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Metformin inhibits androgen-induced IGF-IR up-regulation in prostate cancer cells by disrupting membrane-initiated androgen signaling.

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Metformin Inhibits Androgen-Induced IGF-IR Up-Regulation in Prostate Cancer Cells by Disrupting Membrane-Initiated Androgen Signaling

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We have previously demonstrated that, in prostate cancer cells, androgens up-regulate IGF-I receptor (*IGF-IR*) by inducing cAMP-response element-binding protein (CREB) activation and CREB-dependent *IGF-IR* gene transcription through androgen receptor (AR)-dependent membrane-initiated effects. This IGF-IR up-regulation is not blocked by classical antiandrogens and sensitizes cells to IGF-I-induced biological effects. Metformin exerts complex antitumoral functions in various models and may inhibit CREB activation in hepatocytes. We, therefore, evaluated whether metformin may affect androgen-dependent IGF-IR up-regulation. In the AR⁺ LNCaP prostate cancer cells, we found that metformin inhibits androgen-induced CRE activity and *IGF-IR* gene transcription. CRE activity requires the formation of a CREB-CREB binding protein-CREB regulated transcription coactivator 2 (CRTC2) complex, which follows Ser133-CREB phosphorylation. Metformin inhibited Ser133-CREB phosphorylation and induced nuclear exclusion of CREB cofactor CRTC2, thus dissociating the CREB-CREB binding protein-CRTC2 complex and blocking its transcriptional activity. Similarly to metformin action, CRTC2 silencing inhibited IGF-IR promoter activity. Moreover, metformin blocked membrane-initiated signals of AR to the mammalian target of rapamycin/p70S6Kinase pathway by inhibiting AR phosphorylation and its association with c-Src. AMPK signals were also involved to some extent. By inhibiting androgen-dependent IGF-IR up-regulation, metformin reduced IGF-I-mediated proliferation of LNCaP cells. These results indicate that, in prostate cancer cells, metformin inhibits IGF-I-mediated biological effects by disrupting membrane-initiated AR action responsible for IGF-IR up-regulation and suggest that metformin could represent a useful adjunct to the classical antiandrogen therapy. (*Endocrinology* 155: 1207–1221, 2014)

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Abbreviations: AR, androgen receptor; ARwt, wild-type AR; CBP, CREB binding protein; CRTC2, CREB regulated transcription coactivator 2; CREB, cAMP-response element-binding protein; CRTC2, CREB regulated transcription coactivator 2; FBS, fetal bovine serum; GFP, green fluorescent protein; HEK293, human embryonic kidney 293; IGF-IR, IGF-I receptor; IR, insulin receptor; LKB1, serine/threonine kinase 11; mTORC1, mammalian target of rapamycin complex 1; MTT, methyl thiazolyl tetrazolium; Δ N CREB, dominant-negative CREB; OCT, organic cation transporter; p, phospho; p53, tumor suppressor p53; PKB, protein kinase B; PP2, (4-amino-3-(4-chlorophenyl)-1-(t-butyl)-1H-pyrazolo[3,4-d]pyrimidine, 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine; p70S6K, p70S6 kinase; p90RSK, 90 kDa ribosomal S6 kinase; PTEN, phosphatase and tensin homologue deleted on chromosome ten; R1881, methyltrienolone; REDD1, regulated in development and DNA damage 1; shRNA, short hairpin RNA; siRNA, small interfering RNA; TSC2, Tuberous Sclerosis Complex 2.

Androgen stimulation is critical for growth and resistance to apoptosis in most early-stage prostate carcinomas, which, therefore, are responsive to androgen deprivation. However, the clinical benefits of androgen deprivation are temporary, and these carcinomas may eventually progress to castration-resistant tumors, for which no effective treatment is currently available. The molecular basis of androgen independency is incompletely understood.

In response to androgens, androgen receptors (ARs) regulate transcription by interacting with the androgen response elements located within the promoter regions of target genes and forming a multiprotein complex, which contains coactivators, corepressors, histone acetyltransferases, and histone deacetylases (1). However, increasing evidence suggests that the biological responses to androgens can be additionally mediated by membrane-initiated signals, which trigger rapid intracellular transduction pathways like ERK, phosphoinositide 3-kinase, protein kinase A, and protein kinase C, that may eventually activate gene transcription (2). Membrane-initiated androgen signals appear to be enhanced in malignant prostate cells by various mechanisms, including increased proportion of membrane-associated ARs and increased expression of kinases (eg, c-Src) and/or adaptors that contribute to the formation of multiprotein complexes with AR at the membrane level and trigger the activation of intracellular pathways (3).

Androgen activity itself may contribute to the progression to castration-resistant prostate cancer by up-regulating autocrine loops involving peptide growth factors and their cognate receptors (4).

In this context, we have previously found that androgens induce a selective up-regulation of the IGF-I receptor (IGF-IR) in prostate cancer cells and increase, in this way, cell proliferation and invasiveness in response to IGF-I (5). This effect occurs through the activation of membrane-initiated signals, which require the recruitment of membrane-bound AR to c-Src and subsequent activation of a downstream signaling pathway involving c-Src/ERK/cAMP-response element-binding protein (CREB) that eventually stimulates the activity of the IGF-IR promoter (5, 6). This mechanism may open a new approach to prostate cancer therapy, because it is poorly affected by classic antiandrogens but can be blocked by CREB silencing or by inhibitors of the c-Src/ERK pathway (6). The transcriptional activity of CREB-dependent target genes requires the formation of the CREB-CREB binding protein (CBP)-CREB regulated transcription coactivator 2 (CRTC2) complex (7). In particular, AMPK phosphorylates CRTC2 at Ser171 causing its interaction with 14–3–3 proteins and sequestration in the cytoplasm. Glucose and hor-

mones lead to the dephosphorylation of CRTC2, its dissociation from 14–3–3 proteins, and as a consequence, its translocation to the nucleus, where it binds CREB and promotes CREB-dependent transcription. Metformin may additionally disrupt the CREB-CBP-CRTC2 complex by inducing CBP phosphorylation at Ser436 (7).

In recent years, the biguanide metformin, widely used as antidiabetic drug, has raised much interest for its anticancer potential (8, 9). Indeed, metformin has shown antiproliferative effects in several cancer cells, including prostate cancer cells (10, 11). Interestingly, prostate cancer cells appear to be more sensitive to metformin than normal epithelial prostate cells. In vivo, metformin increases the response of prostate cells xenografts to the antiandrogen bicalutamide (12). Anticancer effects of metformin are mostly attributed to its ability to activate AMPK, which, in turn, down-regulates mammalian target of rapamycin complex 1 (mTORC1) signaling, an essential regulator of cell growth and proliferation (8). Metformin has, however, pleiotropic effects and may additionally inhibit mTORC1 through AMPK-independent pathways (13, 14). Metformin may also target ERK signaling (15), reduce Ca(2+)-dependent protein kinase Ca/ERK and c-Jun N-terminal kinase/activator protein-1 signaling pathways (16, 17), and also inhibit Akt (protein kinase B [PKB]) activity through serine phosphorylation of insulin receptor substrate-1 (18).

Because metformin is able to inhibit multiple signaling pathways, we aimed at evaluating whether, in prostate cancer cells, metformin may also affect the membrane-initiated effects of androgens, which lead to IGF-IR up-regulation and increased sensitivity to IGF-I. Here, we show that metformin inhibits androgen-induced IGF-IR up-regulation and the resulting mitogenic and invasive effects of IGF-I through multiple mechanisms. These include the inhibition of transcriptional activity of CREB-CBP-CRTC2 complex, the inhibition of mTORC1/p70S6 kinase (p70S6K) pathway, and the disruption of AR/c-Src interaction.

Materials and Methods

Materials

The following materials were purchased from the indicated manufacturers: metformin, methyltrienolone (R1881), Src activity inhibitor (4-amino-3-(4-chlorophenyl)-1-(*t*-butyl)-1H-pyrazolo[3,4-*d*]pyrimidine, 4-Amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine) (PP2), and rapamycin from Sigma Chemical Co; fetal calf serum and Opti-MEM from Gibco Laboratories; temsirolimus from DBA; and Metafectene PRO from Biontex Laboratories GmbH. R1881, or methyltrienolone, is a synthetic androgen that is considered the gold stan-

dard AR agonist for in vitro studies because of its excellent stability and its inability to undergo aromatization.

The c-Src/AR interaction inhibitory peptide (S1) has been previously described (19).

LNCaP human prostate cancer cells and human embryonic kidney 293 (HEK293) were obtained from the American Type Culture Collection.

The human AR construct was provided by Dr A.O. Brinkmann. The dominant-negative CREB (Δ N CREB) construct was provided by Dr C. Vinson. Rat IGF-IR promoter sequences cloned into pGL3 vector were provided by Dr C.T. Roberts Jr. The CRE reporter was from SABiosciences. The specific silencer Select Predesigned small interfering RNA (siRNA) for CRCT2, AMPK (α 1- and α 2-subunit), regulated in development and DNA damage 1 (REDD1), the negative control siRNA, and the lipofectamine RNAiMax were purchased from Life Technologies.

Western blot analysis

Subconfluent cells were serum starved and stimulated with 10nM sex steroids for the indicated times and lysed in Laemmli buffer. Cell lysates were subjected to Western blot analysis, as previously described (20), using the following antibodies: anti-IGF-IR, anti-AR (clone 441), anti-IR, anti- β -tubulin, anti-tumor suppressor p53 (p53), and anti-c-Src (B-12) (Santa Cruz Biotechnology, Inc); antiphospho-CREB (clone 1B6), anti-CREB (clone 86B10), anti-phospho (p) Akt(S473), anti-Akt, anti-pERK1/2(T202/Y204), anti-ERK1/2, anti-pp70S6K(T389), anti-p70S6K, and anti-AMPK (Cell Signaling Technology); anti-CRTC2 and anti-CRTC2(S171) (Antibodies-Online); anti-REDD1 (ProteinTech Group, Inc); and anti-phospho tyrosine clone 4G10 (EMD Millipore).

Transient transfection and reporter assays

LNCaP and HEK293 cells were transiently transfected as described previously (6). Twenty-four hours after transfection, cells were serum starved for additional 24 hours and incubated with sex steroids (10nM), metformin (10mM), or vehicle. For luciferase assay, cells were stimulated for 24 hours, lysed, and processed as described previously (6).

