as described under Materials and methods, so that the same blot can be probed with several primary antibodies.

protein synthesis and cell proliferation [18]. In accordance with its anti-proliferative effect, metformin caused a significant inhibition of mTOR phosphorylation in MDA-MB-231 cells cultured in normoglycemic conditions. In contrast, metformin had no effect on mTOR signaling in cells cultured in hyperglycemic conditions, which may help explain the lack of any anti-proliferative effect of metformin in MDA-MB-231 cells in these conditions. In addition, the inhibition of mTOR signaling by metformin in cells cultured in normoglycemic conditions may be attributed to the effect on Raptor. Of interest, it has been shown that Raptor is directly phosphorylated by AMPK in two conserved serine residues, and this inhibitory phosphorylation is required for the inhibition of mTORC1 [27]. In the present work, we not only demonstrate for the first time that metformin significantly inhibits Raptor in MDA-MB-231 cells cultured in normoglycemic conditions and not hyperglycemic conditions, but also confirm that this effect is due to metformin-induced AMPK activation.

Since its discovery, p70S6K has been shown to be a central player in promoting protein synthesis, cell proliferation, and cell survival [28]. Therefore, the mTOR–p70S6K signaling pathway has been an important target of anti-cancer therapies [49] and metformin has been shown to inhibit this pathway in several cell lines [50]. Similarly, our present work demonstrates a very strong inhibitory effect of metformin on p70S6k phosphorylation and activation, which was only achieved when cells were cultured in normoglycemic conditions. Of interest, a relatively low metformin concentration of 0.5 mM was sufficient to significantly inhibit p70S6k and its downstream target ribosomal S6 phosphorylation. While the inhibition of p70S6k by metformin could be attributed to the inhibition of TSC1 through the inhibition of both mTOR and its partner raptor or to ERK1/2 inhibition by metformin (as ERK1/2 can also phosphorylate p70S6k [51]), we also demonstrate that inhibition of p70S6K by metformin is AMPK-dependent, as treatment of cells with the AMPK inhibitor, compound C, completely restored p70S6K phosphorylation. In addition, metformin inhibited p70S6K phosphorylation in WT MEFs but not in AMPK-DKO MEFs, which further confirms that the metformin inhibitory effect on p70S6K is AMPK-dependent. Taking into account the strong proliferative effect of activated p70S6K, it can be concluded that the anti-proliferative effect of metformin in MDA-MB-231 cells cultured in normoglycemic conditions is attributed, at least in part, to the AMPK-dependent p70S6K inhibition. Nevertheless, further work should be performed using cells transfected with constitutively active p70S6K or in p70S6K null cells to confirm the exact contribution of the p70S6K pathway in the anti-proliferative effect of metformin.

Multiple cross-talks and feedback loops have been recently identified between the mTOR and the ERK1/2 pathways [26]. It has been shown that activation of the ERK1/2 pathway leads to enhanced activation of the mTOR–p70S6K pathway [52]. In addition, ERK1/2 has been shown to activate p70S6K either directly [51] or through the p90RSK pathway [53]. Nevertheless, in some scenarios, inhibition of one pathway can lead to a compensatory activation of the other, resulting in a less pronounced anti-proliferative effect [26]. Of interest, in our study, metformin caused a significant inhibition of both the mTOR and the ERK1/2 pathways in MDA-MB-231 cells cultured in normoglycemic conditions and not in hyperglycemic conditions. We speculate that inhibition of ERK1/2 by metformin results from inhibitory crosstalk between AMPK and ERK1/2 (Fig. 11), which has been reported

