

Upregulation of glucose metabolism by NF- κ B2/p52 mediates enzalutamide resistance in castration-resistant prostate cancer cells

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Abstract

Cancer cells reprogram their metabolic pathways to facilitate fast proliferation. Previous studies have shown that overexpression of NF- κ B2/p52 (p52) in prostate cancer cells promotes cell growth and leads to castration resistance through aberrant activation of androgen receptor (AR). In addition, these cells become resistant to enzalutamide. In this study, we investigated the effects of p52 activation on glucose metabolism and on response to enzalutamide therapy. Data analysis of gene expression arrays showed that genes including *GLUT1* (*SLC2A1*), *PKM2*, *G6PD*, and *ME1* involved in the regulation of glucose metabolism were altered in LNCaP cells overexpressing p52 compared with the parental LNCaP cells. We demonstrated an increased amount of glucose flux in the glycolysis pathway, as well as the pentose phosphate pathway (PPP) upon p52 activation. The p52-overexpressing cells increase glucose uptake and are capable of higher ATP and lactate production compared with the parental LNCaP cells. The growth of p52-overexpressing cells depends on glucose in the culture media and is sensitive to glucose deprivation compared with the parental LNCaP cells. Targeting glucose metabolism by the glucose analog 2-deoxy-D-glucose synergistically inhibits cell growth when combined with enzalutamide, and resensitizes p52-overexpressing cells to enzalutamide treatment. These results suggest that p52 modulates glucose metabolism, enhances glucose flux to glycolysis and PPPs, thus facilitating fast proliferation of the cells. Co-targeting glucose metabolism together with AR axis synergistically inhibits cell growth and restores enzalutamide-resistant cells to enzalutamide treatment.

Key Words

- ▶ prostate cancer
- ▶ glucose metabolism
- ▶ NF- κ B2/p52
- ▶ enzalutamide

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Introduction

As the second leading cause of cancer-related death among men in the United States, prostate tumors respond to androgen deprivation therapy initially; however, after

time, relapse occurs and develops into a lethal form of disease, termed castration-resistance prostate cancer (CRPC; Chen *et al.* 2004, Harris *et al.* 2009). The NF- κ B

family has been identified as an important mediator in many cancer-causing processes. The family consists of five members: RelA/p65, NF- κ B1/p50, c-Rel, RelB, and NF- κ B2/p52, in which p65/p50 heterodimer and RelB/p52 heterodimer are involved in the canonical and non-canonical NF- κ B pathways respectively (Fan & Maniatis 1991, Betts & Nabel 1996, Lin *et al.* 1998, Karin & Greten 2005). The non-canonical pathway that involves the processing of NF- κ B2 p100 to p52 is inducible and tightly regulated (Xiao *et al.* 2001a,b, Xiao *et al.* 2004, Nadiminty *et al.* 2006). Overproduction of p52 from p100 causes hyperplastic growth in the liver, lymphocytes, and mammary glands of mice (Ishikawa *et al.* 1997, Connelly *et al.* 2007). In several solid tumors, including breast and prostate, overproduction of p52 has been observed (Cogswell *et al.* 2000, Lessard *et al.* 2006). In previous studies, we have shown that NF- κ B2/p52 induces castration-resistant growth in prostate cancer cells through aberrant activation of androgen receptor (AR) (Nadiminty *et al.* 2008, 2010a). In addition, p52-overexpressing cells are resistant to enzalutamide, a newly approved anti-androgen drug for CRPC patients (Tran *et al.* 2009, Nadiminty *et al.* 2013). Yet, the effect of p52 on cellular metabolism has not previously been studied.