Gene silencing by short hairpin RNA (shRNA) or siRNA

For shRNA experiments, cells were transfected with a mixture containing Opti-MEM, Metafectene PRO, 0.5 μ g of either 4 different shRNAs against CREB or scramble shRNAs. Twenty-four hours after transfection, cells were serum starved and treated with metformin (10mM) with or without R1881 (10nM) for 24 hours and then processed for Western blot analysis. For siRNA experiments, cells were transfected with a mixture containing Opti-MEM, Lipofectamine RNAiMax, and either 10nM scramble siRNA or a mixture of 2 or 3 siRNA specific for the transcript of interest. Twenty-four hours after transfection, cells were incubated with fresh medium and transfected with either IGF-IR-Luc or CRE-Luc (0.5 μ g/well) and green fluorescent protein (GFP) (0.1 μ g/well), using metafectene PRO. After 12 hours, cells were serum starved for 10 hours and treated with metformin (10mM) with or without R1881 (10nM) for 24 hours and then processed for luciferase assay.

Immunoprecipitation

Cells were serum starved for 24 hours and then incubated with metformin (10mM) or PP2 (10 μ M) for the indicated time points, followed by 10nM R1881. Cell lysates were subjected to immunoprecipitation, as previously described (20).

Immunofluorescence

Cells were serum starved and subjected to specific treatments as indicated. Then, cells were fixed in 4% paraformaldehyde, permeabilized with PBS/0.1% Triton X-100, blocked with PBS/0.05% Triton X-100/0.1% BSA, and incubated with primary antibody (CRCT2) for 1 hour. Cells were then washed with PBS and incubated with Alexa-conjugated (Alexa Fluor 594 or 488) secondary antibodies (Molecular Probes) for 1 hour. To visualize the cytoplasm, the cells were incubated with Alexa-conjugated phalloidin (Molecular Probes) for an additional 30 minutes. Cell nuclei were counterstained with Hoechst. Epifluorescence microscopy was performed with an Olympus microscope. The images were digitally acquired with an Orca CCD camera (Hamamatsu) and processed with Image-Pro Plus 4.0 software (Media Cybernetics).

Cell viability assay

Cell viability was measured by the methyl thiazolyl tetrazolium (MTT) test (Amersham Biosciences). Cells were serum starved for 24 hours in medium without phenol red and pretreated for 4 hours with metformin (10mM) followed by R1881 exposure (10nM) for further 24 hours. The medium was then replaced with R1881-free medium containing 1% stripped serum in the presence or not of metformin (10mM). After 4 hours, cells were incubated in the presence or absence of 10nM IGF-I for additional 24 hours. The cells were then incubated with medium containing 0.5-mg/mL MTT. After 4 hours, cells were dissolved in 100 μ L of a solution containing dimethyl sulfoxide plus 2.5% complete medium, and formazan absorbance was read at 405 nm.

Invasion assay

The ability of cells to invade collagen was measured with Boyden's chamber technique (21). Cells, serum starved for 24 hours, were incubated with or without metformin (10mM) and R1881 (10nM) for further 24 hours. Cells were then removed from plates with 0.01% trypsin and allowed to migrate for 18 hours in response to 10nM IGF-I as previously described (22).

Soft-agar colony formation assay

Anchorage-independent growth was assessed by the soft-agar assay. Briefly, 6-well plates were first plated with medium containing 10% fetal bovine serum (FBS) and 0.8% agar. Next, 1000 cells were suspended in medium containing 10% fetal bovine serum (FBS) plus 0.3% agar, and the cell suspension was plated on top of the first layer. Top agar was covered with culture medium alone (basal) or containing metformin (10mM) with or without R1881 (10nM), and the plates were maintained in the tissue-culture incubator. Cells were stimulated twice a week for 3 weeks. Colonies formed were stained with 0.5-mg/mL MTT and photographed.

Cell cycle evaluation

Cells synchronized for 24 hours in serum-free medium without phenol red were pretreated for 4 hours with metformin

(10mM) followed by R1881 exposure (10nM) for 24 hours. The medium was then replaced with R1881-free medium containing 1% stripped serum with or without metformin (10mM). After 4

hours, cells were incubated in the presence or absence of 10nM IGF-I for 8 hours and subjected to fluorescence-activated cell sorting analysis as previously described (22).

Densitometric and statistical analysis

Densitometry results were obtained by using GelEval 1.22 software. Differences between means were evaluated by ANOVA (one- or two-way when appropriate) followed by post hoc analysis of significance (Bonferroni test). The level of significance was set at $P < .05$. Statistical analysis was performed with GraphPad Prism 5 (GraphPad Software). Data are expressed as mean \pm SEM.

Results

Metformin inhibits androgen-mediated IGF-IR up-regulation and promoter activity

LNCaP cells were exposed to R1881 (10nM) for 6, 24, and 48 hours in the presence or absence of metformin (10mM). In accordance with our previous studies (5), IGF-IR was clearly up-regulated after 24 and 48 hours of exposure to R1881, with a maximum increase of approximately 10 ± 3 -fold over basal at 48 hours (Figure 1A). Metformin significantly inhibited R1881-mediated IGF-IR up-regulation (up to $62 \pm 2\%$ and $56 \pm 5\%$ of untreated at 24 and at 48 h, respectively). In contrast, neither R1881 nor metformin affected the expression of the IGF-IR homolog insulin receptor (IR), indicating that these regulatory mechanisms are specific for the IGF-IR (Figure 1A). Dose response experiments indicated that the inhibitory effect of metformin starts at 1mM–3mM and reaches a maximum at 10mM (Figure 1B).

We then evaluated whether metformin could inhibit IGF-IR up-regulation at the level of mRNA and

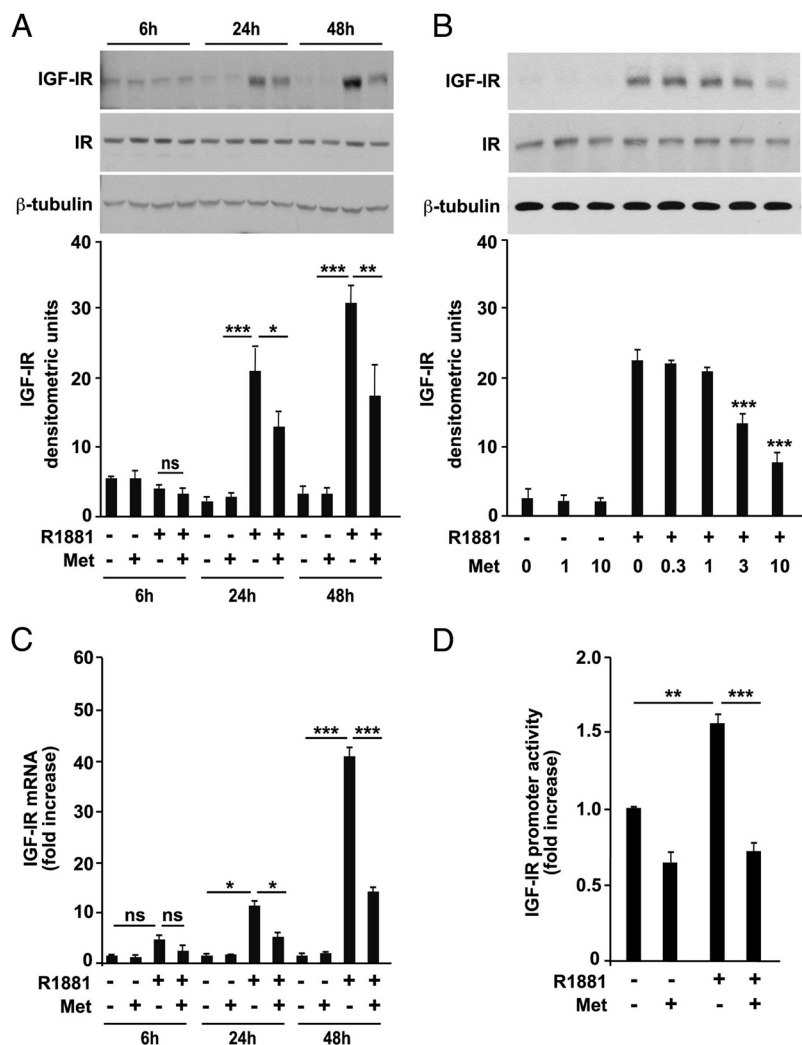


Figure 1. Metformin inhibits androgen-mediated IGF-IR protein, mRNA up-regulation, and IGF-IR promoter activity. **A**, Time-course analysis of IGF-IR protein expression. Cells were serum starved for 24 hours and then preincubated with 10mM metformin (Met) for 4 hours before adding 10nM R1881 for the indicated time points. Cells were therefore solubilized and analyzed by Western blotting. ns, nonstatistically significant ($P > .05$); *, $P < .05$; **, $P < .005$; ***, $P < .0005$ (untreated vs R1881 or R1881 vs R1881 plus metformin). **B**, Dose response of IGF-IR protein expression. Cells were serum starved for 24 hours and then preincubated with different concentrations of metformin 4 hours before adding 10nM R1881 for 24 hours. Cells were then lysed and analyzed by Western blotting. Filters were probed with antibodies specific for IGF-IR and IR. Immunoblotting with anti- β -tubulin was used to control protein loading. ***, $P < .0005$ (R1881 vs R1881 plus 3mM and 10mM metformin). The top panels in **A** and **B** show a representative experiment. The histograms represent the mean \pm SEM (error bars) of densitometric analyses of 4 separate experiments. **C**, IGF-IR mRNA expression. The conditions were as in **A** and relative of IGF-IR mRNA expression measured by SYBR Green Chemistry. β -Actin was used as an endogenous control for normalization. Bars, mean \pm SEM of 4 independent experiments. ns, not statistically significant ($P > .05$); *, $P < .05$; ***, $P < .0005$ (untreated vs R1881 or R1881 vs R1881 plus metformin). **D**, IGF-IR promoter activity. LNCaP cells were transiently transfected with plasmids encoding the full-length IGF-IR promoter-luciferase vector and then pretreated with 10mM metformin before adding 10nM R1881 for 48 hours. Columns, mean of 3 separate experiments, normalized for transfection efficiency with a GFP vector. **, $P < .005$; ***, $P < .0005$ (untreated vs R1881-treated cells, and R1881 vs R1881 plus metformin). Statistical significance was calculated using one-way ANOVA followed by Bonferroni test.

