Metformin and rapamycin have distinct effects on the AKT pathway and proliferation in breast cancer cells

Mahvash Zakikhani · Marie-José Blouin · Esther Piura · Michael N. Pollak

Abstract Rapamycin and its analogues inhibit mTOR, which leads to decreased protein synthesis and decreased cancer cell proliferation in many experimental systems. Adenosine 5′- monophosphate-activated protein kinase (AMPK) activators such as metformin have similar actions, in keeping with the TSC2/1 pathway linking activation of AMPK to inhibition of mTOR. As mTOR inhibition by rapamycin is associated with attenuation of negative feedback to IRS-1, rapamycin is known to increase activation of AKT, which may reduce its anti-neoplastic activity. We observed that metformin exposure decreases AKT activation, an action opposite to that of rapamycin. We show that metformin (but not rapamycin) exposure leads to increased phosphorylation of IRS-1 at Ser^{789}, a site previously reported to inhibit downstream signaling and to be an AMPK substrate phosphorylated under conditions of cellular energy depletion. siRNA methods confirmed that reduction of AMPK levels attenuates both the IRS-1 Ser^{789} phosphorylation and the inhibition of AKT activation associated with metformin exposure. Although both rapamycin and metformin inhibit mTOR (the former directly and the latter through AMPK signaling), our results demonstrate previously unrecognized differences between these agents. The data are consistent with the observation that maximal induction of apoptosis and inhibition of proliferation are greater for metformin than rapamycin.

M. Zakikhani · M.-J. Blouin · M. N. Pollak
Department of Oncology, McGill University, Montreal, QC, Canada

M. Zakikhani · M.-J. Blouin · E. Piura · M. N. Pollak (✉)
Cancer Prevention Center, E-763, Jewish General Hospital, 3755, Cote Ste-Catherine, Montreal, QC H3T 1E2, Canada
e-mail: Michael.pollak@mcgill.ca

Keywords IRS-1 · AMPK · Metformin · Rapamycin · Proliferation

Introduction

Rapamycin and its analogues have antineoplastic activity in many experimental models, and are now used clinically in the treatment of renal cancer cell carcinoma [1]. However, clinical benefit of rapamycin is limited, and one molecular mechanism that may attenuate its antineoplastic activity involves the initially unexpected finding that exposure of tumor cells to rapamycin is associated with increased levels of AKT activation at Ser^{473} [2–6]. This is a consequence of an mTOR-dependent feedback loop that acts to inhibit PI3K/AKT activation by IRS-1. Inhibitory sites of IRS-1 are substrates for mTOR and p70 ribosomal S6 protein kinase 1 (S6K1) [7–10]. Rapamycin, by inhibiting mTOR, not only inhibits protein translation, but also inhibits this feedback loop, leading to increased signaling through IRS-1 resulting in increased AKT activation. This, in turn, may limit some of the antiproliferative actions of rapamycin [3, 11].

It is now recognized that in addition to pharmacologic inhibition of mTOR by rapamycin, mTOR can also be inhibited physiologically as a consequence of AMPK activation through pathways involving raptor and TSC2/1 [12–14]. This is plausible, because activation of AMPK occurs under conditions of cellular energy depletion, and downstream effects of AMPK activation include inhibition of energy-consuming process such as protein translation.

Observations that AMPK activators such as metformin and adiponectin have antiproliferative activity (for example [15–18], reviewed in [19]) have generated interest in possible clinical applications, but comparisons of the effects of

metformin and rapamycin with respect to mTOR inhibition and AKT activation have not been reported.

We confirm that metformin and rapamycin both reduce activation of mTOR and its major substrate S6K1 as expected. However, we show that while rapamycin increases AKT activation as previously described [2–6], metformin reduces AKT activation. We further show that this is related to a direct AMPK-mediated phosphorylation of IRS-1 at Ser^{789}, a site previously described to reduce IRS-1 signaling [20, 21].

Materials and methods

Chemicals

Cell-culture materials were obtained from Invitrogen (Burlington, ON, Canada). Anti-phospho AMPKα (Thr^{172}), anti-AMPKα, anti-phospho-p70S6K (S6K1) (Thr^{389}), anti-phospho-mTOR (Ser^{2448}), anti-mTOR, anti-phospho IRS-1 (Ser^{789}), anti-IRS-1, anti-IGF-1R ß, anti-phospho 4E-BP1 (Ser^{65}), anti-4E-BP1, anti-Poly (ADP-ribose) polymerase (PARP) pAb, and anti-ß-actin were purchased from Cell Signaling Technology (Beverly, MA), and anti-phospho IRS-1 (Tyr^{612}) from Millipore (Billerica, MA). Horseradish peroxidase-conjugated anti-rabbit IgG, anti-mouse IgG, and enhanced chemiluminescene (ECL) reagents were from Pharmacia-Amersham (Baie d'Urfé, QC, Canada). Metformin (1, 1-Dimethylbiguanide hydrchloride) was obtained from Sigma-Aldrich (Oakville, ON, Canada). Rapamycin was purchased from Calbiochem-EMD Biosciences, Inc (La Jolla, CA). siRNA-AMP-activated protein Kinase (AMPK) α1 and negative control siRNA (Alexa Fluor 488) were purchased from QIAGEN (Mississauga, ON, Canada).

Cell lines and culture conditions

Cell lines were purchased from ATCC (Manassas, VA). Cells were cultured in RPM1 1640, supplemented with 10% fetal bovine serum (FBS) and 100 units/ml gentamycin at 37°C and 5% CO_2. Cells were passaged by 0.25% Trypsin-EDTA when they reached ~80% confluence.