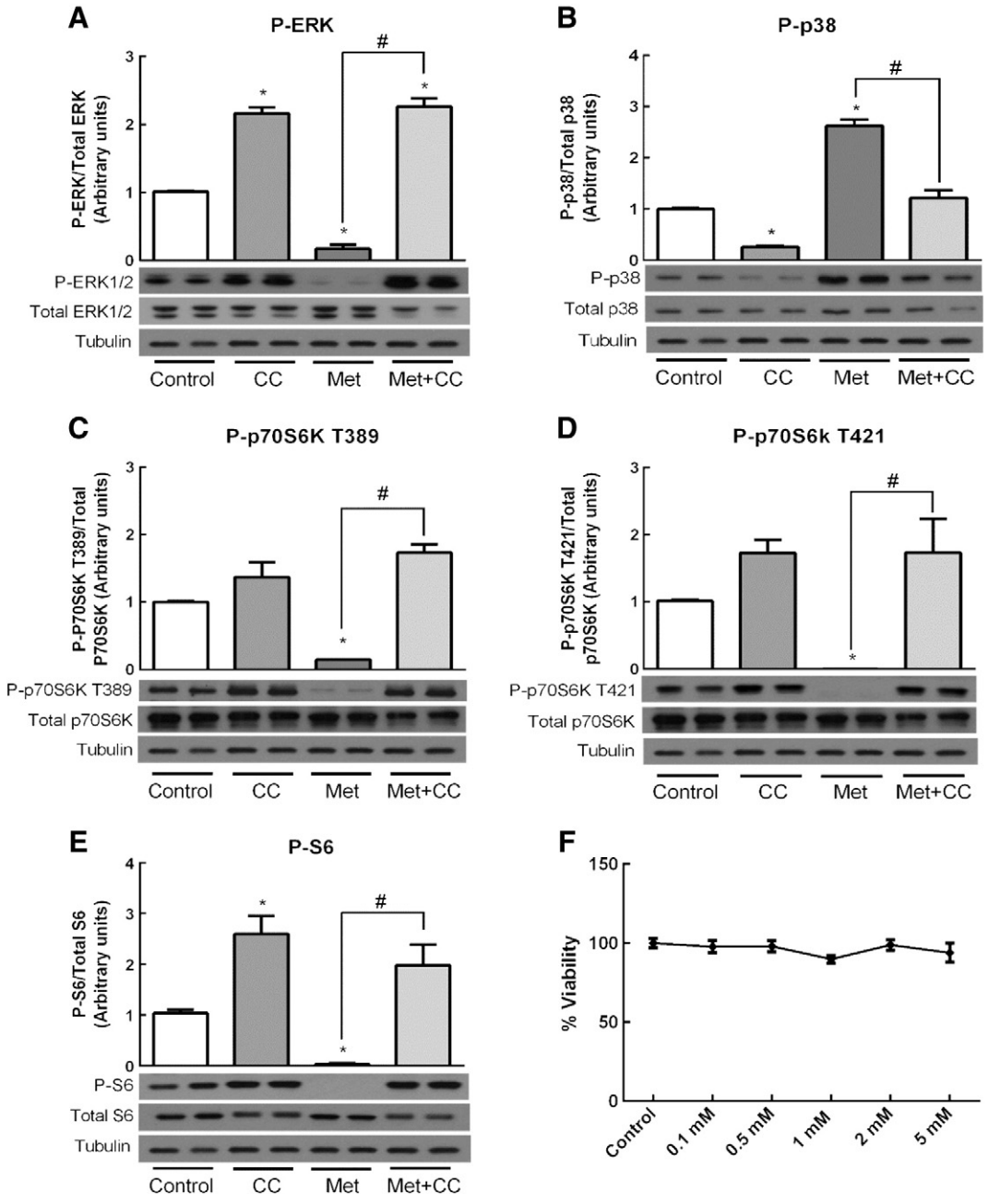
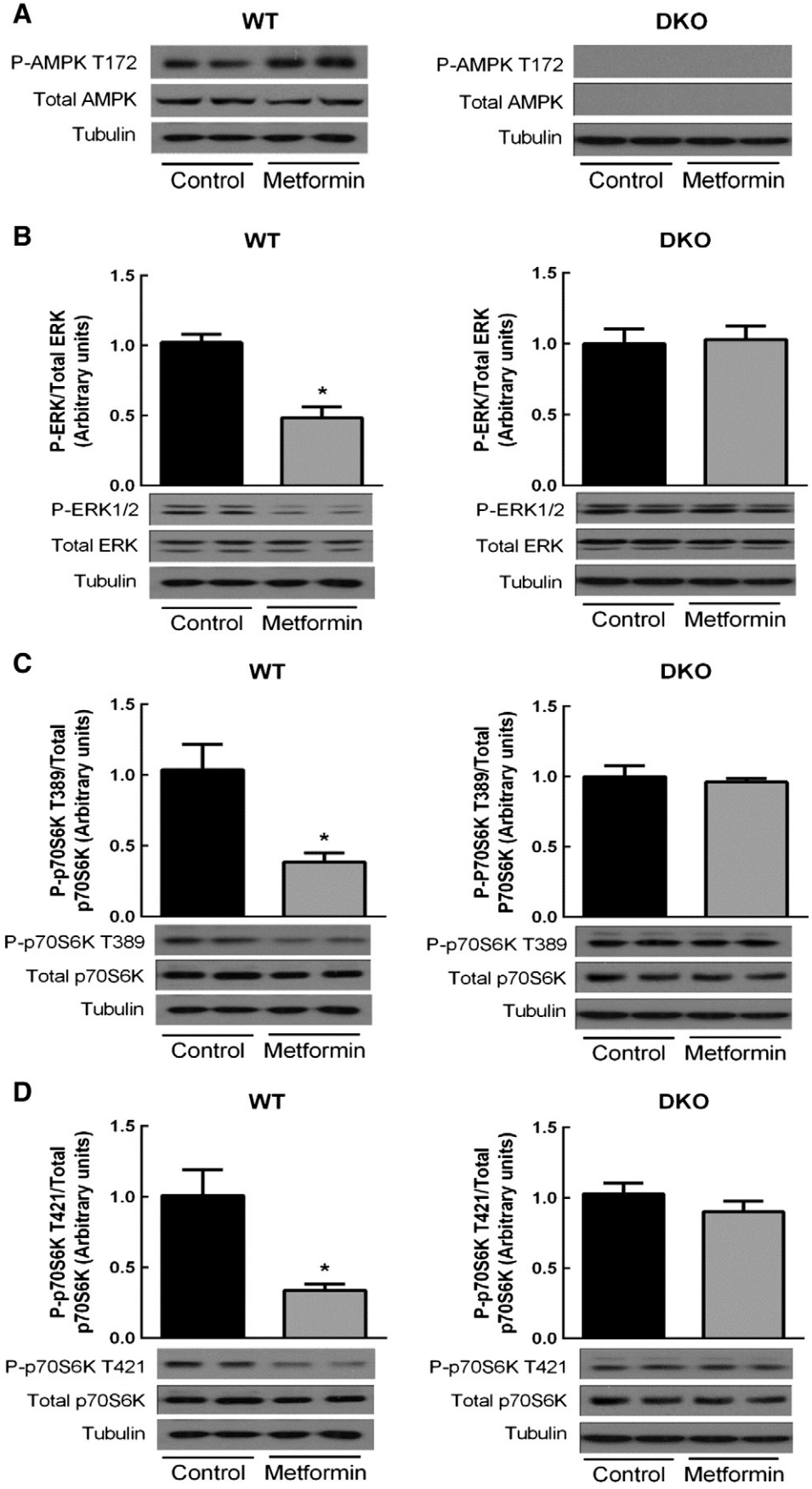


