Metformin, Independent of AMPK, Induces mTOR Inhibition and Cell-Cycle Arrest through REDD1

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Abstract

Metformin is a widely prescribed antidiabetic drug associated with a reduced risk of cancer. Many studies show that metformin inhibits cancer cell viability through the inhibition of mTOR. We recently showed that antiproliferative action of metformin in prostate cancer cell lines is not mediated by AMP-activated protein kinase (AMPK). We identified REDD1 (also known as DDIT4 and RTP801), a negative regulator of mTOR, as a new molecular target of metformin. We show that metformin increases REDD1 expression in a p53-dependent manner. REDD1 invalidation, using siRNA or REDD1−/− cells, abrogates metformin inhibition of mTOR. Importantly, inhibition of REDD1 reverses metformin-induced cell-cycle arrest and significantly protects from the deleterious effects of metformin on cell transformation. Finally, we show the contribution of p53 in mediating metformin action in prostate cancer cells. These results highlight the p53/REDD1 axis as a new molecular target in anticancer therapy in response to metformin treatment. Cancer Res; 71(13); 4366–72. © 2011 AACR.

Introduction

Metformin, a widely used antidiabetic drug, seems to reduce the incidence of cancer in diabetic patients (1) and inhibit cancer cell proliferation and tumor growth in animal models (2–4). Metformin provokes cell-cycle arrest in G0–G1 but does not induce apoptosis or autophagy in prostate cancer cells (3). At the molecular level, metformin regulates the AMP-activated protein kinase (AMPK)/mTOR pathway, as it activates AMPK (5) and consequently inhibits the mTOR pathway via tuberous sclerosis 2 protein (TSC2). mTOR upregulates energy-consuming cellular processes and controls cell growth. Because AMPK activation inhibits energy-consuming pathways and protein synthesis (6), metformin could inhibit cell proliferation through AMPK. In agreement, AMPK inhibition reverses the antiproliferative effects of metformin in breast and ovarian cancer cells (7, 8). In contrast, we showed that downregulation of AMPK did not affect metformin action on prostate cancer cell growth and mTOR inhibition (3), suggesting a role for an alternative pathway.

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10% FBS, 100 U/mL penicillin, 100 mg/L streptomycin, and 2 mmol/L glutamine.

**Cell transfection**

LNCaP cells were transfected with siRNA targeting REDD1, p53, α1 and α2 AMPK, TSC2, or a control siRNA (Ambion) using Lipofectamine RNAi max (Invitrogen). Metformin (Sigma) was added 48 hours after transfection. DU145 cells were transfected with Flag-REDD1 plasmid using Lipofectamine 2000.

**Cell analysis**

Cell extracts were prepared using lysis buffer (3). Immunoblotting was carried out with antibodies against REDD1 (ProteinTech); Thr172P-AMPK, Phospho-S6 ribosomal protein, S6 ribosomal protein, AMPK, and TSC2 (Cell Signalling technology); p53 and HSP90 (Santa Cruz Biotechnology); cyclin D1 (BD Bioscience); and α tubulin (Sigma). Cell cycle was analyzed by flow cytometry (3).

**Real-time quantitative PCR**

The relative amount of REDD1 mRNA was quantified by real-time quantitative PCR using ABI Prism 7000 (Applied Biosystems; ref. 11). The primer sequences are available upon request.

**Bromodeoxyuridine incorporation assay**

Cells were incubated for 24 hours in medium containing 10% FBS and then treated in the presence or absence of metformin (5 or 10 mmol/L) for 24 hours before bromodeoxyuridine (BrdUrd) incorporation assay (3).

**Results and Discussion**

**Metformin increases REDD1 expression in a p53-dependent manner**

Effects of metformin on mTOR and cell growth are not mediated by AMPK in prostate cancer cells (3). Because of its role in mTOR regulation, we asked whether REDD1 could mediate metformin effects. REDD1 expression markedly increased from 4 to 24 hours after metformin treatment in LNCaP cells and REDD1 mRNA levels increased after 4 hours (Fig. 1B). A similar effect of metformin occurred in breast (MCF7) and lung cancer (A549) cells (Fig. 1C).