Cell proliferation assay

The effect of metformin and/or rapamycin on cell lines was evaluated by the indicator dye Alamar Blue (Biosource International, Camarilo, CA). Cells were plated at $3–5 \times 10^3$ per well in triplicate in 96-well plates and incubated in medium containing 10% FBS. After 24 h, the complete medium was replaced with test medium containing vehicle control or various doses of metformin and/or rapamycin for 72 h at 37°C. Alamar Blue was then added to plates which were incubated at 37°C and a colorimetric change was measured according to the methods provided by the supplier.

Flow cytometry for apoptosis induction

After 72 h treatment; adherent cells were briefly trypsinized, detached, and combined with floating cells from the original growth medium, centrifuged, and washed twice with phosphate-buffered saline (PBS). Approximately 10^6 cells (for each condition) were stained for 30 min with annexinV-FITC and propidium iodide using the Annexin V-FITC kit (Invitrogen, Burlington, ON, Canada). Analysis was conducted on a FACSCalibur flow cytometer (BD Biosciences, Burlington, MA) with CellQuest software (BD Biosciences Immunocytometry Systems, Franklin Lakes, NJ). For quantification of caspase-3 activation, cells (approximately 0.5×10^6) were obtained as for testing with annexinV and propidium iodide analyses, but were washed in media, resuspended in 150 µl media containing 10% FBS and 0.5 µl Red-DEVD-FMK (Caspase-3 detection kit, Calbiochem-EMD Biosciences, La Jolla, CA), and incubated for 30 min at 37°C in a cell-culture incubator with 5% CO_2. The stained cells were centrifuged, washed twice with the wash buffer provided in the kit, resuspended in 500 µl of the same buffer, and analyzed for fluorescence on a FACSCalibur flow cytometer using CellQuest software. All apoptosis tests were conducted in triplicate and results shown are representative of three independent experiments.

Cell transfection

MCF-7 cells were transfected with small interfering RNA (siRNA) targeting the AMPKα1, or a negative control siRNA using Pipette-type electroporator (MicroPorator MP-100, Digital Bio Technology Co., Ltd., Seoul, Korea) as described by the manufacturer's instructions. Cell cultures were incubated for 24 h with various concentrations of siRNA prior to metformin treatment.

Protein extraction and western blot analysis

Cells were washed three times with ice-cold PBS and lysed in 100–400 µl lysis buffer (20 mM Tris–HCl (pH 7.5)), 150 mM NaCl, 2.5 mM sodium pyrophosphate, 1 mM ß-glycerol phosphate, 1 mM Na_3VO_4, 1 mM EGTA, 1% Triton, and complete protease inhibitor mixture inhibitors from Roche Diagnostic (Laval, QC, Canada). Cellular debris was removed by centrifugation at $14{,}000 \times g$ for 20 min at 4°C. Following assay for total protein (Bio-Rad, Mississauga, ON, Canada), clarified protein lysates from each experimental condition (40–50 µg) were boiled for 5 min and subjected to electrophoresis in denaturing 8%

SDS-polyacrylamide gel for mTOR, 16% for 4E-BP1, or 10% SDS-PAGE for other proteins. Separated proteins were transferred to a nitrocellulose membrane and after blocking, the membranes were probed with antibodies of interest. In some cases, developed blots were stripped in stripping buffer (62 mM Tris–HCL (pH 6.8), 100 mM ß-mercaptoethanol, 2% SDS) to confirm equal protein loading. Horseradish peroxidase-conjugated anti-rabbit IgG and anti-mouse IgG were used as secondary antibodies. The position of proteins was visualized using the enhanced chemiluminescene reagent ECL.

Statistical analysis

Prior to statistical analysis, data were square-root transformed to normalize the distribution and to obtain variance homogeneity. Statistical significance was evaluated using GLM Procedure, and least-squares means post-hoc for multiple unpaired comparisons of means (LSMEANS statement with Bonferroni correction) was applied. All statistical analyses were performed using Statistical Analysis System software, version 9.1.3 (SAS Institute, Cary, NC). P values <0.05 were considered significant.

Results

Effects of metformin and/or rapamycin on breast cancer cell growth

Results of dose-response studies are shown in Fig. 1. MCF-7 and T47D (ER-positive), and Hs-578T as well as MDA-231 (ER-negative) breast cancer cell growth were inhibited by both metformin and rapamycin. However, for all cell lines, while increasing concentrations of metformin led to increased growth inhibition in a linear fashion, increasing rapamycin concentration 500 fold had little effect on growth.

Metformin treatment increases levels of apoptosis in MCF-7 cells

Results of flow cytometric analyses of MCF-7 cells treated with metformin, rapamycin, or both for 3 days and then

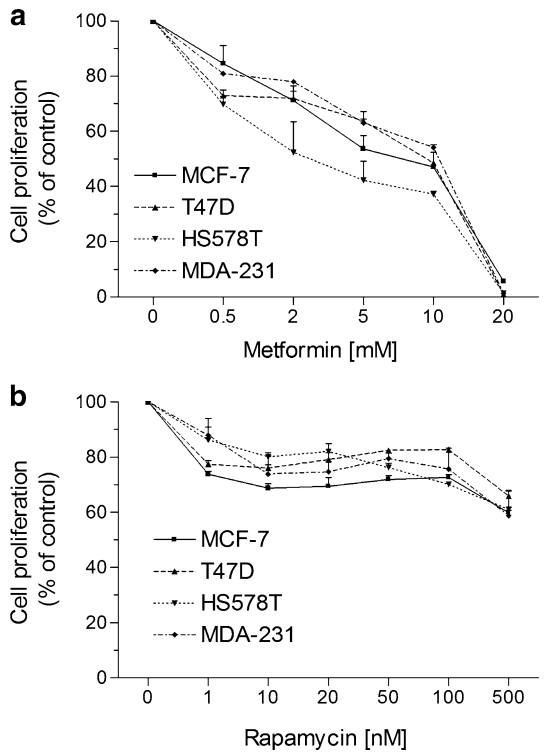

Fig. 1 Inhibition of MCF-7, T47D (ER-positive), Hs-578T and MDA-231 (ER-negative) breast cancer cells growth by **a** metformin and **b** rapamycin. Cells in exponential stages of growth (5000/well) were seeded into 96-well plates with 10% FBS and after 24 h exposed to increasing concentrations of metformin or rapamycin in RPMI containing 1% FBS for 72 h. Cell proliferation in each well was measured by Alamar Blue dye reduction. Data are presented as mean ± S.E. from 3 independent experiments. Triplicates were used for each dose combination for each experiment