Different from highly differentiated normal cells, cancer cells reprogram their metabolism to facilitate fast proliferation. One typical feature is known as the 'Warburg effect', also called aerobic glycolysis, where cancer cells mainly depend on glycolysis for their energy production in the presence of oxygen (Warburg 1956). This process produces far less energy than that oxidative phosphorylation exhibited in normal cells; however, it produces more intermediate products for anabolic building blocks and much less oxidative stress to keep the redox balance (Cairns *et al.* 2011, Vander Heiden 2011, DeBerardinis & Thompson 2012, Teicher *et al.* 2012). It has been reported that metabolic enzymes such as pyruvate kinase can be alternatively spliced to isoform M2, which supports anabolic growth and promotes tumorigenesis (Christofk *et al.* 2008a, Sun *et al.* 2011). Activation of many oncogenes, such as *PI3K* (*PIK3CA*), *AKT*, *mTORC1*, and *MYC*, can also impact metabolism of mitochondria (Berwick *et al.* 2002, Buzzai *et al.* 2005, Hatzivassiliou *et al.* 2005, Cunningham *et al.* 2007).

In this study, we demonstrated through gene expression array data analysis that genes involved in glucose metabolic pathways in p52-overexpressing LNCaP cells were upregulated. Further analysis on glucose metabolism revealed enhancements in both the

glycolysis and pentose phosphate pathways (PPPs) accompanied by higher glucose consumption and lactate production. Moreover, targeting glucose metabolism by glucose analog 2-deoxy-D-glucose (2-DG) resensitized p52-overexpressing prostate cancer cells' response to enzalutamide.

Materials and methods

Cell culture and reagents

LNCaP and C4-2B cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 0.1 mg/ml streptomycin and maintained at 37 °C in a humidified incubator with 5% CO₂. LNCaP-neo and LNCaP-p52 stable cells are generated as described previously (Nadiminty *et al.* 2010b). C4-2B MDVR cells were generated by culturing C4-2B cells in media containing 20 μ M enzalutamide until the cells became resistant to enzalutamide as described previously (Nadiminty *et al.* 2013). The cells were seeded in 24-well plates and treated with 20 μ M enzalutamide and/or 1 mM/2 mM 2-DG after 24 h. After 48–72 h of treatment, they were counted and harvested; medium was collected for glucose consumption, lactate production, and PSA concentration assays. 2-DG was purchased from Sigma–Aldrich. ATP assay kit was purchased from PerkinElmer (Santa Clara, CA, USA). Glucose consumption, lactate production, pyruvate kinase activity, and NADPH/NADP assay kits were all purchased from Biovision (Milpitas, CA, USA). PSA ELISA kit was purchased from Abnova (Walnut, CA, USA).

Microarray pathway analysis

Metabolic pathways of NF- κ B2/p52 were analyzed using Ingenuity Pathway Analysis Software (Ingenuity). This method uses gene identities in conjunction with controlled, vocabulary-based data mining of literature associations, protein–protein interaction databases, and a metabolism pathway database.

Preparation of whole-cell extracts

Cells were washed twice with PBS and lysed for 20 min on ice with cell lysis buffer containing 10 mM HEPES, pH 7.9, 250 mM NaCl, 1 mM EDTA, 1% NP-40, supplemented with 1 mM dithiothreitol, 1 mM phenylmethylsulphonyl fluoride, 5 mM NaF, 1 mM Na₃VO₄, and protease inhibitors (Roche).

Western blot analysis

The cell extracts were obtained and separated by SDS-PAGE and the proteins transferred onto nitrocellulose membranes. The membranes were blocked by 5% non-fat milk in PBS/0.05%Tween-20, followed by probing with appropriate primary and secondary antibodies.

Cell transfection

CWR22Rv1 cells were transiently transfected with HA tag-labeled p52 plasmid. The cells were plated at density of 1×10^5 /ml 1 day before transfection, which was done by Attractene (Qiagen) following the manufacturer's instruction.

Measurement of ATP production, glucose consumption, and lactate production

ATP levels in the cell lysate were determined by luminescence-based assay kit (ATPlite; PerkinElmer) according to the manufacturer's instruction. The cells were seeded in the culture dishes and the medium was changed after 12 h. The culture medium was collected for the measurement of glucose and lactate concentration after 24–48 h. Glucose levels were determined by comparing difference in the collected medium and fresh medium using a glucose assay kit (Biovision). Lactate levels were measured by lactate assay kit (Biovision). ATP production, glucose consumption, and lactate production levels were normalized to cell number.