Fig. 9. Compound C reverses the effect of metformin on MAPK and p70S6K signaling. MDA-MB-231 cells were harvested 24 h after incubation with 1 mM metformin (Met) in the absence or presence of 5 μM of the AMPK inhibitor compound C (CC) in normoglycemic conditions. Cell lysates were blotted with anti-phospho-ERK1/2(Thr202/Tyr204), anti-total-ERK1/2, and anti-tubulin antibodies (A); with anti-phospho-p38(Thr180/Tyr182), anti-total-p38, and anti-tubulin antibodies (B); with anti-phospho-p70S6K(T389 and T421), anti-total-p70S6K, and anti-tubulin antibodies (C and D, respectively); or with anti-phospho-S6(Ser240/244), anti-total-S6, and anti-tubulin (E). Results are presented as mean ± SE of densitometry measurements of the phospho/total proteins; *, $p < 0.05$ versus control; # $p < 0.05$ versus metformin-treated cells ($n = 4$; three independent experiments). The blots were stripped and cut as described under Materials and methods, so that the same blot can be probed with several primary antibodies. F. Loss of metformin anti-proliferative effect in the presence of compound C. MDA-MB-231 cells were cultured in the presence of the AMPK inhibitor, compound C (5 μM) under normoglycemic conditions. After 2 h, the cells were treated with increasing metformin concentrations (0.1–5 mM), and cell growth was assessed by the MTT assay after 48 h as described under Materials and methods. The percentage of cell viability was calculated relative to control wells designated as 100% viable cells. The values are presented as mean ± standard error of the mean (SE) and analyzed by ANOVA ($n = 8$ from three independent experiments).