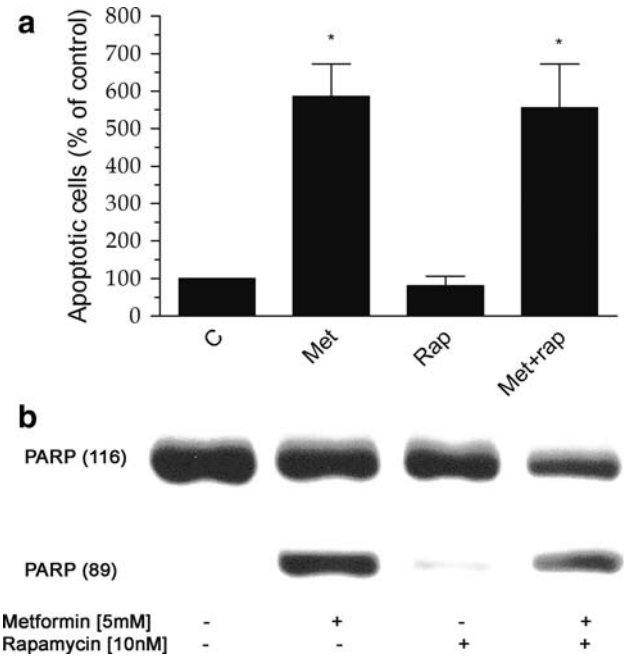

Fig. 2 Effects of metformin and/or rapamycin on apoptosis of MCF-7 breast cancer cells. **a** Flow cytometric analysis of apoptosis in cells stained with annexin V and propidium iodide was performed after 72-h treatment of breast cancer cells with metformin (5 mM) and/or rapamycin (10 nM) in RPMI containing 1% FBS. * indicates significant differences between control and treated cells with metformin and/or rapamycin (One-way ANOVA, $P < 0.0001$). **b** Western blot analyses of MCF-7 cells using PARP antibody. The full length 116 kD product and the lower band represents the 89 kD cleaved PARP which is associated with apoptosis. Experiments were repeated 3 times with similar results