IGF-IR promoter activity. In time-course experiments, R1881 increased IGF-IR mRNA with an effect that starts at 6 hours (5 ± 0.8 -fold over basal) and reaches a maximum at 48 hours (43 ± 8 -fold over basal) (Figure 1C). Metformin (10mM) significantly reduced this R1881 effect both at 24 and 48 hours (Figure 1C). At 48 hours, IGF-IR transcript was approximately 40% of peak stimulation obtained by R1881. This effect was also specific for the IGF-IR, because IR mRNA expression was neither affected by R1881 nor by metformin incubation (data not shown).

To ascertain whether metformin inhibits R1881 effect at the level of IGF-IR promoter, a full-length IGF-IR promoter-luciferase construct was transfected in LNCaP cells. Promoter activation was evaluated as luciferase activity after stimulation with R1881 for 6, 24, and 48 hours in the presence or absence of metformin. R1881 increased the activity of the IGF-IR promoter starting at 6 hours (data not shown), with a maximum of 1.5 ± 0.7 -fold over basal at 48 hours ($P < .0005$) (Figure 1D). Metformin significantly inhibited IGF-IR promoter activation by R1881 (Figure 1D).

We previously reported that androgens are also able to up-regulate IGF-IR protein and increase IGF-IR promoter activity in other cell models, including HEK293 cells transfected with a wild-type AR (ARwt) expression construct (5). Here, we asked whether metformin was also able to block these effects in ARwt-transfected HEK293 cells as well as in a second AR⁺ human prostate cancer cell line, VCaP cells. In both cell lines, R1881 induced marked IGF-IR up-regulation, which was strongly inhibited by metformin (Supplemental Figure 1A, published on The Endocrine Society's Journals Online web site at <http://endo.endojournals.org>). Similarly, in both cell lines, R1881 significantly increased IGF-IR promoter activity, which was completely blocked by metformin (Supplemental Figure 1B).

Metformin inhibits androgen-dependent CREB activity and phosphorylation

We previously demonstrated that, in LNCaP cells, androgens activate CREB and that CREB-dependent stimulation of IGF-IR promoter activity is essential for androgen-induced IGF-IR up-regulation (6). According to these data, CREB silencing by specific shRNA abolished IGF-IR up-regulation in response to R1881 (Figure 2A). We then evaluated whether the inhibitory effect of metformin may be mediated by the inhibition of androgen-induced CRE activity. To this aim, a full-length CRE-Luc construct was cotransfected together with H2BGFP vector, and CRE activation was measured as luciferase activity after stimulation with 10nM R1881. We found that R1881 stimulates

CRE activity by approximately 2-fold over the basal and that either metformin or a Δ N CREB construct completely blocks this activation. Metformin had no additional effect in the presence of Δ N CREB (Figure 2B). Similar results were obtained in HEK293 cells cotransfected with an AR expression construct and a full-length CRE-Luc plasmid (Supplemental Figure 1C).

We then evaluated whether metformin inhibits CREB Ser133 phosphorylation, which is associated with CREB activation. We found that metformin affects CREB Ser133 phosphorylation in a different way, depending on the time of drug exposure. Indeed, in cells preincubated for 6 hours, both metformin and R1881 significantly increased CREB phosphorylation, and the combination of the 2 drugs further increased it. After 24 hours of exposure, metformin alone increased CREB phosphorylation slightly but induced a reduction of R1881-dependent CREB phosphorylation. At 48 hours, metformin did not affect basal CREB phosphorylation but strongly inhibited R1881-induced CREB phosphorylation (Figure 2C).

Taken together, these data indicate that the inhibition of CREB phosphorylation and activity is crucial for metformin-induced inhibition of androgen-induced IGF-IR up-regulation.

Metformin affects the compartmentalization of CREB cofactor CRTC2

The transcriptional activity of CREB-dependent target genes is affected by CREB binding to its coactivators CBP and CRTC2. In hepatocytes, AMPK, induced by starvation or metformin, phosphorylates CRTC2 at Ser171 causing its interaction with 14-3-3 proteins and sequestration in the cytoplasm. By contrast, glucose and hormones lead to CRTC2 dephosphorylation and dissociation from 14-3-3 proteins. Dephosphorylated CRTC2 translocates into the nucleus and promotes CREB-dependent transcription of gluconeogenic gene expression (7). We hypothesized, therefore, that in LNCaP cells, metformin may block androgen-induced IGF-IR up-regulation by inducing the dissociation of the CREB-CBP-CRTC2 complex via CRTC2 phosphorylation, which would lead to its nuclear exclusion and degradation.

We first evaluated whether metformin could affect the cellular compartmentalization of CRTC2 in LNCaP cells treated with R1881, by using immunofluorescence and confocal microscopy. Both in basal conditions and after metformin exposure, CRTC2 was exclusively localized in the cytoplasm, whereas it translocated into the nucleus in androgen-stimulated cells (Figure 3A). Coincubation with metformin prevented CRTC2 nuclear translocation (Figure 3A). We then studied the phosphorylation status of CRTC2 after metformin and R1881 and found that met-

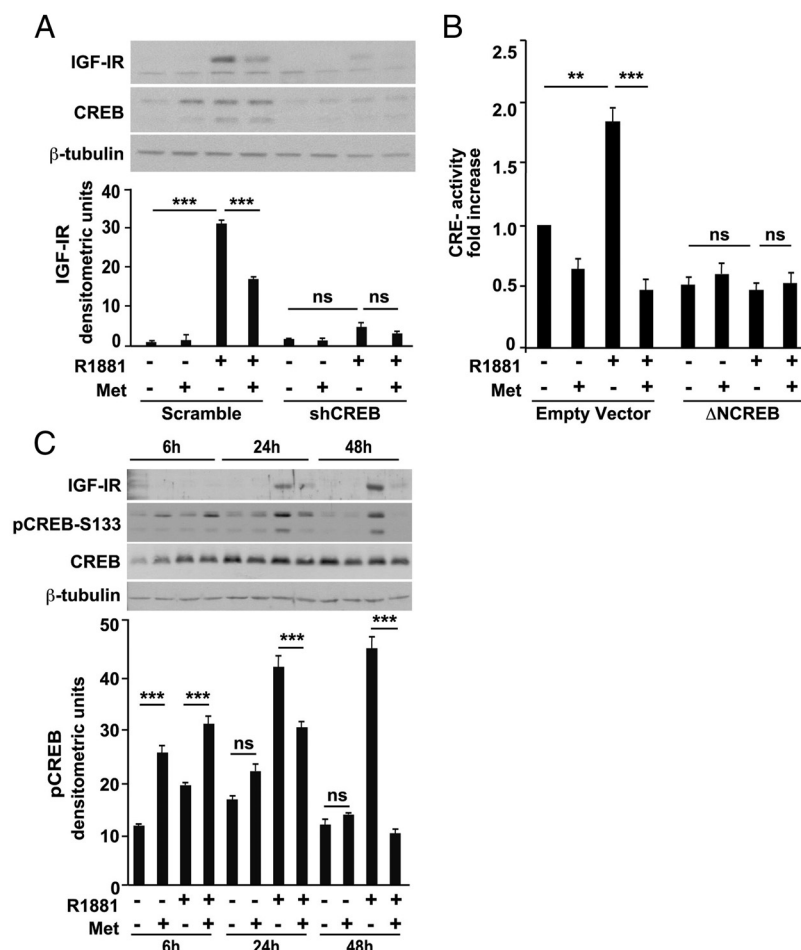


Figure 2. Metformin inhibits androgen-induced IGF-IR up-regulation by inhibiting CREB activity and phosphorylation. **A**, Androgen-induced IGF-IR up-regulation after CREB silencing. LNCaP cells were transiently transfected with shRNAs direct to CREB or scrambled shRNAs. After 24 hours, cells were serum starved for 24 hours and then were treated with metformin (10mM) in association or not with R1881 (10nM) for further 24 hours. Cells were lysed and analyzed by Western blotting. Filters were probed with antibodies specific for IGF-IR and CREB. Immunoblotting anti- β -tubulin was used to control protein loading. The histogram represents the mean \pm SEM (error bars) of densitometric analyses of 3 separate experiments. ns, not statistically significant ($P > .05$); ***, $P < .0005$ (untreated vs R1881, and R1881 vs R1881 plus metformin). **B**, Effect of Δ NCREB mutant on metformin effect. LNCaP cells were transiently cotransfected with plasmids encoding Δ NCREB mutant in the presence of a CRE-Luc construct. Cells were serum starved for 24 hours and then pretreated with 10mM metformin (10mM) 4 hours before adding R1881 (10nM) for further 24 hours. After drug exposure, CRE activity was measured. Columns, mean of 3 independent experiments normalized with a GFP vector. ns, not statistically significant ($P > .05$); **, $P < .005$; ***, $P < .0005$ (untreated vs R1881, and R1881 vs R1881 plus metformin). **C**, Time course of CREB phosphorylation by metformin with or without R1881. Cells were serum starved for 24 hours and then pretreated with 10mM metformin (Met) for 4 hours before adding 10nM R1881 for the indicated time points. Cells were, therefore, solubilized and analyzed by Western blotting. The top panel shows a representative experiment. The histogram represents the mean \pm SEM (error bars) of densitometric analysis of 3 separate experiments. ns, not statistically significant ($P > .05$); ***, $P < .0005$ (untreated vs metformin or R1881 vs R1881 plus metformin). Statistical significance was calculated using one-way ANOVA followed by Bonferroni test.

formin stimulates CRTC2 S171 phosphorylation, whereas R1881 decreases it. The combination treatment prevented R1881 effect (Figure 3B). Next, we evaluated the effect of CRTC2 silencing on androgen-induced IGF-IR and CRE promoter activation. We found that

CRTC2 silencing reduces R1881 stimulation on both IGF-IR and CRE promoter activities (Figure 3, C and D). Taken together, these data suggest that metformin may affect the transcriptional activity of the CREB-CBP-CRTC2 complex on IGF-IR promoter by inducing CRTC2 S171 phosphorylation.

Involvement of AMPK/mTORC1/p70S6K and p53/REDD1 pathways in the inhibitory effect of metformin

Metformin pleiotropic actions mostly arise from the activation of the serine/threonine kinase 11 (LKB1)/AMPK pathway, which, in turn, inhibits the mTORC1/p70S6K pathway. Indeed, in LNCaP cells, metformin does inhibit the mTORC1/p70S6K pathway, although this effect has been reported to be AMPK independent and to require p53-mediated REDD1 up-regulation (14). Because the mTORC1/p70S6K pathway may regulate CREB transcriptional activity, we evaluated the possible involvement of this pathway in metformin-mediated inhibition of IGF-IR up-regulation.