previously in MCF-7 breast cancer cells [54], skeletal muscle [55], and cardiac fibroblasts [56]. Interestingly, the AMPK inhibitor, compound C, completely restored ERK1/2 phosphorylation in metformin-treated cells. Furthermore, metformin inhibited ERK1/2 phosphorylation in WT MEFs but not in AMPK-DKO MEFs, which confirms that ERK1/2 inhibition by metformin is also AMPK-dependent. In addition to its role in controlling cell proliferation, ERK1/2 pathway is a crucial regulator of cell survival, motility, and invasiveness [57]. For instance, ERK1/2 signaling regulates members of the BCL-2 family of apoptotic regulators to protect against apoptosis and promote cell survival [58]. Despite the striking inhibition of ERK1/2 signaling in the current work, we could not detect any evidence of metformin-induced apoptosis in MDA-MB-231 cells under the current experimental conditions. Of interest, higher metformin concentrations (10–40 mM) have been shown to induce apoptosis in MDA-MB-231 cells via both the intrinsic and extrinsic pathways [59]. Therefore, metformin may have a dual concentration-dependent effect on MDA-MB-231 cells: an anti-proliferative effect at low clinically relevant concentrations, and a pro-apoptotic effect at higher concentrations.

In contrast to the ERK1/2 pathway, we demonstrate that metformin treatment caused a significant increase of the phosphorylation and


A
WT
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P-ERK/Total ERK (Arbitrary units)
1.5
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Tubulin
Control
Metformin
C
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P-p70S6K T389/Total p70S6K (Arbitrary units)
P-P70S6K T389/Total P70S6K (Arbitrary units)
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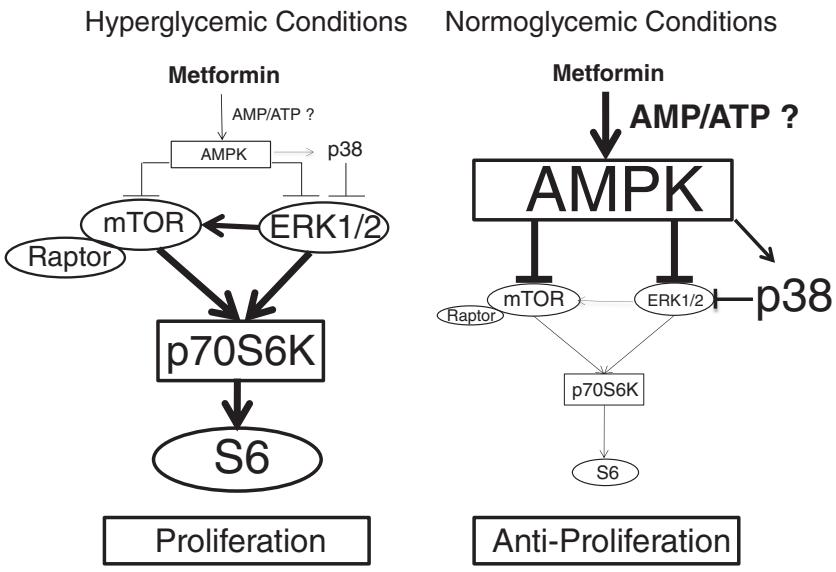

Fig. 11. A schematic diagram representing the proposed mechanism of the anti-proliferative effect of metformin under normoglycemic conditions. Metformin treatment coupled with limited glucose supply causes a striking activation of AMPK, most probably due to a very high AMP/ATP ratio. This substantial AMPK activation inhibits mTOR and ERK1/2 signaling pathways, while it activates the p38 MAPK pathway. As a downstream target for both mTOR and ERK1/2, metformin treatment significantly inhibits p70S6K and ribosomal S6 proteins, switching off protein synthesis and cell proliferation. On the contrary, in hyperglycemic conditions, a surplus of glucose supply rescues the cells from the aforementioned metformin effects, most probably by generating enough ATP through aerobic glycolysis.

activation of p38 MAPK, which was much more pronounced in MDA-MB-231 cells cultured in normoglycemic conditions. In agreement with our results showing AMPK activation is upstream of p38 MAPK signaling, AMPK has been shown to activate p38 MAPK in human lung cancer cells [30], human airway epithelial cells [60], and mouse cardiac fibroblasts [61]. That said, AMPK has also been shown to down-regulate p38 MAPK signaling in paclitaxel-treated human lung cancer cells [62], squamous cell carcinoma xenograft [50], and MCF-7 breast cancer cells [54], leaving open the possibility of cell-specific differences in AMPK–p38 MAPK signaling. To the best of our knowledge, the effect of metformin on p38 MAPK has never been reported in the TNBC MDA-MB-231 cells. In addition, whether p38 activation promotes or inhibits cell proliferation and survival is still controversial. For instance, it has been shown that p38 can cause growth arrest and induce apoptosis through several pathways including the inhibition of the pro-survival ERK1/2 pathway [29,63,64]. On the other hand, inhibition of p38 MAPK activity reduced the proliferation, survival, and invasion of several cancer cell lines [65,66]. Therefore, further research is needed to elucidate whether p38 MAPK activation contributes to the anti-proliferative effect of metformin in TNBC MDA-MB-231 cells.