NADPH/NADP assay

The cells were harvested after 48 h of culture. NADP total (NADPH and NADP) levels and NADPH levels were determined by a commercially available assay kit (Biovision). NADP levels were determined by the difference between NADP total and NADPH levels. The ratio was calculated thereafter.

Glucose deprivation assay

The cells were seeded into 12-well plates 1 day before switching to an RPMI-1640 glucose-free medium containing 10% dialyzed FBS. The cells were counted 3 days after changing the medium.

PSA ELISA assay

Culture medium was collected 2 days after seeding cells or drug treatment. PSA levels in the medium were measured

by a commercially available kit, following the manufacturer's instruction. Data are expressed as mean \pm s.d. from three independent experiments.

Statistical analysis

All data presented are shown as mean \pm s.d. Student's *t*-test was used to compare differences between two groups. $P < 0.05$ was considered significant.

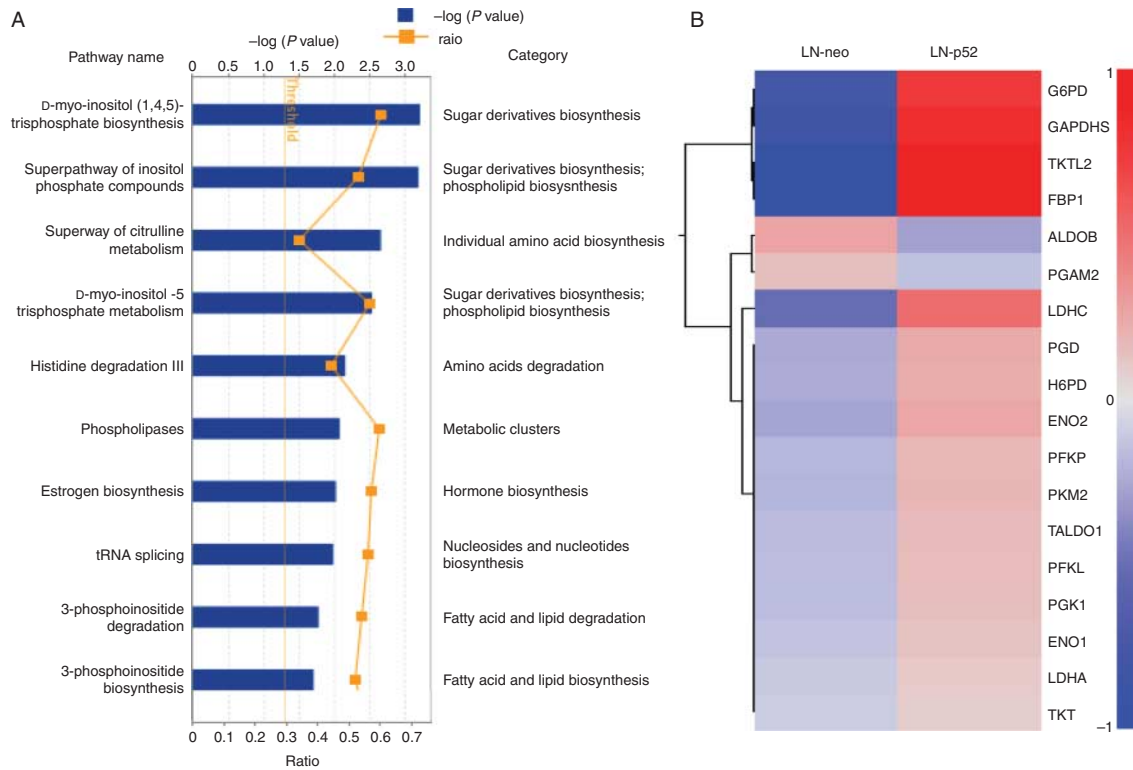
Results

Alteration of glucose pathways by p52

Our previous studies have demonstrated that p52-overexpressing LNCaP prostate cancer cells are more aggressive in terms of tumour growth, resistance to androgen deprivation, and enzalutamide treatment (Nadiminty *et al.* 2006, 2008, 2010a,b, 2013). As it is well documented that cancer cells reprogram their metabolism to facilitate fast proliferation, we examined the metabolic pathways in p52-overexpressing LNCaP cells by analyzing the gene expression microarray data generated from p52 retrovirus-infected cells (Nadiminty *et al.* 2010b).