First, we found that R1881 markedly stimulates p70S6K phosphorylation, used as a read out of mTORC1 activity, whereas metformin inhibits p70S6K phosphorylation dose dependently, in strict correlation with the inhibition of IGF-IR up-regulation (Figure 4A). The activity of the TORC1 complex is regulated by Akt (PKB) but also by other kinases, including 90 kDa ribosomal S6 kinase (p90RSK) (23). In LNCaP cells, Akt (PKB) is constitutively activated, due to a phosphatase and tensin homologue deleted on chromosome ten (PTEN) mutation, and Akt (PKB) phosphorylation was only slightly enhanced by R1881 (Figure 4A). However, metformin, especially at 10mM, inhibited Akt (PKB) phosphorylation (Figure 4A). We found that inhibition of the mTORC1/p70S6K pathway by rapamycin reduced androgen-depen-

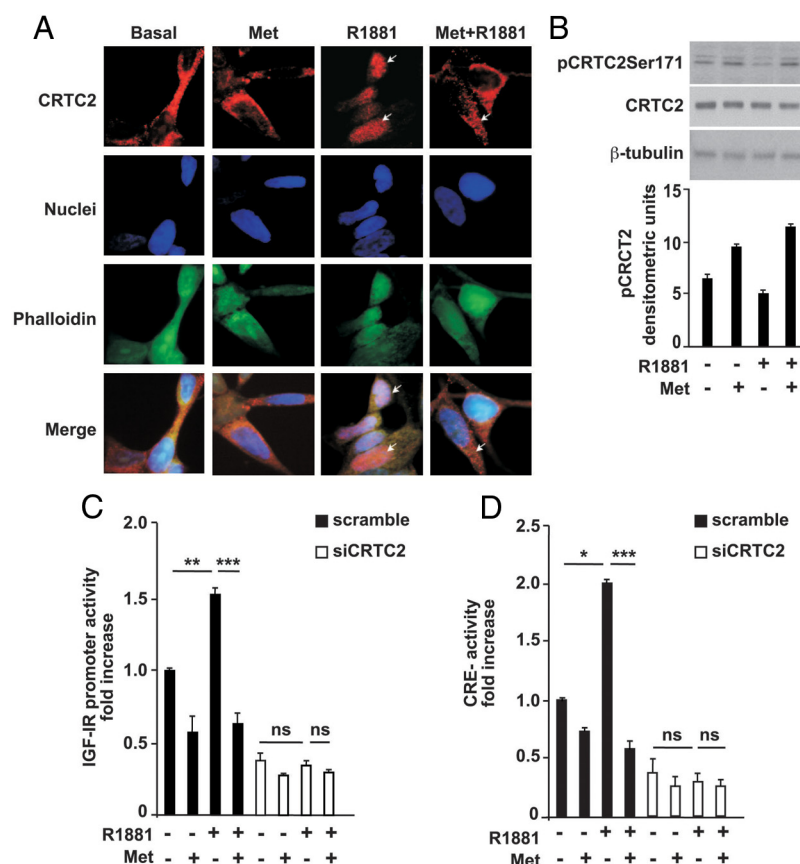


Figure 3. Metformin inhibition of androgen-dependent IGF-IR up-regulation requires CREB cofactor CRT2. **A**, CRT2 localization by immunofluorescence. LNCaP cells were serum starved for 24 hours and then pretreated with metformin (Met) (10mM) for 4 hours followed by R1881 exposure (10nM for 24 h). Cells were then plated on cover slips, fixed, and stained for CRT2 (red) and filamentous actin (phalloidin, green). Nuclei were visualized with Hoechst (blue). The images presented are representative of most cells examined for that treatment. **B**, Effect of metformin treatment in presence or absence of R1881 on CRT2 phosphorylation at Ser171. LNCaP cells were serum starved for 24 hours and then pretreated with metformin (30mM) for 4 hours followed by 30 minutes of R1881 (10nM) exposure. Cells were, therefore, solubilized and analyzed by Western blotting. The top panel shows a representative experiment. The histogram represents the mean \pm SEM (error bars) of densitometric analysis of 3 separate experiments. Activity of IGF-IR promoter (**C**) and CRE (**D**) after CRT2 silencing. LNCaP cells were transiently transfected with siRNAs to CRT2 or scrambled shRNAs and, after 24 hours, were transfected again with plasmids encoding the full-length IGF-IR promoter-luciferase vector (**C**) or CRE-Luc construct (**D**). Twelve hours after transfection, cells were serum starved for 10 hours and then pretreated with metformin (10mM) before adding R1881 (10nM) for further 24 hours. Columns, means of 3 separate experiments, normalized for transfection efficiency with a GFP vector. In **C** and **D**, ns, not statistically significant ($P > .05$); *, $P < .05$; **, $P < .005$; ***, $P < .0005$ (untreated vs R1881, and R1881 vs R1881 plus metformin, one-way ANOVA followed by Bonferroni test).

dent IGF-IR up-regulation both at the protein (Figure 4B) and mRNA levels (Figure 4C), mirroring metformin action. Rapamycin also reproduced metformin effects on the IGF-IR promoter (Figure 4D), indicating that mTORC1/p70S6K activity might be involved in mediating metformin effects. A second inhibitor of the mTORC1/p70S6K pathway, temsirolimus, showed similar effects than rapamycin (data not shown). We then silenced each of the 2 pathways, AMPK and p53/REDD1, possibly involved in the inhibition of mTORC1/p70S6K activity, and

evaluated whether this could revert the effects of metformin. We found that AMPK silencing partially affected metformin-dependent inhibition of R1881-induced IGF-IR overexpression (Figure 5A) and promoter activation (Figure 5B). REDD1 silencing only marginally affected IGF-IR overexpression (Figure 5, C and D). Because REDD1 is downstream p53 (24), we evaluated whether p53 silencing elicited similar effects. However, p53 silencing did not affect IGF-IR expression and promoter activity in response to either R1881 or metformin, and the combination of REDD1 and p53 silencing was not different from REDD1 silencing alone (Figure 5, C and D). Altogether, these results indicate that, in LNCaP cells, mTOR/p70S6K activity plays a role in androgen-mediated IGF-IR up-regulation and that metformin inhibits androgen-dependent mTORC1/p70S6K activity through mechanisms that are only partially dependent on AMPK activation and marginally affected by the p53/REDD1 pathway.

Metformin inhibits c-Src recruitment to AR

The activation of membrane-initiated AR signaling is originated by androgen-induced recruitment of AR to c-Src, subsequent c-Src activation, and AR/c-Src complex interaction with other relevant receptors and signaling molecules (19). According to our previous results, AR recruitment to c-Src and c-Src activation are crucial steps in mediating CREB activation and IGF-IR promoter activity through the ERK/p90RSK pathway (6). In fact, Src kinase activity inhibition with PP2 was able to block IGF-IR up-regulation by androgens (5). We then evaluated whether metformin was able to prevent AR recruitment to c-Src. Indeed, cell exposure to 10mM metformin for either 1 or 24 hours completely inhibited the formation of the AR/c-Src complex and downstream activation of p70S6K (Figure 6, A and B). As expected, a similar effect was obtained after Src kinase activity inhi-

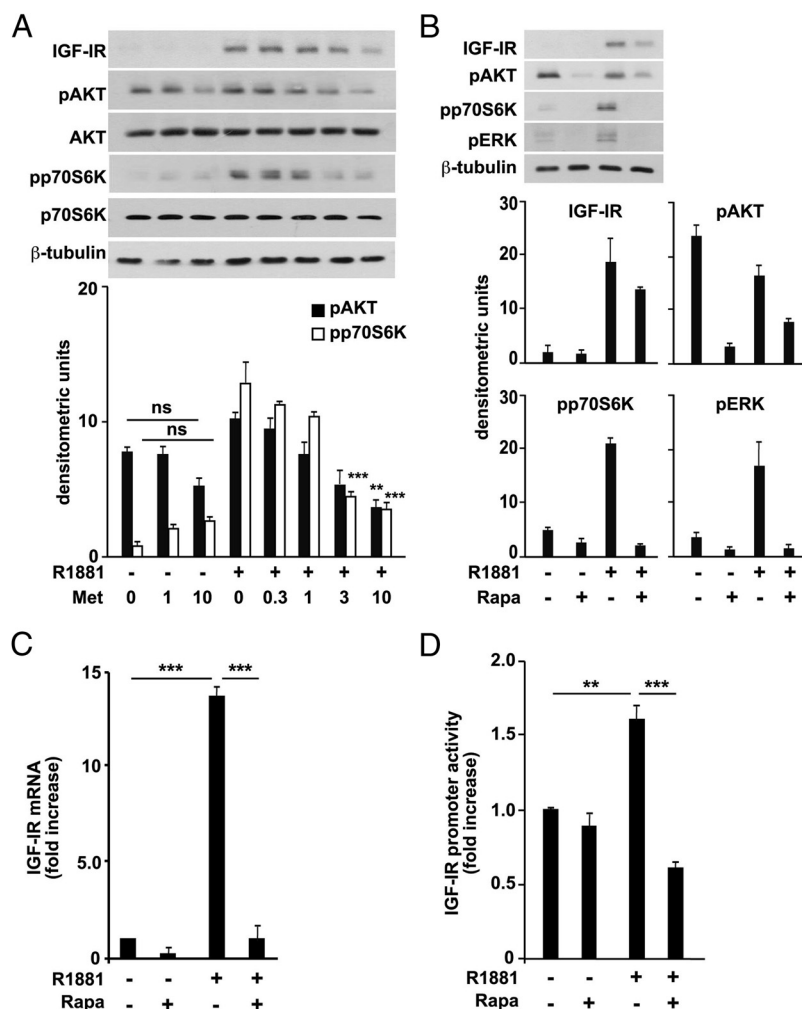


Figure 4. Involvement of mTORC1/p70S6K pathway in the inhibitory effect of metformin. A, Akt (PKB) and p70S6K activation in response to metformin (Met) in cells incubated or not with R1881. Cells were treated as in Figure 1B, lysed, and analyzed by Western blotting. Filters were probed with antibodies specific for the phosphorylated form (p) of AKT (PKB) and p70S6K. The same blots were probed with anti-AKT (PKB), anti-p70S6K, and anti- β -tubulin to control for protein loading. The top panel shows a representative experiment. The histogram represents the mean \pm SEM (error bars) of densitometric analyses of 4 separate experiments after normalization of each phosphopeptide against the total nonphosphorylated form. ns, not statistically significant ($P > .05$) (untreated vs 10mM metformin, pAKT, and pp70S6K analyses); *, $P < .005$ (R1881 vs R1881 plus 10mM metformin, pAKT analysis); ***, $P < .0005$ (R1881 vs R1881 plus 3mM or 10mM metformin, pp70S6K analysis). B, Involvement of the mTOR pathway in IGF-IR up-regulation by R1881. Cells were serum starved for 24 hours and then pretreated with 10nM rapamycin (Rapa) for 4 hours followed by 10nM R1881 for further 24 hours. Cells were, therefore, solubilized and analyzed by Western blotting. Filters were probed with antibodies specific for IGF-IR and the phosphorylated form (p) of AKT (PKB), p70S6K, and pERK. Immunoblotting for β -tubulin was used to control for protein loading. The top panel shows a representative experiment. The histogram represents the mean \pm SEM (error bars) of densitometric analyses of 3 separate experiments. C, IGF-IR mRNA relative quantification. The conditions were as in B. IGF-IR mRNA expression was measured by quantitative RTPCR in SYBR Green Chemistry. β -Actin was used as an endogenous control for normalization. Bars, mean \pm SEM of 3 independent experiments. ***, $P < .0005$ (untreated vs R1881, and R1881 vs R1881 plus rapamycin). D, Cells were transiently transfected with plasmids encoding the full-length IGF-IR promoter-luciferase vector and then pretreated with 10nM rapamycin before adding 10nM R1881 for 24 hours. Columns, means of 3 separate experiments, normalized for transfection efficiency with a GFP vector. **, $P < .005$; ***, $P < .0005$ (untreated vs R1881, and R1881 vs R1881 plus rapamycin). Statistical significance was calculated using one-way ANOVA followed by Bonferroni test.