In addition to hyperglycemia, hyperinsulinemia is a primary characteristic of type 2 diabetes [67]. Both hyperglycemia and hyperinsulinemia are thought to promote the growth of MDA-MB-231 cells [23]. Therefore, it was important to investigate whether metformin will have similar effects in MDA-MB-231 cells in the presence of insulin. In the current study, we have investigated the effect of metformin in the presence of a wide range of insulin concentrations. Similar to our previous results, metformin had no effect on ERK1/2 or p70S6K signaling pathways in the presence of insulin in MDA-MB-231 cells cultured in hyperglycemic conditions (25 mM glucose). On the other hand, metformin significantly inhibited ERK1/2 and p70S6K signaling pathways in MDA-MB-231 cells cultured in normoglycemic conditions (5 mM glucose) in the presence of all the insulin concentrations tested, which suggests that metformin effect is dependent on glucose but not on insulin concentration. Of importance, in normoglycemic conditions, higher p70S6K activation has been observed in MDA-MB-231 cells treated with high insulin concentrations as compared to the cells treated with the physiological insulin concentration. This is consistent with previous reports which demonstrated that insulin can promote the growth of MDA-MB-231 cells [23].

Taken together, the present work demonstrates for the first time that culturing MDA-MB-231 cells in normoglycemic conditions sensitizes these aggressive cells to relatively low metformin concentrations (Fig. 11). The lowest effective metformin concentration (0.5 mM) reported in the current work is approximately 10 times lower than effective metformin concentrations reported previously in breast cancer cell lines [18,68]. Although plasma concentration of metformin in diabetic patients is still lower than this value, it has been reported that metformin accumulates in the tissues and reaches a tissue concentration that is 10 to 20 times higher than its plasma concentration [69]. Therefore, although concentrations of metformin in tumors in diabetic cancer patients have never been determined, metformin concentrations of 0.1–1 mM could be considered clinically relevant when used for in vitro studies. Of interest, these findings are not limited to breast cancer cells. Similar to our results, it has been recently shown that clinically relevant metformin concentrations inhibit the proliferation of pancreatic cancer cells when they are cultured in physiological glucose concentration [21]. Similarly, metformin induced apoptosis in p53-deficient HCT116 cells under nutrient deprivation conditions [70]. In addition, phenformin, a biguanide similar to metformin, has been shown to exhibit a greater inhibitory effect on neuroblastoma cells proliferation when cultured in low glucose conditions [71]. These results demonstrate the necessity of using medium containing physiological glucose concentration (5 mM) for future experiments that investigate the anti-cancer effect of metformin in all cancer cell types. From a clinical prospective, these findings suggest that tight normoglycemic control may enhance the anti-proliferative effect of metformin in diabetic

Fig. 10. Metformin inhibits ERK1/2 and p70S6K signaling in WT but not in AMPK-DKO MEFs. WT and AMKP-DKO MEFs were harvested after 24 h incubation with 2 mM metformin in normoglycemic conditions. Lysates from WT and AMPK-DKO MEFs were blotted with anti-phospho-AMPK(T172), anti-total-AMPK, and anti-tubulin antibodies (A); anti-phospho-ERK1/2(Thr202/Tyr204), anti-total-ERK1/2, and anti-tubulin antibodies (B); anti-phospho-p70S6K(T389 and T421), anti-total-p70S6K, and anti-tubulin antibodies (C and D, respectively). Results are presented as mean ± SE of densitometry measurements of the phospho/total proteins; *, $p < 0.05$ versus control ($n = 6$; three independent experiments). The blots were stripped as described under Materials and methods, so that the same blot can be probed with several primary antibodies.

cancer patients; however, further research is needed to confirm this in a clinical setting. In addition, these findings provide the mechanistic basis for the ongoing clinical trials that investigate the anti-cancer effect of metformin in non-diabetic cancer patients [72,73].

Acknowledgements

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