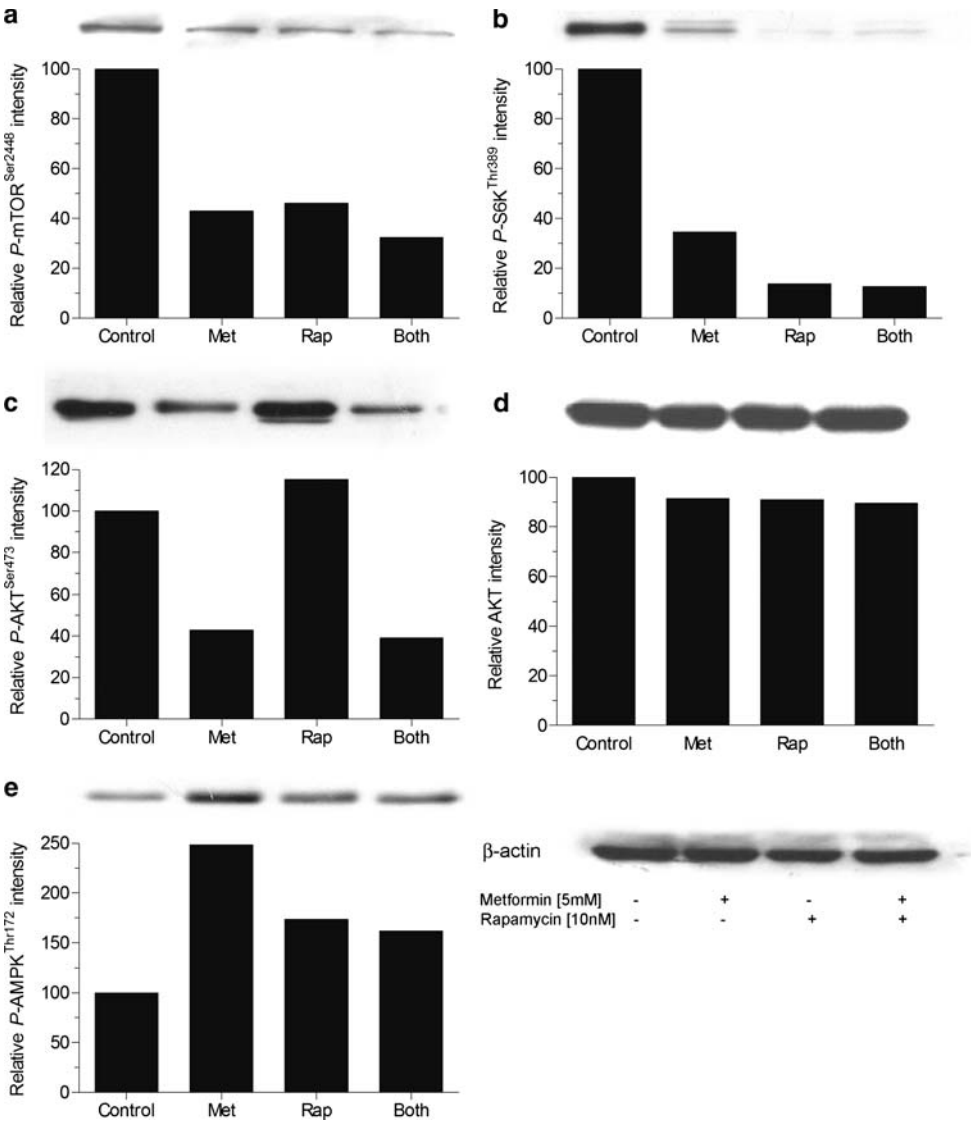

Fig. 3 The effect of metformin and/or rapamycin on mTOR, AKT, and AMPK phosphorylation in MCF-7 breast cancer cells. Cells were exposed to the indicated concentrations of metformin and/or rapamycin in RPMI containing 1% FBS for 72 h. After harvesting, cells were lysed and prepared for immunoblot analyses using antibodies against **a** phospho-mTOR (Ser^{2448}), **b** phospho-S6K (Thr^{389}), **c** phospho-AKT (Ser^{473}), **d** total AKT and **e** phospho-AMPK (Thr^{172}). ß-actin is shown as a loading control. Relative levels of phosphorylated mTOR, p70S6K, AKT, AMPK, and total AKT were quantified by scanning densitometry and normalized by ß-actin. Experiments were repeated 3 times with similar results

stained with annexinV and propidium iodide are shown in Fig. 2a. Apoptotic cell death was also assessed by measuring cleaved poly (ADP-ribose) polymerase (Fig. 2b). Both methods showed induction of apoptosis by metformin but not by rapamycin (Fig. 2).

Alterations in AKT and mTOR signaling

Phosphorylation of S6K1 by mTOR enhances its kinase activity and phosphorylation of downstream targets. Results in Fig. 3a and b demonstrate that metformin, like rapamycin, decreased mTOR phosphorylation and prevented the phosphorylation of S6K1. While the drugs had similar effects on phospho-mTOR (Ser^{2448}), rapamycin was associated with a somewhat greater reduction in phosphoS6K (Thr^{389}) than metformin. The major finding, however, was that metformin exposure resulted in a decrease in AKT Ser^{473} phosphorylation, in contrast to the increase in phosphorylation of this site on exposure of cells to rapamycin (Fig. 3c and d). The AMPK activator AICAR also decreased AKT Ser^{473} phosphorylation (data not shown). We confirmed our prior observation [15, 16] that metformin exposure led to markedly increased AMPK (Thr^{172}) phosphorylation and also noted a smaller effect in the same direction on rapamycin exposure (Fig. 3e).

Metformin induces dephosphorylation of 4E-BP1

4E-BP1 is a translational repressor whose action is attenuated when phosphorylated by mTOR. As expected, metformin (which inhibits more through AMPK-TSC2/1) reduced phospho-4E-BP1 levels as shown in the Fig. 4, consistent with repression of translation. Interestingly, at the time point studied (72 h) we observed no effect of rapamycin on phospho-4E-BP1 levels, even though classically in short time period experiments, rapamycin is

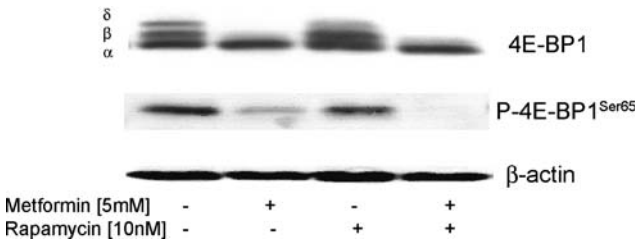

Fig. 4 Effect of metformin and/or rapamycin on 4E-BP1. Cells were exposed to the indicated concentrations of metformin and/or rapamycin for 72 h in RPMI containing 1% FBS. After harvesting, cells were lysed and prepared for immunoblot analyses using antibodies against 4E-BP1, and phospho-4E-BP1 (Ser^{65}). ß-actin is shown as a loading control. Experiments were repeated 3 times with similar results

known to reduce phospho-4E-BP1 levels. Our data concerning rapamycin in this respect are consistent with a prior report by Choo et al. [22].

Metformin stimulates phosphorylation of IRS-1 at Ser^{789} and down-regulates IGF-I receptor and IRS-1 levels

As shown in Fig. 5a and b, metformin reduced level of the insulin-like growth factor (IGF)-I receptor as well as IRS-1, actions which might be expected to reduce downstream signaling and AKT activation. Furthermore, metformin exposure increased IRS-1 phosphorylation at Ser^{789} (despite the reduction in total IRS-1) (Fig. 5c). This is