bition with PP2 (Figure 6, C and D). These data indicate that metformin may inhibit the R1881-induced AR/c-Src complex formation and Src kinase activity, which is the very early step of membrane-initiated AR effects, to a similar degree than the Src kinase inhibitor PP2. Similarly, metformin inhibited the formation of R1881-induced AR/c-Src complex also in VCaP and in ARwt-transfected HEK293 cells (Supplemental Figure 1D).

To confirm that the inhibitory effect of metformin on androgen-induced IGF-IR promoter activity was dependent on c-Src association with AR, we used a peptide (S1 peptide), encompassing the 377–386 amino acid AR sequence, that inhibits c-Src recruitment to AR by inhibiting the interaction between the AR prolin-rich sequence and the Src Homology 3 Domain of Src kinase (19). We found that S1 peptide, similarly to metformin, abolished IGF-IR promoter activity induced by R1881 (Figure 6E).

AR tyrosine phosphorylation is an important event for prostate tumor growth, sensitivity to IGF-I responses, and progression to castration-resistant stage (25). We, therefore, evaluated the effect of R1881, metformin, and the combination of both drugs on AR tyrosine phosphorylation status. We found that, in LNCaP cells, AR is constitutively tyrosine phosphorylated, and this phosphorylation was significantly enhanced by 10nM R1881 for 2 minutes. Metformin (10mM for 1 or 24 h) slightly reduced basal AR phosphorylation and markedly inhibited R1881-induced AR phosphorylation (Figure 6, F and G). Altogether, these results suggest an additional mechanism of metformin inhibitory action on R1881 effects. Figure 7 summarizes the multiple levels of membrane-initiated androgen effects, which are affected by metformin.

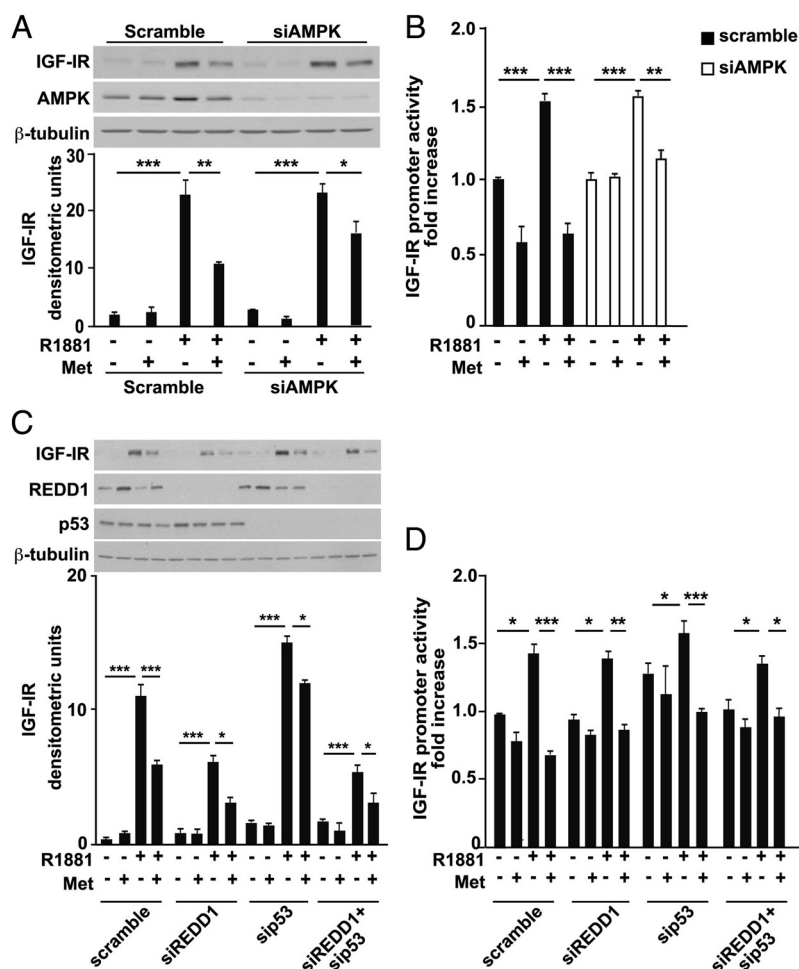


Figure 5. Involvement of AMPK and p53/REDD1 pathways in the inhibitory effect of metformin. **A**, Androgen-stimulated IGF-IR up-regulation after AMPK silencing. Cells were transiently transfected with siRNAs direct to AMPK or scrambled siRNAs. After 24 hours, cells were serum starved for 24 hours and then treated with metformin (Met) (10mM) 4 hours before the incubation or not with R1881 (10nM) for further 24 hours. Then cells were lysed and analyzed by Western blotting. Filters were probed with antibodies specific for IGF-IR and AMPK. Anti- β -tubulin immunoblotting was used to control protein loading. The histogram represents the mean \pm SEM (error bars) of densitometric analyses of 3 separate experiments. *, $P < .05$; **, $P < .005$; ***, $P < .0005$ (untreated vs R1881 and R1881 vs R1881 plus metformin, one-way ANOVA followed by Bonferroni test). **B**, Androgen-stimulated IGF-IR promoter activity after AMPK silencing. Cells were transiently transfected with siRNAs direct to AMPK or scrambled siRNAs. After 24 hours, cells were transiently transfected with plasmids encoding the full-length IGF-IR promoter-luciferase vector. Twelve hours after transfection cells were serum starved for 10 hours and then pretreated with metformin (10mM) followed by 10nM R1881 for further 24 hours. Columns, mean of 3 separate experiments, normalized for transfection efficiency with a GFP vector. **, $P < .005$; ***, $P < .0005$ (untreated vs R1881 and R1881 vs R1881 plus metformin, one-way ANOVA followed by Bonferroni test). **C**, Androgen-stimulated IGF-IR up-regulation after p53 and/or REDD1 silencing. Cells were transiently transfected with siRNAs direct to p53 and/or REDD1 or scrambled siRNAs and then treated as in **A**. Filters were probed with antibodies specific for IGF-IR, p53, and REDD1. Immunoblotting anti- β -tubulin was used to control protein loading. The histogram represents the mean \pm SEM (error bars) of densitometric analyses of 3 separate experiments. *, $P < .05$; ***, $P < .0005$ (untreated vs R1881, and R1881 vs R1881 plus metformin, one-way ANOVA followed by Bonferroni test). **D**, Androgen-stimulated IGF-IR promoter activity after p53 and/or REDD1 silencing. Cells were transiently transfected with siRNAs direct to p53 and/or REDD1 or scrambled siRNAs and then treated as in **B**. Columns, means of 3 separate experiments, normalized for transfection efficiency with a GFP vector. *, $P < .05$; **, $P < .005$; ***, $P < .0005$ (untreated vs R1881 and R1881 vs R1881 plus metformin). Statistical significance was calculated by one-way ANOVA followed by Bonferroni test.

Metformin inhibits the androgen-mediated enhancement of IGF-I biological effects

In prostate cancer cells, the IGF-IR up-regulation induced by androgens sensitizes cells to IGF-I-mediated biological effects (5). We, therefore, evaluated whether metformin, by inhibiting androgen-dependent IGF-IR up-regulation, was able to prevent cell sensitization to IGF-I biological effects. Serum-starved cells were preincubated or not with R1881 (10nM) for 24 hours. After medium change, cells were exposed to IGF-I (10nM) in the presence or absence of metformin (10mM). In cells preincubated with R1881, cell proliferation and invasion in response to IGF-I were significantly higher than in cells nonpreincubated with R1881 (Figure 8, A and B). Interestingly, this R1881 priming effect on both IGF-I actions was blocked by metformin (proliferation decreased from $215 \pm 2\%$ to $118 \pm 5\%$, and cell invasion decreased from $225 \pm 5\%$ to $120 \pm 3\%$) (Figure 7, A and B). Similar results were observed in cell cycle analysis experiments (Figure 8C), in which cell cycle progression, observed after IGF-I in androgen preincubated cells, was almost abrogated by metformin (S+G₂/M phase decreased from $135 \pm 4\%$ to $100 \pm 3\%$). Furthermore, in soft-agar assay, both LnCaP cells (Figure 8D) and VCaP cells (Supplemental Figure 1E) cotreated with metformin and R1881 formed fewer and smaller colonies compared with cells treated with R1881 alone, indicating a decrease in anchorage-independent growth. Altogether, these data suggest that metformin by inhibiting androgen-mediated IGF-IR up-regulation severely reduced both cell proliferation, invasion, and clonogenic capacity.