Because REDD1 is a direct p53 transcriptional target (10), we asked whether p53 regulates REDD1 expression upon metformin treatment. LNCaP cells harbor wild-type (WT) p53, whereas DU145 and PC3 cells are p53 mutated and null, respectively. Whereas REDD1 expression increased in LNCaP cells, it remained unchanged in cells lacking a functional p53 (Fig. 2A). In LNCaP cells, metformin slightly increased AMPK phosphorylation, whereas it induced a stronger activation in DU145 and PC3 cells. Conversely, it strongly decreased the phosphorylation of S6 ribosomal protein in LNCaP cells and only moderately in DU145 and PC3 cells (Fig. 2A). In agreement, REDD1 did not increase anymore in response to metformin following p53 invalidation using siRNA in

![Figure 1](cancerres.aacrjournals.org) Metformin increases REDD1 expression in different cancer cell lines. LNCaP (prostate cancer; A and B), MCF7 (breast cancer), and A549 (lung cancer) cells (C) were treated with 5 mmol/L metformin as indicated before immunoblotting with REDD1 or mRNA quantification. C, control; M5, 5 mmol/L metformin.
Therefore, REDD1 appears as a p53-mediated molecular target of metformin. Similarly, REDD1 is a p53 target upon DNA damage but is regulated independently of p53 in response to alkylating agents (10). We have shown that HIF-1 controls REDD1 expression in response to insulin and hypoxia in adipocytes (11). However, HIF does not appear to be implicated in metformin-induced REDD1 expression, as HIF-1 expression was not affected in LNCaP cells (data not shown).

We then determined whether p53 is implicated in metformin antiproliferative action in LNCaP cells. As shown in Fig. 2C, metformin decreased BrdUrd incorporation by 45% in cells transfected with a control siRNA. Transfection with a p53 siRNA partially prevented the inhibitory effect of metformin with a 20% decrease. Similarly, metformin was not effective in p53−/− MEFs (Supplementary Fig. S1). BrdUrd incorporation was then compared in PC3, DU145, and LNCaP cells. Metformin was significantly more powerful to inhibit cell proliferation in LNCaP cells (52% decrease) than in DU145 (26%) and PC3 (22%), two p53-deficient cell lines (Fig. 2D). We highlight here the important role of p53 in the antiproliferative effect of metformin in prostate cancer cells.

Our results are at variance with those from Buzzai and colleagues, who have shown that metformin selectively impairs the growth of HCT116 colon cancer cells, which are deficient for p53 (4). This discrepancy may depend on cancer cell types; indeed, metformin induces autophagy in a p53-dependent manner in colon cancer cells (4), whereas it inhibits 2-deoxyglucose–induced autophagy in prostate cancer cells (15). Finally, our observation shows that the p53 status may influence the efficiency of metformin in cancer therapy.

REDD1 mediates mTOR inhibition in response to metformin in LNCaP cells

We then asked whether REDD1 mediates effects of metformin on mTOR activity in LNCaP cells. When REDD1 was
downregulated, using siRNA, S6 phosphorylation was significantly less decreased by metformin treatment than in control cells (siCT), whereas the phosphorylation of AMPK was not affected (Fig. 3A). In agreement, metformin did not affect S6 phosphorylation in REDD1+/−/+/− MEFs compared with 5 mmol/L metformin before immunoblotting. Whatever the status of REDD1, metformin activated AMPK. These data show that REDD1 negatively regulates mTOR activity in prostate cancer cells and show for the first time that metformin inhibits mTOR via REDD1.