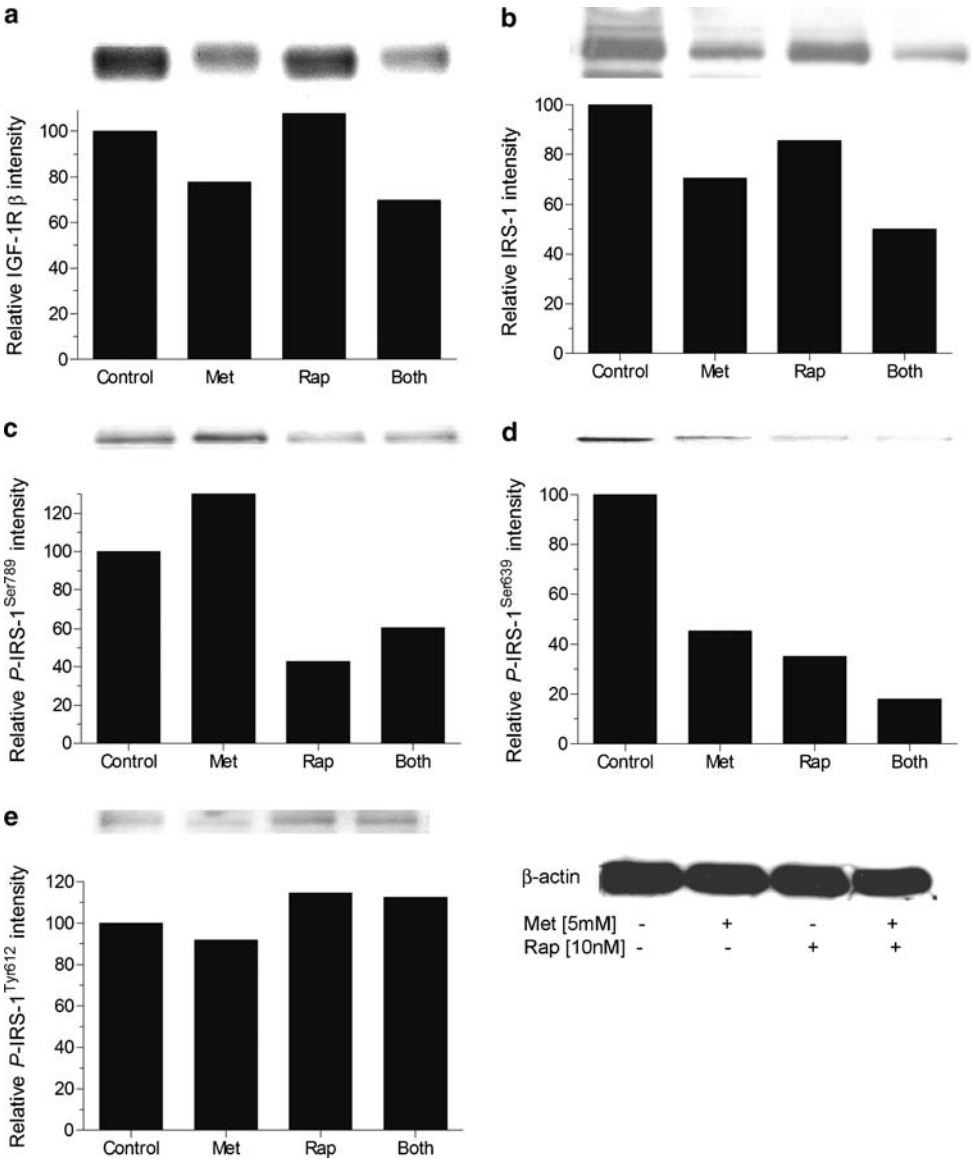

Fig. 5 Metformin stimulates phosphorylation of IRS-1 at Ser^{789} in MCF-7 breast cancer cells. Cells were exposed to the indicated concentrations of metformin and/or rapamycin for 72 h in RPMI containing 1% FBS. After harvesting, cells were lysed and prepared for immunoblot analyses using antibodies against **a** IGF-1Rβ, **b** IRS-1, **c** phospho-IRS-1 (Ser^{789}) **d** phospho-IRS-1 (Ser^{639}) and **e** phospho-IRS-1(Tyr^{612}). ß-actin is shown as a loading control. Relative levels of phosphorylated IRS-1, total IRS-1, and IGF-1Rβ were quantified by scanning densitometry and normalized by ß-actin. Experiments were repeated 3 times with similar results

plausible because IRS-1 Ser^{789} has previously been recognized [21, 23] as a substrate for AMPK. This phosphorylation event would be expected to further decrease downstream signaling, as IRS-1 Ser^{789} phosphorylation inhibits IRS-1 signal transduction [20, 24], and thereby contributes to the reduction in AKT activation by metformin exposure shown in Fig. 3c. Interestingly, rapamycin exposure led to a substantial decrease in phosphorylation of IRS-1 Ser^{789}, which may contribute to the increased AKT Ser^{473} phosphorylation shown in Fig. 3c and in prior reports [2, 3].

In keeping with the fact that both metformin (indirectly via AMPK) and rapamycin (directly) reduce mTOR and S6K activity and the fact that the inhibitory Ser^{639} site of IRS-1 is a substrate for S6K, we observed reduced IRS-1 Ser^{639} phosphorylation in the presence of either metformin or rapamycin (Fig. 5d). While metformin decreases phosphorylation of the inhibitory Ser^{639} site in a manner similar to rapamycin, in keeping with inhibition of mTOR, its separate action to increase phosphorylation of the inhibitory Ser^{789} site appears to be dominant, given the observation of reduced AKT activation on metformin exposure. In contrast to the findings for IRS-1 Ser^{789}, no important changes in the phosphorylation of IRS-1 at Tyr^{612} were seen on exposure to either metformin or rapamycin (Fig. 5e).

Suppression of AMPKα1 by siRNA blocks the inhibitory effect of metformin on AKT activation

In order to determine if activation of AMP kinase by metformin is required for the effects on AKT Ser^{473} activation, we carried out experiments with siRNA against AMP kinase. As shown in Fig. 6, suppression of AMPK led to a decrease in IRS-1 phosphorylation on Ser^{789} and an attenuation of the metformin-induced decrease in AKT phosphorylation on Ser ^{473}.