Of the top 30 altered pathways by overexpression of p52 in LNCaP cells compared with the parental LNCaP cells, the intermediate or final products of 11 pathways, including the top 2 pathways, are involved in sugar metabolism, fatty acid synthesis, and/or lipid biosynthesis and degradation. Five pathways are correlated to amines, polyamines, amino acids biosynthesis, and degradation. In addition, pathways involved in the biosynthesis and degradation of nucleoside and nucleotide as well as biosynthesis of vitamin, NAD, and polysaccharides are all significantly enhanced. The most altered metabolic pathways are listed in Fig. 1A. These findings suggest that in p52 cells, anabolic metabolism pathways are enhanced, providing additional byproducts necessary for cell division, thus facilitating quicker cell proliferation.

In correspondence with the enhanced macromolecular biosynthesis, glycolysis processes including fermentation of pyruvate to lactate are at top of the list. Many key enzymes within glycolysis, such as PFKP, GAPDHS, PGK1, ENO1, ENO3, PKM2, and LDHA, have elevated expression levels in LNCaP-p52 cells compared with the parental LNCaP cells (Fig. 1B). In addition to the upregulated genes in glycolysis, genes within the PPP, such as *G6PD*, *PGD*, *TALDO1*, and *TKT*, are also upregulated (Fig. 1B).

**Figure 1**

Metabolic pathways and genes altered in LNCaP-p52 cells. (A) Top 10 altered metabolic pathways are listed. Blue boxes show $-\log(P)$ value. Yellow dots show in \log_2 (fold change). Fold change threshold is set as 1.2. (B) Heat map of gene expression within glycolysis and pentose phosphate

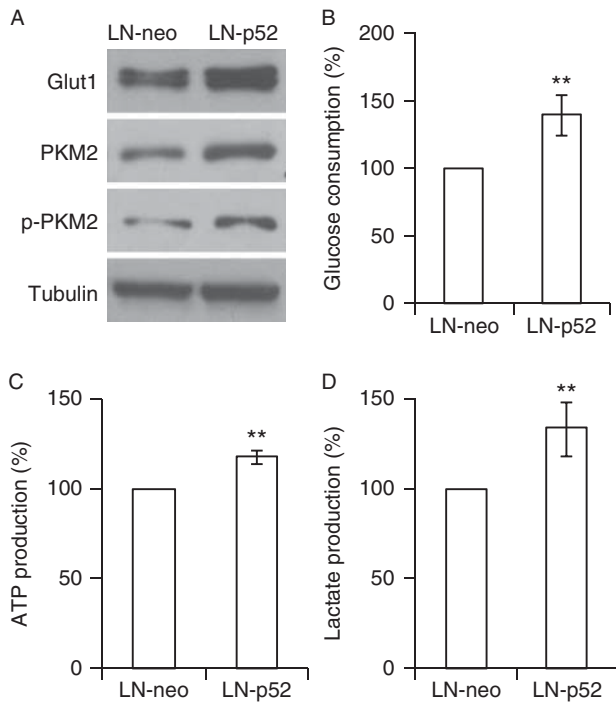
pathways. All genes of the glycolysis and pentose phosphate pathways with a >1.2 -fold change are included in the heat map. Color scale shows the format of \log_2 (fold change).