To reinforce the role of REDD1, we overexpressed REDD1 in DU145 cells, which harbor a nonfunctional p53. We observed a stronger inhibition of S6 phosphorylation in cells overexpressing REDD1 and treated with metformin compared with control conditions (Fig. 3C). Similarly, the re-expression of REDD1 in REDD1+/−/− cells restored the metformin-induced inhibition of mTOR (Supplementary Fig. S2). Altogether, these results show that REDD1 potentiates the inhibitory effect of metformin on mTOR. The TSC2 negatively regulates mTOR when it is phosphorylated on T1227 and S1345 by AMPK (16). To determine its role in response to metformin, we used TSC2 siRNA. Following TSC2 invalidation, metformin no longer inhibited S6 phosphorylation (Fig. 3D).

Our results are in accordance with studies showing that REDD1 regulates mTOR in response to hypoxia, 2-deoxyglucose, and nutrient depletion (13). It is noteworthy that the downregulation of S6 phosphorylation is less important in DU145 and PC3 than in LNCaP cells (Fig. 2A), and overexpression of REDD1 in DU145 cells potentiates the effect of metformin on mTOR. These results suggest that REDD1 plays an important role in mTOR inhibition. They also show that mTOR inhibition by metformin is mediated by TSC2 and is not dependent on REDD1 in p53-deficient cells. Because the effects of metformin on mTOR were not mediated by AMPK in prostate cancer cells (3), REDD1 may represent one alternative route that downregulates mTOR.

Figure 3. Metformin mediates mTOR inhibition through REDD1. A, LNCaP cells were transfected with REDD1 siRNA and treated with 5 mmol/L metformin for 8 hours before immunoblotting. Phospho-S6 (P-S6) was quantified in 3 experiments. *, P < 0.04. B, REDD1+/−/+/− MEFs were treated for 8 hours with 5 mmol/L metformin before immunoblotting. C, DU145 cells overexpressing REDD1 or transfected with the empty vector (PCMV) were treated with 5 mmol/L metformin before immunoblotting. D, LNCaP cells transfected with siCt or siTSC2 were treated with 5 mmol/L metformin before immunoblotting.
Figure 4. REDD1, but not AMPK, is required for metformin antiproliferative effects. A, LNCaP cells transfected with control, REDD1, or AMPK (α1/α2) siRNA and treated with 5 or 10 mmol/L metformin were used for BrdUrd incorporation assay. B, flow cytometry of LNCaP cells transfected with control or REDD1 siRNA and treated with 5 mmol/L metformin. C, soft agar assay in LNCaP cells transfected with CT or REDD1 siRNA and treated for 7 days with metformin (1, 2, and 5 mmol/L; left); photographs of colonies stained with MTT (magnification ×100). M1, 1 mmol/L metformin; M2, 2 mmol/L metformin; M5, 5 mmol/L metformin; M10, 10 mmol/L metformin.
REDD1 regulates metformin antiproliferative effect and cyclin D1 decrease independently of AMPK

To determine whether REDD1 is implicated in metformin-induced inhibition of cell viability, LNCaP cells were transfected with REDD1 siRNA and treated with metformin. BrdUrd incorporation was affected by only 18% in cells transfected with REDD1 siRNA compared with 76% in cells transfected with control siRNA (Fig. 4A) in the presence of 5 mmol/L metformin. In parallel, the inhibition of AMPK had a minor impact on metformin effects with a reduction of BrdUrd incorporation of 60% and 80% compared with 76% and 82% with control siRNA in the presence of 5 and 10 mmol/L of metformin, respectively (Fig. 4A). These results show that REDD1 but not AMPK mediates the antiproliferative effects of metformin in LNCaP cells. Metformin-induced cell-cycle arrest and cyclin D1 decrease are not dependent on AMPK in LNCaP cells (3). To determine the implication of REDD1 in this effect, we analyzed cyclin D1 expression and cell cycle in cells transfected with siREDD1. Cyclin D1 was no longer decreased, and S6 phosphorylation was significantly less affected after metformin treatment compared with control siRNA conditions (Supplementary Fig. S3). Furthermore, the cell cycle was not blocked in 

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