Discussion

The antiproliferative effects of rapamycin are significant but limited in magnitude. In our experimental system, this agent did not induce apoptosis and, as previously proposed by others [2–6], this may relate at least in part to the fact that rapamycin exposure results in increased AKT activation, which is known to stimulate cell survival pathways

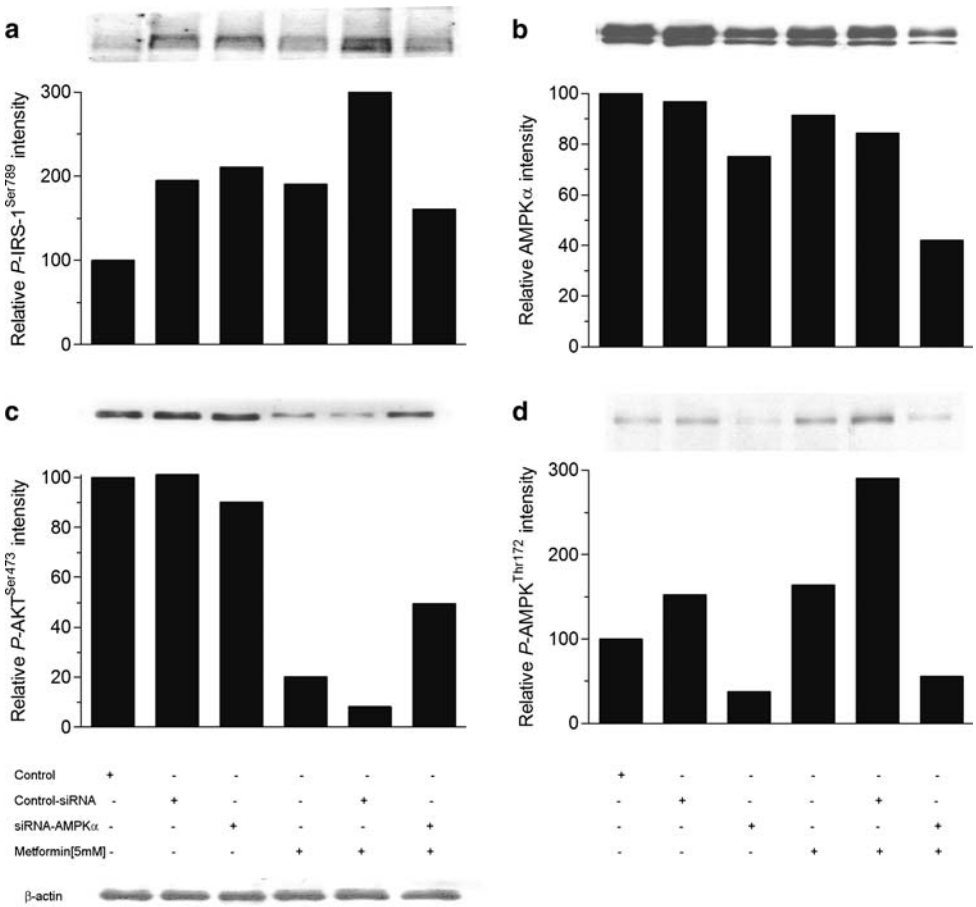

Fig. 6 Suppression of AMPKα1 by siRNA blocks the inhibitory effect of metformin on AKT activation. MCF-7 cells were transfected with 50 nM AMPKα1-siRNA or with control siRNA. Twenty-four hours after transfection under 10% FBS, cells were treated with metformin (5 mM) or rapamycin (10 nM) and incubated for 72 h in RPMI containing 1% FBS. After harvesting, cells were lysed and prepared for immunoblot analyses using antibodies against **a** phospho-IRS-1 (Ser^{789}), **b** AMPKα, **c** phospho-AKT (Ser^{473}) and **d** phospho-AMPK (Thr^{172}). ß-actin is shown as a loading control. Relative levels of phosphorylated IRS-1, AKT, AMPK and total AMPK were quantified by scanning densitometry and normalized by ß-actin. Experiments were repeated 3 times with similar results

and to inhibit apoptosis [25, 26]. Prior reports [20, 21] provide strong evidence that IRS-1 Ser^{789} is an AMPK substrate. Our results suggest that an explanation for the difference between the effect of rapamycin and metformin on AKT activation in MCF-7 cells concerns IRS-1 (Fig. 7). While both compounds reduce mTOR activation and thus reduce phosphorylation of the IRS-1 $Ser^{636/639}$ inhibitory sites, only metformin, through AMPK activation, phosphorylates the Ser^{789} inhibitory site of IRS-1. Thus, in the presence of insulin or IGF-I receptor activation, IRS-1 mediated signal transduction to PI3K/AKT would be exaggerated in the presence of rapamycin due to reduced inhibitory site (Ser^{789}, $Ser^{636/639}$) phosphorylation by mTOR and S6K, but in the case of metformin, AMPK-mediated phosphorylation of IRS-1 at Ser^{789} acts to limit downstream signaling. However, other mechanisms, including the dephosphorylation of S6K and 4E-BP1 in MCF-7 by metformin and the observed reduction of level of IGF-IR that occurs on exposure of cells to metformin, may also contribute to inhibitory effect of this drug on AKT activation and the anti-proliferative and pro-apoptotic activity of metformin.

The basis for reduced phosphorylation of the inhibitory IRS-1 Ser^{789} site by rapamycin remains unclear. This site does not have the characteristics of a S6K substrate, although it clearly is an AMPK substrate. Rapamycin exposure did not decrease AMPK activation (Fig. 3e), so a pathway linking rapamycin exposure to AMPK activity and thereby to decrease IRS-1 Ser^{789} phosphorylation seems unlikely. There is a precedent for rapamycin-induced upregulation of protein phosphatase 2 (PP2A) activity [27], and a mechanistic role for serine phosphatase actions [28, 29] on IRS-1 is a possibility under study.

AKT activation is well recognized as an important cell survival signal [25, 26]. The observed differences between rapamycin and metformin with respect to AKT activation correlate with the observed differences in the slope of dose response curves in proliferation assays and with observed differences in apoptosis induction. Each compound was studied at concentrations achieving ~50% growth inhibition; while metformin exhibited a linear antiproliferative effect and induced apoptosis, rapamycin did not induce apoptosis and increasing the dose 500 fold beyond that which achieved ~50% growth inhibition induced no further antiproliferative effect.