Discussion

We have previously shown that, in prostate cancer cells, androgens in-

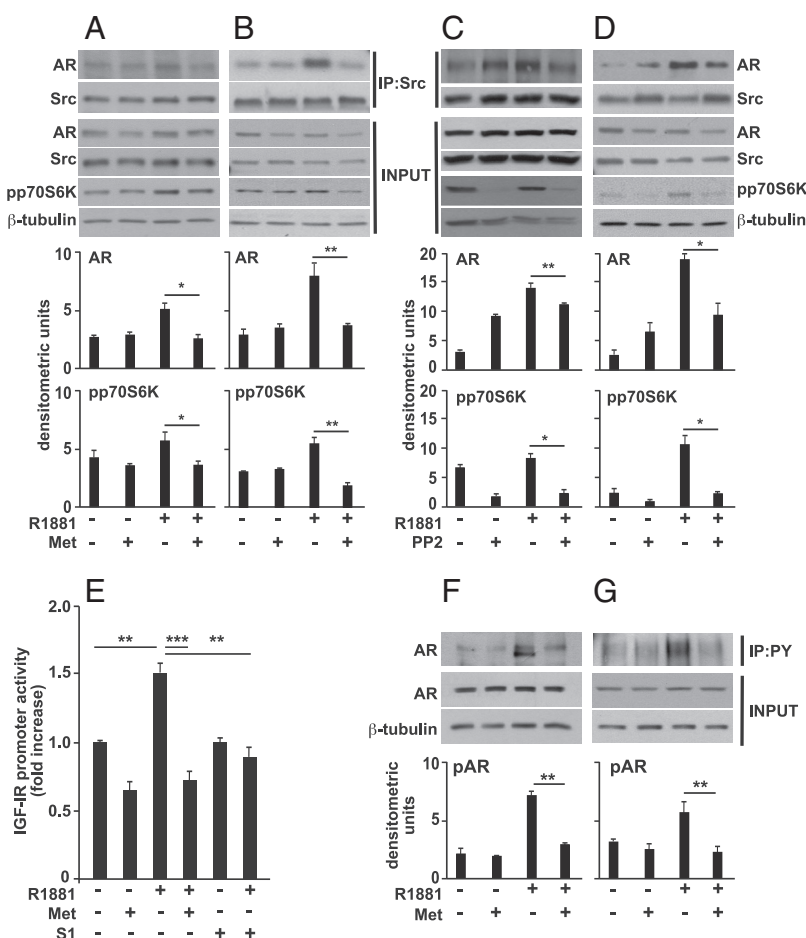


Figure 6. c-Src recruitment to AR. A and B, c-Src binding with AR after metformin (Met) treatment with or without R1881. Cells were serum starved for 24 hours and then treated with metformin (10mM) for 1 hour (A) or for 24 hours (B) followed by the exposure to 10nM R1881 for 2 minutes. Cells were then solubilized, and the lysates were immunoprecipitated with an anti-Src-specific antibody and analyzed by Western blotting. Total lysates (input) were also evaluated as a control. Filters were probed with anti-AR, anti-Src, anti-pp70S6K, and anti- β -tubulin antibodies as indicated. The panel shows a representative experiment of 3. The histogram represents the mean \pm SEM (error bars) of densitometric analyses of AR and pp70S6K for the 3 experiments. *, $P < .05$; **, $P < .005$ (R1881 vs R1881 plus metformin). C and D, Cells were serum starved for 24 hours and then treated with the Src inhibitor PP2 (10 μ M) for 1 hour (C) or for 24 hours (D) followed by the exposure to 10nM R1881 for 2 minutes. Cells were then harvested as in A. *, $P < .05$; **, $P < .005$ (R1881 vs R1881 plus PP2). E, IGF-IR promoter activity induced by androgens is inhibited by c-Src/AR interaction inhibitory peptide (S1). LNCaP cells were transiently transfected with plasmids encoding the full-length IGF-IR promoter-luciferase vector. Cells were serum starved for 24 hours and then pretreated with 10mM metformin or 1nM the c-Src/AR interaction peptide (S1) before adding 10nM R1881 for 24 hours. Columns, means of 3 separate experiments, normalized for transfection efficiency with a GFP vector. **, $P < .005$; ***, $P < .0005$ (untreated vs R1881-treated cells, and R1881 vs R1881 plus metformin or peptide S1). F and G, AR phosphorylation after metformin treatment in presence or absence of R1881. Cells were serum starved for 24 hours and then treated with metformin (10mM) for 1 hour (F) or for 24 hours (G) followed by 10nM R1881 exposure for 2 minutes. Cells were then solubilized, and the lysates were immunoprecipitated (IP) with an anti-phosphotyrosine (pY) (4G10)-specific antibody and analyzed by Western blotting. Total lysates (input) were also evaluated as a control and immunoblotted with anti-AR and anti- β -tubulin antibodies, as indicated. The panel shows a representative experiment of 3. The histogram represents the mean \pm SEM (error bars) of densitometric analyses of the experiments. **, $P < .005$ (R1881 vs R1881 plus metformin). Statistical significance was determined by one-way ANOVA followed by Bonferroni test.

duce significant up-regulation of the IGF-IR, which sensitizes cells to IGF-I-induced growth and invasion (5, 6). This androgen-dependent effect occurs through membrane-initiated events, which involve the formation of multiprotein complexes containing AR and Src and subsequent activation of c-Src/ERK/p90RSK cascade that ultimately induces CREB-dependent activation of IGF-IR promoter and increased IGF-IR mRNA transcription (6).

In the present study, we found that, in LNCaP prostate cancer cells, metformin inhibits androgen-induced IGF-IR up-regulation as well as IGF-I protumor effects. Metformin inhibited the stimulation of CREB phosphorylation and activity induced by androgens with consequent inhibition of androgen-induced IGF-IR promoter activity. These metformin effects were not exclusive of LNCaP cells but also observed in a second AR⁺ prostate cancer cell line, VCaP, and in AR-transfected HEK293.

Metformin effects are believed to mostly involve the activation of the LKB1/AMPK pathway. AMPK activation by metformin appears to be directly involved in the inhibition of mTORC1 complex activity, the decrease of cyclin D levels, and the up-regulation of p53 (10, 11, 14). Recent studies have suggested, however, that metformin may also have AMPK-independent effects (10, 13, 14). However, the effects of metformin at the level of membrane-initiated androgen actions are unknown, and the understanding of these effects may show great potential in prostate cancer management. Given the pleiotropic effects of metformin, we aimed at evaluating which mechanism is primarily associated with the inhibition of androgen-mediated up-regulation of IGF-IR.

When we evaluated the possible AMPK involvement on the metformin inhibitory action, we found

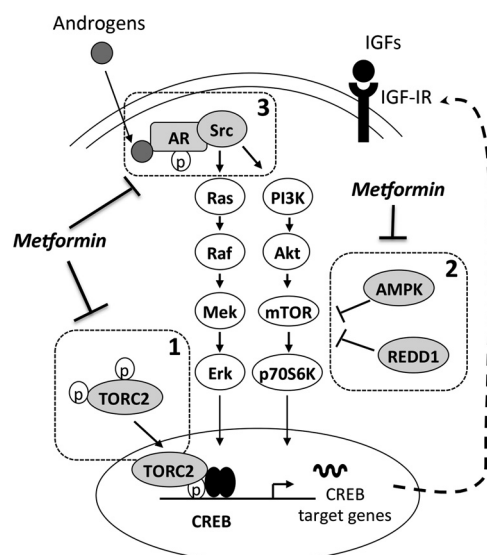


Figure 7. Cross talk between metformin, androgens, and IGF system and molecular mechanisms of metformin inhibitory effects. In prostate cancer cells, androgens up-regulate IGF-IR by inducing CREB transcriptional activation through membrane-initiated activation of the Src/MAPK/phosphoinositide 3-kinase (PI3K) pathway. The molecular mechanisms by which metformin may disrupt this androgen effect include: 1) inhibition of androgen-induced TORC2 dephosphorylation and nuclear translocation with consequent reduction in CREB/TORC2 complex transcription activity; 2) inhibition of mTOR/p70S6K signaling, partially through the AMPK and REDD1 pathways; and 3) Disruption of AR/c-Src interaction and inhibition of AR phosphorylation. Ras, rat sarcoma; Raf, rapidly accelerated fibrosarcoma; Mek, mitogen-activated protein kinase kinase.

that AMPK silencing was associated with some reduction of metformin effect, indicating that this is only partially dependent on AMPK activation. AMPK may inhibit CREB transcriptional activity by 2 different mechanisms involving both the CREB-CBP-CRCT2 complex (26–28) and the mTORC complex (16, 29). According to the first mechanism, described in hepatocytes, metformin-induced inhibition of CREB transcriptional activity involves AMPK-dependent phosphorylation of CREB cofactor CRCT2 at Ser171 causing its sequestration in the cytoplasm by interaction with 14–3–3 proteins (26–28). On the other hand, CRCT2 dephosphorylation causes its translocation to the nucleus, where it contributes to the formation of the CREB-CBP-CRCT2 complex and CREB-dependent transcription (7).

In unstimulated cells, we found that CRCT2 is phosphorylated at Ser171 and almost entirely located in the cytoplasmic compartment. Cells exposure to R1881 induced CRCT2 dephosphorylation and nuclear translocation. Both events were prevented by metformin. Furthermore, CRCT2 silencing reduced R1881 stimulation of IGF-IR and CRE promoter activities. This mechanism, therefore, may partially account for the inhibitory effect of metformin, and for the first time, it is described in cancer

cells. Metformin also contributed to inhibit CRE activity by inhibiting androgen-induced CREB Ser133 phosphorylation despite a transient increase in CREB phosphorylation. Although the mechanism(s) by which metformin transiently increase CREB phosphorylation is unclear, it is accepted that transient CREB phosphorylation is a poor inducer of CREB-mediated transcription, which requires prolonged and strong CREB phosphorylation (17).

Next, we found that mTORC1 complex is a key mediator of androgen-induced IGF-IR up-regulation, because mTORC1 inhibition by rapamycin or temsirolimus blocked IGF-IR up-regulation and its promoter activity. It is well known that metformin may inhibit mTORC1 through AMPK (29). AMPK inhibits mTORC1 by inducing Tuberous Sclerosis Complex 2 (TSC2) Ser1345 phosphorylation, which stimulates its GTPase activity (30), but also by inducing raptor phosphorylation, which causes raptor dissociation from mTOR (31). Although mTORC1 inhibition by metformin is usually AMPK dependent, in LNCaP cells, metformin may inhibit mTORC1 activity by an AMPK-independent mechanism, which involves p53-dependent up-regulation of REDD1 (14). REDD1, in turn, suppresses mTORC1 activity by displacing 14–3–3 proteins from TSC2, thus releasing TSC2 from inhibition (32). We, therefore, evaluated whether these mechanisms may account for metformin effects.