p52 enhances glucose metabolism

Gene expression array data suggest the alteration of glucose metabolic pathway by p52 in LNCaP cells. To further understand reprogramed glucose metabolism by p52, we assayed several indicators within the glucose metabolic pathways. Glucose uptake is the first step in glucose metabolism, which can be measured by the expression levels of glucose transporter, GLUT1, and by glucose consumption assays. LNCaP-p52 cells expressed GLUT1 at higher levels compared with the control (Fig. 2A), consistent with higher glucose consumption in p52 overexpression cells compared with the control (Fig. 2B). These data suggest an increased glucose uptake and consumption by the cells overexpressing p52. As a rate-limiting final step of glycolysis, catalysis of PEP to pyruvate by PKM2, a splice isoform of pyruvate kinase, plays important roles in cancer metabolism. Expression of PKM2 has a growth advantage for tumor cells *in vivo* (Christofk et al. 2008a,b). Our gene expression array data indicated an increased expression of *PKM2* mRNA by

overexpression of p52. To confirm whether p52 enhances PKM2 protein expression, we analyzed the expression of PKM2 and phosphorylated PKM2. As shown in Fig. 2A, the protein levels of both PKM2 and phosphorylated PKM2 were upregulated in LNCaP-p52 cells compared with the control. As cancer cells mainly generate energy from aerobic glycolysis of glucose, we measured ATP production as an indicator of aerobic glycolysis. The p52-overexpressing LNCaP cells are capable of generation of higher ATP production compared with the parental LNCaP cells (Fig. 2C). In addition to ATP production, lactate production was also increased in LNCaP-p52 cells compared with the parental LNCaP cells (Fig. 2D).

PPP is a branch shunt from glycolysis, which provides intermediate products for nucleoside synthesis, and more importantly provides reductants such as NADPH to maintain the redox balance of fast proliferating cells. The enzyme involved in the first step of PPP flux, G6PD, was upregulated in LNCaP-p52 cells (Fig. 3A). In addition, the NADPH:NADP ratio was also much higher in LNCaP-p52 cells than control cells (Fig. 3B), suggesting an overall

**Figure 2**

p52 increases LNCaP cells' glucose uptake and aerobic glycolysis. (A) Western blots for glucose transporter 1 (GLUT1), PKM2, phospho-PKM2. Tubulin was used as a loading control. Glucose consumption (B), ATP production (C), lactate production (D) of LNCaP-p52 cells compared with LNCaP-neo cells. Data are presented as mean \pm s.d. of at least three independent experiments. ** $P < 0.01$.

enhanced PPP in LNCaP-p52 cells. To test whether p52-mediated glucose metabolism is not LNCaP cell specific, CWR22Rv1 cells were transiently transfected with p52. As shown in Fig. 3C, transient transfection of p52 increased PKM2 expression and glucose consumption in CWR22Rv1 cells (Fig. 3C). Collectively, these data suggest that overexpression of p52 enhances glucose metabolism in LNCaP cells.

Overexpression of p52 increases cell sensitivity to glucose deprivation and 2-DG treatment