Our results justify further in vitro and in vivo evaluation of similarities and differences of the consequences of exposure to rapalogs, metformin (or other AMPK

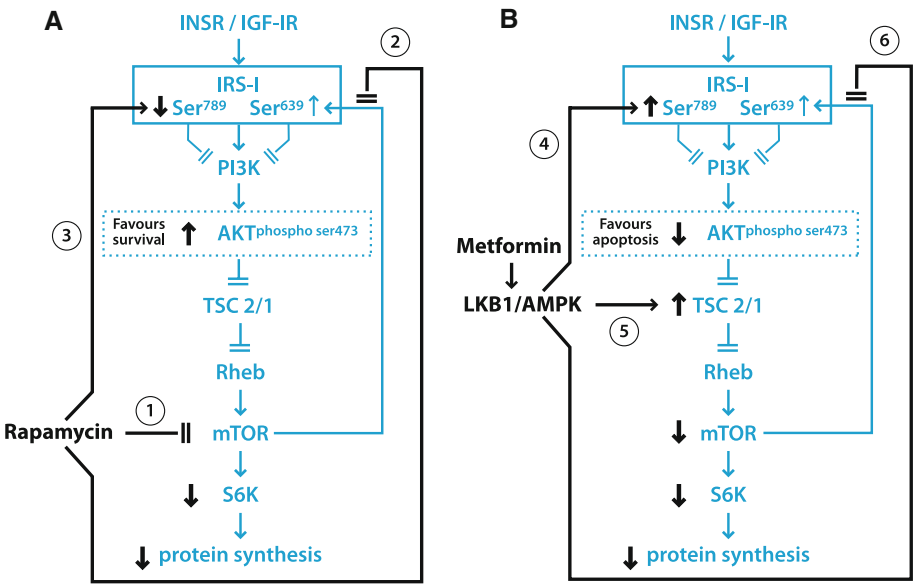

Fig. 7 Similarities and differences in effects of metformin and rapamycin on AKT activation in MCF-7 cells. A simplified model of the relevant signaling network including the previously described [8, 9] negative feedback loop by which mTOR activation leads to phosphorylation of the inhibitory Ser^{639} site of IRS-1 and thereby limits activation of AKT, is shown in *blue*. **a** *Rapamycin*, by blocking mTOR [1], deactivates this feedback inhibition [2], leading to decreased phosphorylation of the inhibitory IRS-1 Ser^{639} site and increased AKT activation, which favors cell survival [25, 26], despite antiproliferative effects related to inhibition of protein synthesis. Rapamycin exposure also leads to decreased activation of the inhibitory Ser^{789} site of IRS-1 [3] which would be expected to further increase AKT activation. **b** *Metformin*, via AMPK activation, phosphorylates the inhibitory Ser^{789} site of IRS-1 [4], and this is associated with decreased AKT activation (despite the fact that metformin also has actions [5] which lead to reduced mTOR activation (and reduced protein synthesis [15, 16], and reduced feedback inhibition via IRS Ser^{639} [6]. The opposite effects of rapamycin and metformin on AKT activation are compatible with the observation that metformin, but not rapamycin, induces apoptosis in our experimental system (Fig. 2)

activators), or combinations of these classes of drugs. It is important to recognize that it is unknown if the concentration of metformin used (5 mM) would be found in neoplastic tissue of patients receiving tolerable dose of the drug (typically $\sim$1.5 g/day). This issue cannot be definitely resolved by measuring serum drug concentrations, as metformin is actively transported into cells via an organic cation transporter 1 (OCT1) [30] which is present in MCF-7 cells (data not shown). However, the metformin concentration associated with inhibition of AKT activation in our experiments was considerably lower than those used in other in vitro studies [31, 32].

While metformin and rapamycin exposure led to similar degrees of inhibition of mTOR activation in our model, the consequences in terms of effects on AKT activation, 4E-BP1 dephosphorylation, proliferation, and apoptosis differed markedly; this suggests these may be advantages to inhibition of mTOR in the context of AMPK activation, as compared to direct inhibition with agents that act only at mTOR. In view of insights into the complexity of the mTOR signaling network [11, 33], further studies to compare consequences of mTOR inhibition and AMPK activation strategies will be of interest.

Acknowledgments We thank Ms. L. Lui for valuable assistance in manuscript preparation. This study was supported by the Translational Acceleration grant #16512 from the Canadian Breast Cancer Research Alliance and the Translational Cancer Research Award ref: 2008-08 from the Terry Fox Research Institute.