Metformin inhibited R1881-dependent p70S6K phosphorylation, used as readout of mTORC1 activity. However, AMPK silencing only partially affected the inhibitory effects of metformin on IGF-IR overexpression and promoter activation, whereas REDD1 silencing had only a marginal effect. Because REDD1 induction by metformin is downstream of p53, we also evaluated the effect of p53 silencing alone or in combination with REDD1 depletion. Neither of these conditions, however, significantly affected metformin inhibitory action. Basal IGF-IR levels were up-regulated by p53 silencing, in agreement with the central role played by p53 as negative regulator of IGF-IR promoter (33). These data are in partial agreement with previous findings showing that, in LNCaP cells, mTORC1 inhibition by metformin is independent by AMPK (14). However, in contrast to our results, these authors found that metformin effect was mediated by REDD1 up-regulation.

Taken together, our data show that, in LNCaP cells, AMPK is a weak inhibitor of androgen-stimulated mTORC1 activity and that REDD1 has a marginal role. One possible interpretation of these data is based on the well-known presence of an activating mutation of PTEN in LNCaP cells (34). PTEN mutation causes constitutive activation of Akt (PKB), which phosphorylates TSC2 at multiple sites, thus inactivating its Ras homolog enriched in brain GTPase activity and inducing constitutive

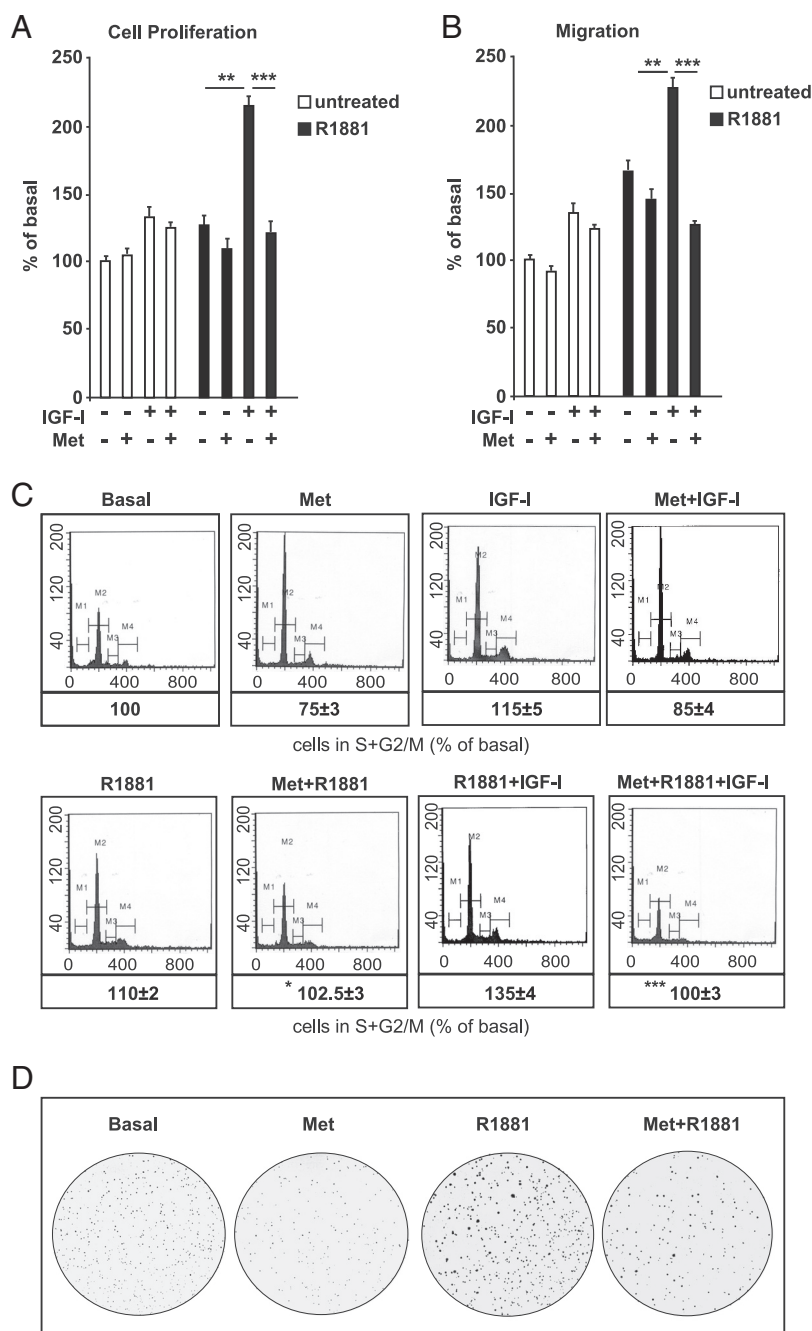


Figure 8. Metformin inhibits the androgen-mediated enhancement of IGF-I biological effects. A, Cell proliferation. LNCaP cells were grown in 96-well plates, serum starved for 24 hours, and preincubated with or without R1881 (10nM) in the presence or absence of metformin (Met) (10mM). After 24 hours, the medium was then replaced with R1881-free medium containing 1% stripped serum, and cells were incubated with or without 10nM IGF-I for additional 24 hours, in presence or absence of metformin (10mM). Cell viability was evaluated by MTT assay. Values are expressed as percentages of untreated cells and represent the mean \pm SEM (errors bars) of 2 separate experiments performed in triplicate. **, $P < .005$; ***, $P < .0005$ (IGF-I vs basal and IGF-I vs IGF-I+metformin, respectively). B, Invasion. Subconfluent LNCaP cells, serum starved for 24 hours, were preincubated with or without R1881 (10nM) and in the presence or absence of metformin (10mM). Then, cells were removed from plates with 0.01% trypsin and seeded on polycarbonate filters. Cells were allowed to migrate to the underside of the top chamber for 18 hours in response to 10nM IGF-I added to the lower chamber and in the presence or absence of metformin (10mM). Values are mean \pm SEM (errors bars) of 2 separate experiments done in triplicate and are expressed as percent of untreated cells. **, $P < .05$; ***, $P < .0005$ (IGF-I vs basal and IGF-I vs IGF-I+metformin, respectively). C, Cell cycle progression. LNCaP cells were treated as in A and then analyzed for their cell cycle profiles. Cell populations positive for propidium iodine staining were evaluated by fluorescence-activated cell sorting (FACS) analysis, and G_0/G_1 and G_2/M phases were scored. Panels show the cell cycle profile of untreated and stimulated cells of a representative experiment. Below each cell cycle profile the percentage of cells in S and G_2/M phases is indicated. Values are expressed as percent of basal and are the mean \pm SEM (errors bars) of 2 separate experiments. *, $P < .05$; ***, $P < .0005$ (R1881 vs R1881 plus metformin in presence or absence of IGF-I, respectively). Statistical significance was calculated using one-way ANOVA followed by Bonferroni test. D, Colony formation after metformin treatment with or without R1881. The soft-agar colony assay of LNCaP cells was performed as described in Material and Methods. Cells were cultured for 3 weeks, and the colonies formed were stained with MTT and then photographed.

mTORC1 activation (35–37). It seems likely that this constitutive phosphorylation of TSC2 by Akt (PKB) reduces the activity of AMPK and of REDD1, which also act on TSC2 (14).

We then hypothesized that the inhibition of androgen-induced p70S6K stimulation by metformin could result by the disruption of the AR-c-Src complex. As a result of AR-c-Src association, androgens induce downstream signaling, which involves ERK1/2 and p70S6K. Notably, metformin was able to block AR association with c-Src. Disruption of the AR association with c-Src at the cell membrane appears, therefore, to play an essential role in metformin antagonism on membrane-initiated androgen effects.

It has been reported that AR may be tyrosine phosphorylated in response to growth factors. This AR modification may be important for prostate tumor growth, sensitivity to IGF-I effects, and progression to castration-resistant stage (25). Thus, we explored the effect of metformin and androgens on AR tyrosine phosphorylation. We found the R1881 mediated AR tyrosine phosphorylation and that metformin inhibited it, suggesting an additional mechanism of metformin inhibitory action on R1881 effects.

Interestingly, in FBS-containing semisolid agar, metformin was able also to inhibit R1881-induced enhancement of colony formation, an effect closely related to malignant cell growth and cancer invasion. These data are reminiscent of previous results showing that metformin enhances the antiproliferative and apoptotic effect of bicalutamide in prostate cancer cells (12). Several other studies have provided evidence that metformin may have therapeutic potential in prostate cancer (10, 14). A recent retrospective clinical study has shown that metformin may improve recurrence-free survival, distant metastases-free survival, cancer-specific mortality, and development of castration-resistant prostate cancer (38). However, the molecular mechanisms involved are unclear, and authors mostly refer to the inhibiting effect of LKB1/AMPK on mTORC1 (8, 9, 11). We now show that, in LNCaP cells exposed to metformin, AMPK plays a minor role in inhibiting mTORC1, whereas metformin strongly interferes with membrane-initiated effects of androgens by disrupting the very early step of this signaling cascade, which involves AR association with c-Src, and AR tyrosine phosphorylation, and also inhibit CRCT2 nuclear translocation and CREB activation.

Future studies are needed to establish to what extent our mechanistic results are generalizable to *in vivo* models.

Metformin started to be efficacious at a concentration of 1mM with a maximal effect observed at 10mM, a concentration well within the range used by most *in vitro*

studies using various cancer cells, including prostate cancer cells (1mM–100mM, most often 10mM–20mM) (10–12, 17). Although, these concentrations are clearly higher than metformin levels observed in diabetic patients (mostly <20μM) (39), it has been previously shown that many organs are exposed to metformin concentrations significantly higher than those present in the bloodstream. For instance, metformin concentration is much higher in the portal vein than in the general circulation. Therefore, liver is exposed to metformin concentration in the millimolar range (40). Moreover, metformin is also able to concentrate in several other organs/tissues in dependence on its pharmacokinetics and the expression of transporters, such as organic cation transporter (OCT)1, OCT2, and OCT3. Therefore, salivary glands, stomach, small intestine, kidney, and other organs/tissues are all exposed to metformin concentrations appreciably higher than that present in the bloodstream (41). Finally, because of its positive charge, metformin may concentrate by approximately 1000-fold in mitochondria, to reach millimolar concentrations in the mitochondrial matrix (42). Furthermore, recent studies have reported that breast stem cancer cells are sensitive to micromolar concentrations of metformin (43), suggesting that subsets of cancer cells with stem-like features might be able to exquisitely concentrate metformin. To our knowledge, studies investigating metformin concentrations in prostate and prostate cancer tissue are still lacking and are certainly needed, also because metformin use is associated with all-cause and prostate cancer-specific mortality among men with diabetes (44).