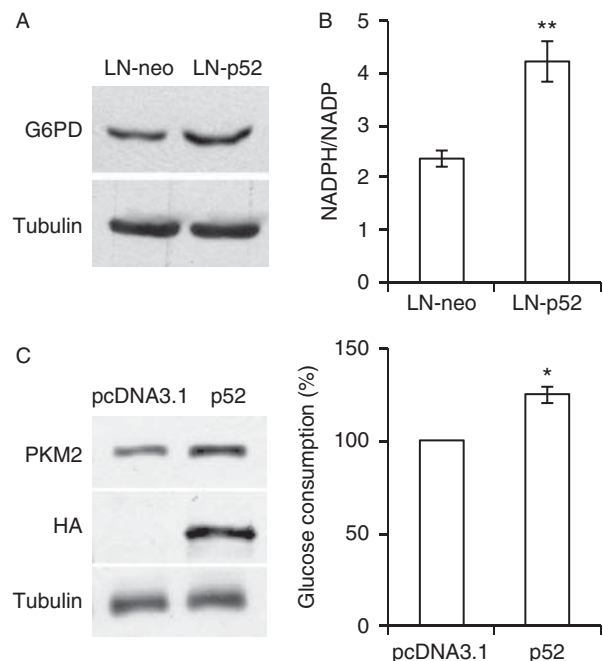
As LNCaP-p52 cells have higher glucose uptake and rate of glucose metabolism, we hypothesized that p52-overexpressing LNCaP cells might be dependent on glucose for survival, and were more sensitive to glucose deprivation than parental LNCaP cells. To test that, we monitored cell growth in the absence of glucose. As shown in Fig. 4A, more cells were dead among p52-overexpressing LNCaP cells compared with the parental LNCaP cells when they grew in the media deprived of glucose. To further confirm this observation, we treated the cells with an analog of

glucose, 2-DG, an inhibitor of glucose metabolism. As shown in Fig. 4B, p52-overexpressing LNCaP cells were more sensitive to 2-DG treatment than parental LNCaP cells. These results suggest that p52-overexpressing LNCaP cells are more sensitive to glucose deprivation than parental LNCaP cells.

Targeting glucose metabolism by 2-DG resensitizes LNCaP-p52 cells to enzalutamide treatment

Our previous studies have shown that LNCaP-p52 cells were resistant to enzalutamide treatment (Nadiminty et al. 2013). As LNCaP-p52 cells exhibit enhanced glucose consumption and are more sensitive to glucose deprivation and 2-DG treatment, we combined 2-DG with enzalutamide to examine whether the combination treatment could restore the cells' sensitivity to enzalutamide. As shown in Fig. 5A, a low dose of 2-DG (1 mM) combined with 20 μ M enzalutamide dramatically reduced LNCaP-p52 cell number.

We previously generated enzalutamide-resistant C4-2B MDVR cells (Nadiminty et al. 2013). Similar to

**Figure 3**

(A) Western blots for G6PD of LNCaP-neo and LNCaP-p52 cells. Tubulin was used as a loading control. (B) NADPH:NADP ratio of LNCaP-p52 cells compared with LNCaP-neo cells. (C) Transient transfection of p52 enhances glucose metabolism in CWR22Rv1 cells. Immunoblots of HA-tagged p52, PKM2, and tubulin in transiently transfected LNCaP cells (left). Glucose consumption assay in transient vehicle and p52 transfected CWR22Rv1 cells (right). Data was presented as mean \pm s.d. of three independent experiments. * $P < 0.05$, ** $P < 0.01$.

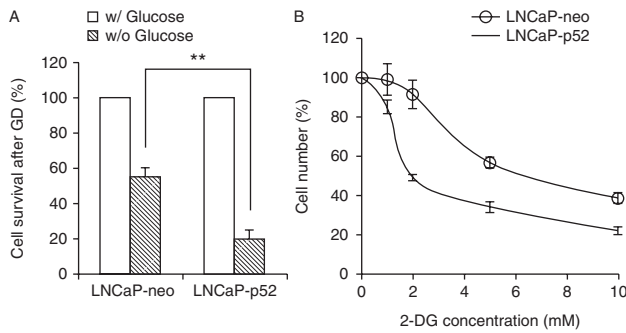


Figure 4 LNCaP-p52 cells are more sensitive to glucose deprivation and 2-DG treatment. (A) Cell growth comparison after 3 days of glucose deprivation. Cell numbers of LNCaP-neo and LNCaP-p52 cells in absence of glucose are normalized to the ones with glucose respectively. (B) Cell growth of LNCaP-p52 and LNCaP-neo at different concentrations of 2-DG. Data are presented as mean \pm s.d. in three independent experiments. ** $P < 0.01$.