References

1. Rini B, Kar S, Kirkpatrick P (2007) Temsirolimus. Nat Rev Drug Discov 6:599–600
2. Sun SY, Rosenberg LM, Wang X, Zhou Z, Yue P, Fu H, Khuri FR (2005) Activation of Akt and eIF4E survival pathways by rapamycin-mediated mammalian target of rapamycin inhibition. Cancer Res 65:7052–7058
3. O'Reilly KE, Rojo F, She QB, Solit D, Mills GB, Smith D, Lane H, Hofmann F, Hicklin DJ, Ludwig DL, Baselga J, Rosen N (2006) mTOR inhibition induces upstream receptor tyrosine kinase signaling and activates Akt. Cancer Res 66:1500–1508
4. Bhaskar PT, Hay N (2007) The two TORCs and Akt. Dev Cell 12:487–502
5. Guertin DA, Sabatini DM (2007) Defining the role of mTOR in cancer. Cancer Cell 12:9–22
6. Wang X, Yue P, Kim YA, Fu H, Khuri FR, Sun SY (2008) Enhancing mammalian target of rapamycin (mTOR)-targeted cancer therapy by preventing mTOR/raptor inhibition-initiated, mTOR/rictor-independent Akt activation. Cancer Res 68:7409–7418
7. Easton JB, Kurmasheva RT, Houghton PJ (2006) IRS-1: auditing the effectiveness of mTOR inhibitors. Cancer Cell 9:153–155
8. Ozes ON, Akca H, Mayo LD, Gustin JA, Maehama T, Dixon JE, Donner DB (2001) A phosphatidylinositol 3-kinase/Akt/mTOR pathway mediates and PTEN antagonizes tumor necrosis factor inhibition of insulin signaling through insulin receptor substrate-1. Proc Natl Acad Sci USA 98:4640–4645
9. Tzatsos A, Kandror KV (2006) Nutrients suppress phosphatidylinositol 3-kinase/Akt signaling via raptor-dependent mTOR-mediated insulin receptor substrate 1 phosphorylation. Mol Cell Biol 26:63–76
10. Harrington LS, Findlay GM, Gray A, Tolkacheva T, Wigfield S, Rebholz H, Barnett J, Leslie NR, Cheng S, Shepherd PR, Gout I, Downes CP, Lamb RF (2004) The TSC1-2 tumor suppressor controls insulin-PI3K signaling via regulation of IRS proteins. J Cell Biol 166:213–223
11. Guertin DA, Sabatini DM (2009) The pharmacology of mTOR inhibition. Sci Signal 2:e24
12. Hardie DG (2008) AMPK and Raptor: matching cell growth to energy supply. Mol Cell 30:263–265
13. Gwinn DM, Shackelford DB, Egan DF, Mihaylova MM, Mery A, Vasquez DS, Turk BE, Shaw RJ (2008) AMPK phosphorylation of raptor mediates a metabolic checkpoint. Mol Cell 30:214–226
14. Shaw RJ (2009) LKB1 and AMP-activated protein kinase control of mTOR signalling and growth. Acta Physiol (Oxf) 196:65–80
15. Zakikhani M, Dowling R, Fantus IG, Sonenberg N, Pollak M (2006) Metformin is an AMP kinase-dependent growth inhibitor for breast cancer cells. Cancer Res 66:10269–10273
16. Zakikhani M, Dowling RJ, Sonenberg N, Pollak MN (2008) The effects of adiponectin and metformin on prostate and colon neoplasia involve activation of AMP-activated protein kinase. Cancer Prev Res (Phila Pa) 1:369–375
17. Dowling RJ, Zakikhani M, Fantus IG, Pollak M, Sonenberg N (2007) Metformin inhibits mammalian target of rapamycin-dependent translation initiation in breast cancer cells. Cancer Res 67:10804–10812
18. Algire C, Zakikhani M, Blouin M-J, Shuai JH, Pollak M (2008) Metformin attenuates the stimulatory effect of a high energy diet on in vivo H59 carcinoma growth. Endocr Relat Cancer 15:833–839
19. Pollak M (2008) Insulin and insulin-like growth factor signalling in neoplasia. Nat Rev Cancer 8:915–928
20. Tzatsos A, Tsichlis PN (2007) Energy depletion inhibits phosphatidylinositol 3-kinase/Akt signaling and induces apoptosis via AMP-activated protein kinase-dependent phosphorylation of IRS-1 at Ser-794. J Biol Chem 282:18069–18082
21. Jakobsen SN, Hardie DG, Morrice N, Tornqvist HE (2001) 5′-AMP-activated protein kinase phosphorylates IRS-1 on Ser-789 in mouse C2C12 myotubes in response to 5-aminoimidazole-4-carboxamide riboside. J Biol Chem 276:46912–46916
22. Choo AY, Yoon SO, Kim SG, Roux PP, Blenis J (2008) Rapamycin differentially inhibits S6Ks and 4E-BP1 to mediate cell-type-specific repression of mRNA translation. Proc Natl Acad Sci USA 105:17414–17419
23. Boura-Halfon S, Zick Y (2009) Phosphorylation of IRS proteins, insulin action, and insulin resistance. Am J Physiol Endocrinol Metab 296:E581–E591
24. Gual P, Marchand-Brustel Y, Tanti JF (2005) Positive and negative regulation of insulin signaling through IRS-1 phosphorylation. Biochimie 87:99–109
25. Manning BD, Cantley LC (2007) AKT/PKB signaling: navigating downstream. Cell 129:1261–1274
26. Vivanco I, Sawyers CL (2002) The phosphatidylinositol 3-Kinase AKT pathway in human cancer. Nat Rev Cancer 2:489–501
27. Hartley D, Cooper GM (2002) Role of mTOR in the degradation of IRS-1: regulation of PP2A activity. J Cell Biochem 85:304–314
28. Virshup DM, Shenolikar S (2009) From promiscuity to precision: protein phosphatases get a makeover. Mol Cell 33:537–545
29. Padmanabhan S, Mukhopadhyay A, Narasimhan SD, Tesz G, Czech MP, Tissenbaum HA (2009) A PP2A regulatory subunit regulates C. elegans insulin/IGF-1 signaling by modulating AKT-1 phosphorylation. Cell 136:939–951

30. Shu Y, Sheardown SA, Brown C, Owen RP, Zhang S, Castro RA, Ianculescu AG, Yue L, Lo JC, Burchard EG, Brett CM, Giacomini KM (2007) Effect of genetic variation in the organic cation transporter 1 (OCT1) on metformin action. J Clin Invest 117: 1422–1431
31. Zhuang Y, Miskimins WK (2008) Cell cycle arrest in Metformin treated breast cancer cells involves activation of AMPK, down-regulation of cyclin D1, and requires p27Kip1 or p21Cip1. J Mol Signal 3:18
32. Liu B, Fan Z, Edgerton SM, Deng XS, Alimova IN, Lind SE, Thor AD (2009) Metformin induces unique biological and molecular responses in triple negative breast cancer cells. Cell Cycle 8:2031–2040
33. Huang J, Wu S, Wu CL, Manning BD (2009) Signaling events downstream of mammalian target of rapamycin complex 2 are attenuated in cells and tumors deficient for the tuberous sclerosis complex tumor suppressors. Cancer Res 69:6107–6114