In conclusion, we have identified a novel potential antitumor effect of metformin operating in AR⁺ prostate cancer cells and involving the inhibition of membrane-initiated androgen effects, adding to the list of the numerous antitumor effects of metformin observed in several *in vitro* and *in vivo* model systems. We have especially characterized the action of metformin in blocking androgen-induced IGF-IR up-regulation and IGF-I-mediated biological effects. However, given that metformin inhibits a very early step of membrane-initiated androgen effects, ie, the association between Src and AR and AR phosphorylation, there is the possibility that metformin may also block other membrane-initiated effects of androgens. Several evidences indicate that membrane-initiated androgen effects are enhanced in cancer cells and may play a role in tumor progression (1–3). Our present findings, therefore, open the way to a deeper understanding of the potential effects of metformin in prostate cancer.

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References

- McKenna NJ, O'Malley BW. Combinatorial control of gene expression by nuclear receptors and coregulators. *Cell*. 2002;108:465–474.
- Castoria G, Migliaccio A, D'Amato L, et al. Integrating signals between cAMP and MAPK pathways in breast cancer. *Front Biosci*. 2008;13:1318–1327.
- Michels G, Hoppe UC. Rapid actions of androgens. *Front Neuroendocrinol*. 2008;29:182–198.
- De Bellis A, Ghiandi P, Comerci A, et al. Epidermal growth factor, epidermal growth factor receptor, and transforming growth factor- α in human hyperplastic prostate tissue: expression and cellular localization. *J Clin Endocrinol Metab*. 1996;81:4148–4154.
- Pandini G, Mineo R, Frasca F, et al. Androgens up-regulate the insulin-like growth factor-I receptor in prostate cancer cells. *Cancer Res*. 2005;65:1849–1857.
- Genua M, Pandini G, Sisci D, et al. Role of cyclic AMP response element-binding protein in insulin-like growth factor-I receptor up-regulation by sex steroids in prostate cancer cells. *Cancer Res*. 2009;69:7270–7277.
- He L, Sabet A, Djedjos S, et al. Metformin and insulin suppress hepatic gluconeogenesis through phosphorylation of CREB binding protein. *Cell*. 2009;137:635–646.
- Dowling RJ, Niraula S, Stambolic V, Goodwin PJ. Metformin in cancer: translational challenges. *J Mol Endocrinol*. 2012;48:R31–43.
- Emami Riedmaier A, Fisel P, Nies AT, Schaeffeler E, Schwab M. Metformin and cancer: from the old medicine cabinet to pharmacological pitfalls and prospects. *Trends Pharmacol Sci*. 2013;34:126–135.
- Ben Sahra I, Laurent K, Loubat A, et al. The antidiabetic drug metformin exerts an antitumoral effect in vitro and in vivo through a decrease of cyclin D1 level. *Oncogene*. 2008;27:3576–3586.
- Ben Sahra I, Le Marchand-Brustel Y, Tanti JF, Bost F. Metformin in cancer therapy: a new perspective for an old antidiabetic drug? *Mol Cancer Ther*. 2010;9:1092–1099.
- Colquhoun AJ, Venier NA, Vandersluis AD, et al. Metformin enhances the antiproliferative and apoptotic effect of bicalutamide in prostate cancer. *Prostate Cancer Prostatic Dis*. 2012;15:346–352.
- Kalender A, Selvaraj A, Kim SY, et al. Metformin, independent of AMPK, inhibits mTORC1 in a rag GTPase-dependent manner. *Cell Metab*. 2010;11:390–401.
- Ben Sahra I, Regazzetti C, Robert G, et al. Metformin, independent of AMPK, induces mTOR inhibition and cell-cycle arrest through REDD1. *Cancer Res*. 2011;71:4366–4372.
- Malki A, Youssef A. Antidiabetic drug metformin induces apoptosis in human MCF breast cancer via targeting ERK signaling. *Oncol Res*. 2011;19:275–285.
- Kim HG, Hien TT, Han EH, et al. Metformin inhibits P-glycoprotein expression via the NF- κ B pathway and CRE transcriptional activity through AMPK activation. *Br J Pharmacol*. 2011;162:1096–1108.
- Hwang YP, Jeong HG. Metformin blocks migration and invasion of tumour cells by inhibition of matrix metalloproteinase-9 activation through a calcium and protein kinase C α -dependent pathway: phorbol-12-myristate-13-acetate-induced/extracellular signal-regulated kinase/activator protein-1. *Br J Pharmacol*. 2010;160:1195–1211.
- Kumar N, Dey CS. Metformin enhances insulin signalling in insulin-dependent and-independent pathways in insulin resistant muscle cells. *Br J Pharmacol*. 2002;137:329–336.
- Migliaccio A, Varricchio L, De Falco A, et al. Inhibition of the SH3 domain-mediated binding of Src to the androgen receptor and its effect on tumor growth. *Oncogene*. 2007;26:6619–6629.
- Pandini G, Frasca F, Mineo R, Sciacca L, Vigneri R, Belfiore A. Insulin/insulin-like growth factor I hybrid receptors have different biological characteristics depending on the insulin receptor isoform involved. *J Biol Chem*. 2002;277:39684–39695.
- Sieg DJ, Ili D, Jones KC, Damsky CH, Hunter T, Schlaepfer DD. Pyk2 and Src-family protein-tyrosine kinases compensate for the loss of FAK in fibronectin-stimulated signaling events but Pyk2 does not fully function to enhance FAK- cell migration. *EMBO J*. 1998;17:5933–5947.
- Malaguarnera R, Sacco A, Voci C, Pandini G, Vigneri R, Belfiore A. Proinsulin binds with high affinity the insulin receptor isoform A and predominantly activates the mitogenic pathway. *Endocrinology*. 2012;153:2152–2163.
- Chong ZZ, Shang YC, Zhang L, Wang S, Maiese K. Mammalian target of rapamycin: hitting the bull's-eye for neurological disorders. *Oxid Med Cell Longev*. 2010;3:374–391.
- Ellisen LW, Ramsayer KD, Johannessen CM, et al. REDD1, a developmentally regulated transcriptional target of p63 and p53, links p63 to regulation of reactive oxygen species. *Mol Cell*. 2002;10:995–1005.
- Guo Z, Dai B, Jiang T, et al. Regulation of androgen receptor activity by tyrosine phosphorylation. *Cancer Cell*. 2006;10:309–319.
- Conkright MD, Canettieri G, Screaton R, et al. TORCs: transducers of regulated CREB activity. *Mol Cell*. 2003;12:413–423.
- Screaton RA, Conkright MD, Katoh Y, et al. The CREB coactivator TORC2 functions as a calcium- and cAMP-sensitive coincidence detector. *Cell*. 2004;119:61–74.
- Dentin R, Liu Y, Koo SH, et al. Insulin modulates gluconeogenesis by inhibition of the coactivator TORC2. *Nature*. 2007;449:366–369.
- Horike N, Sakoda H, Kushiya A, et al. AMP-activated protein kinase activation increases phosphorylation of glycogen synthase kinase 3 β and thereby reduces cAMP-responsive element transcriptional activity and phosphoenolpyruvate carboxykinase C gene expression in the liver. *Journal Biol Chem*. 2008;283:33902–33910.
- Inoki K, Zhu T, Guan KL. TSC2 mediates cellular energy response to control cell growth and survival. *Cell*. 2003;115:577–590.
- Gwinn DM, Shackelford DB, Egan DF, et al. AMPK phosphorylation of raptor mediates a metabolic checkpoint. *Mol Cell*. 2008;30:214–226.

32. DeYoung MP, Horak P, Sofer A, Sgroi D, Ellisen LW. Hypoxia regulates TSC1/2-mTOR signaling and tumor suppression through REDD1-mediated 14-3-3 shuttling. *Genes Dev.* 2008;22:239–251.
33. Werner H, Shalita-Chesner M, Abramovitch S, Idelman G, Shaharabani-Gargir L, Glaser T. Regulation of the insulin-like growth factor-I receptor gene by oncogenes and antioncogenes: implications in human cancer. *Mol Genet Metab.* 2000;71:315–320.
34. Vlietstra RJ, van Alewijk DC, Hermans KG, van Steenbrugge GJ, Trapman J. Frequent inactivation of PTEN in prostate cancer cell lines and xenografts. *Cancer Res.* 1998;58:2720–2723.
35. Inoki K, Li Y, Zhu T, Wu J, Guan KL. TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. *Nat Cell Biol.* 2002;4:648–657.
36. Manning BD, Tee AR, Logsdon MN, Blenis J, Cantley LC. Identification of the tuberous sclerosis complex-2 tumor suppressor gene product tuberlin as a target of the phosphoinositide 3-kinase/akt pathway. *Mol Cell.* 2002;10:151–162.
37. Huang J, Manning BD. A complex interplay between Akt, TSC2 and the two mTOR complexes. *Biochem Soc Trans.* 2009;37:217–222.
38. Spratt DE, Zhang C, Zumsteg ZS, Pei X, Zhang Z, Zelefsky MJ. Metformin and prostate cancer: reduced development of castration-resistant disease and prostate cancer mortality. *Eur Urol.* 2013;63:709–716.
39. Frid A, Sterner GN, Löndahl M, et al. Novel assay of metformin levels in patients with type 2 diabetes and varying levels of renal function: clinical recommendations. *Diabetes Care.* 2010;33:1291–1293.
40. Owen MR, Doran E, Halestrap AP. Evidence that metformin exerts its anti-diabetic effects through inhibition of complex 1 of the mitochondrial respiratory chain. *Biochem J.* 2000;348 Pt 3:607–614.
41. Wilcock C, Bailey CJ. Accumulation of metformin by tissues of the normal and diabetic mouse. *Xenobiotica.* 1994;24:49–57.
42. Carvalho C, Correia S, Santos MS, Seica R, Oliveira CR, Moreira PI. Metformin promotes isolated rat liver mitochondria impairment. *Mol Cell Biochem.* 2008;308:75–83.
43. Hirsch HA, Iliopoulos D, Tsiachlis PN, Struhl K. Metformin selectively targets cancer stem cells, and acts together with chemotherapy to block tumor growth and prolong remission. *Cancer Res.* 2009;69:7507–7511.
44. Margel D, Urbach DR, Lipscombe LL, et al. Metformin use and all-cause and prostate cancer-specific mortality among men with diabetes. *J Clin Oncol.* 2013;31:3069–3075.



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