LNCaP-p52 cells, C4-2B MDVR cells have enhanced glucose consumption and ATP and lactate production (Fig. 5B). As 2-DG can greatly resensitize the response of LNCaP-p52 cells to enzalutamide, we tested whether 2-DG would have a similar effect to MDV-resistant cells. Combination

treatment with 2-DG and enzalutamide significantly decreased cell number in C4-2B MDVR cells (Fig. 5C and D). Collectively, these data suggest that targeting glucose metabolism can resensitize enzalutamide treatment in enzalutamide-resistant prostate cancer cells.

Discussion

Our previous studies have demonstrated that p52-overexpressing LNCaP cells grew significantly larger tumors *in vivo*, became castration-resistant through aberrant AR activation and resistant to enzalutamide treatment. Cancer cells have reprogrammed metabolism to facilitate fast proliferation. In the present study, we analyzed glucose metabolism in p52-overexpressing prostate cancer cells, and found that glycolysis and PPP are both upregulated, indicating upregulation of glucose metabolism and ATP production by p52. Targeting glucose metabolism by 2-DG resensitizes the cell's response to enzalutamide of not only LNCaP-p52 cells, but also other enzalutamide-resistant cells, like C4-2B MDVR cells.

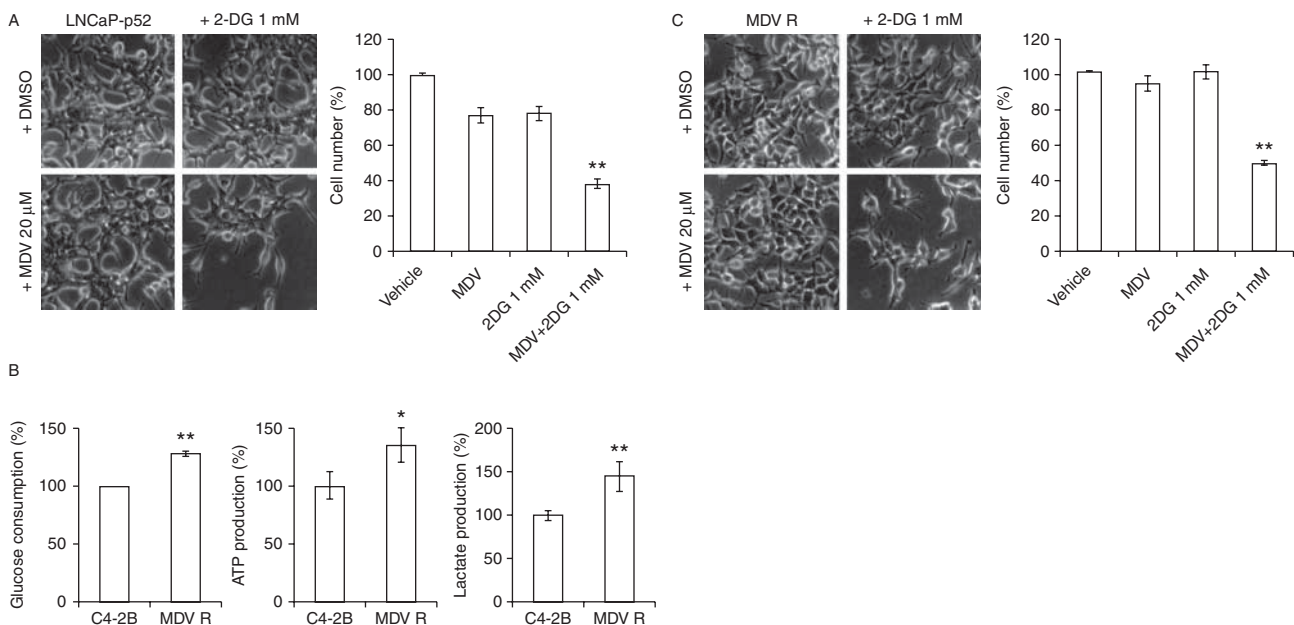


Figure 5 2-DG resensitizes cells to enzalutamide treatment. (A) Cell morphology of LNCaP-p52 cells was shown after 2 days treatment with 2-DG in combination with or without enzalutamide. Vehicle control and 2-DG treatment were shown in top panel. Enzalutamide and combination treatment with 1 mM 2-DG were shown in bottom panel. (Right panel) Cell growth after 2 days of treatment. All numbers were normalized to vehicle group. Data were presented as mean \pm s.d. in at least three independent

experiments. (B) Glucose consumption (left), ATP production (middle), and lactate production (right) in C4-2B MDVR enzalutamide-resistant cells compared with parental C4-2B enzalutamide-sensitive cells. (C) 2-DG resensitizes C4-2B MDVR enzalutamide-resistant cells' response to enzalutamide treatment. Cell numbers were normalized to vehicle group. Data were presented as mean \pm s.d. in three independent experiments; * $P < 0.05$, ** $P < 0.01$.

The Warburg effect is a widely observed feature in tumors with elevated glucose uptake, glucose consumption, and lactate production. Several enzymes including GLUT1, PKM2, and LDHA are critically involved in glucose metabolism. We have shown that the levels of GLUT1 (*SLC2A1*), PKM2, and LDHA gene expression were upregulated in p52-overexpressing LNCaP cells, which correlated with higher glucose metabolism. The upregulation of these rate-limiting glucose metabolic enzymes may play a critical role in the p52-induced Warburg effect, featuring elevated glucose consumption and higher lactate production. One typical feature of cancer cells is switching energy production from oxidative phosphorylation to glycolysis to generate additional precursors for macromolecular biosynthesis. Gene expression microarray data analysis showed an enhanced macromolecular biosynthesis in p52-overexpressing LNCaP cells compared with the parental LNCaP cells, suggesting an enhanced production of building blocks for these macromolecules derived from glucose metabolites. This is in consistence with the upregulated glucose metabolism pathways observed in p52-overexpressing LNCaP cells.

Due to an altered glucose metabolism that cancer cells usually have, targeting glucose metabolism to inhibit cancer progression is an attractive approach for cancer therapy. 2-DG, a glucose analog, is the most widely investigated drug in experimental and clinical oncology (Dwarkanath & Jain 2009). 2-DG is competitively taken up by cells through the same transporter as glucose and transformed by hexokinase to 2-DG-6-phosphate which cannot be metabolized further, therefore decreasing the glucose flux to glycolysis and PPPs. However, as a single therapeutic agent, the clinical trial of 2-DG has been discontinued due to persistent side effects such as diaphoresis and disturbance of the CNS (Dwarkanath et al. 2009, Gupta et al. 2009). A potential promising approach is to combine 2-DG therapy with radiation or chemotherapy drugs (Gupta et al. 2009). Enzalutamide, a newly approved anti-androgen drug, can effectively inhibit CRPC cell growth *in vivo* (Tran et al. 2009). Despite its successes and continuing widespread use, development of resistance is inevitable. Our previous studies have demonstrated that p52-overexpressing LNCaP cells were resistant to enzalutamide (Nadiminty et al. 2013). In the present study, we combined 2-DG and enzalutamide and found that a low dose of 2-DG resensitized the response of p52-overexpressing LNCaP cells to enzalutamide treatment. The combination of these two drugs has profound synergistic effects on the inhibition of cell

growth. We further validated the synergistic effects of combination treatment of 2-DG with enzalutamide in another enzalutamide-resistant C4-2B-MDVR cells.

In summary, these results suggest that p52 modulates glucose metabolism and enhances glucose flux to glycolysis and PPPs, thus facilitating fast proliferation of the cells. Targeting glucose metabolism by deprivation of glucose or using glucose analog, such as 2-DG, inhibits cell growth. We found that combination treatment of 2-DG with enzalutamide could resensitize enzalutamide-resistant prostate cancer cells to enzalutamide treatment, suggesting a potential therapeutic approach for CRPC patients, by co-targeting glucose metabolism and AR pathways.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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