ORIGINAL ARTICLE

Cancer Science WILEY

Upregulation of glucocorticoid receptor-mediated glucose transporter 4 in enzalutamide-resistant prostate cancer

Seiji Hoshi ⓘ | Satoru Meguro | Hitomi Imai | Yuta Matsuoka | Yuki Yoshida | Akihumi Onagi | Ryo Tanji | Ruriko Honda-Takinami | Kanako Matsuoka | Tomoyuki Koguchi | Junya Hata | Yuichi Sato | Hidenori Akaihata | Masao Kataoka | Soichiro Ogawa | Yoshiyuki Kojima

Department of Urology, Fukushima Medical University School of Medicine, Fukushima, Japan

Correspondence
Seiji Hoshi, Department of Urology, Fukushima Medical University School of Medicine, Fukushima, Japan.
Email: uro-hosi@fmu.ac.jp

Abstract

Enzalutamide (Enz) is a second-generation androgen receptor (AR) antagonist for castration-resistant prostate cancer (CRPC) therapy, and it prolongs survival time in these patients. However, during Enz treatment, CRPC patients usually acquire resistance to Enz and often show cross-resistance to other AR signaling inhibitors. Although glucocorticoid receptor (GR) is involved in this resistance, the role of GR has not yet been clarified. Here, we report that chronic Enz treatment induced GR-mediated glucose transporter 4 (GLUT4) upregulation, and that upregulation was associated with resistance to Enz and other AR signaling inhibitors. Additionally, inhibition of GLUT4 suppressed cell proliferation in Enz-resistant prostate cancer cells, which recovered from Enz resistance and cross-resistance without changes in GR expression. Thus, a combination of Enz and a GLUT4 inhibitor could be useful in Enz-resistant CRPC patients.

KEYWORDS
androgen receptor, enzalutamide, glucocorticoid receptor, glucose transporter 4, prostate cancer

1 | INTRODUCTION

Enzalutamide and Abi are second-generation small-molecule inhibitors of AR signaling, and both are useful for the treatment of CRPC. However, during treatment with these inhibitors, most patients acquire resistance to them and often show cross-resistance to other AR signaling inhibitors. A recent cross-over trial showed that Enz retained clinical activity as a second-line drug following Abi treatment, whereas Abi retained low second-line activity following Enz treatment, even though Abi and Enz have similar first-line activity for metastatic CRPC.[1] Chronic Enz treatment could make CRPC cells acquire resistance to AR signaling inhibitors more easily than Abi.

Although several mechanisms of Enz resistance, including AR mutations, the induction of AR splice variants, and AR-related gene upregulation, have been reported,[2-5] the proposed mechanisms cannot fully explain Enz resistance. In recent reports, GR

Abbreviations: 2-DG, 2-deoxyglucose; Abi, abiraterone; AR, androgen receptor; AR V7, androgen receptor splice variant 7; Bcl, bicalutamide; cd-FBS, charcoal/dextran-treated FBS; CRPC, castration-resistant prostate cancer; DHT, dihydrotestosterone; Enz, enzalutamide; FDG, fluorodeoxyglucose; GLUT, glucose transporter; GR, glucocorticoid receptor; PCa, prostate cancer; Rit, ritonavir.

---

upregulation has been identified as a driver of Enz resistance.[6-8] Activation of AR signals by GR binding to the AR gene,[8,9] or activation of an AR-independent pathway by GR, could contribute to Enz-resistance[6,8]; however, the detailed mechanism of how GR-regulated pathways contribute to Enz resistance has not yet been clarified.

In general, anticancer drug resistance is closely related to increased metabolism of glucose[10] as well as lipids and amino acids.[11] Although lipid and glutamine metabolism are considered to play an important role in the process by which PCa cells acquire resistance to anticancer agents,[12-15] the role of glucose metabolism has remained unclear.[16-18] Cancer cells consume a lot of glucose in the process of acquiring drug resistance,[13,17] and GLUTs are expressed on cell surfaces to supplement the consumed glucose.[19] Glucose transporters are 12-transmembrane glucose transport proteins that have been reported to have 14 isoforms. In PCa, upregulation of GLUT1, 3, 4, and 12 has been observed.[16] Of these GLUTs, both GLUT1 and GLUT4 are related to AR signaling-dependent cell proliferation.[16,20] However, the detailed role of GLUTs in the process of acquiring drug resistance in PCa and CRPC has remained unknown.

In skeletal muscle and adipocytes, inhibition of GR suppresses GLUT4 expression and reduces glucose uptake from blood into muscle and adipocytes.[21,22] Hence, we considered that GLUT4 might be regulated by GR in PCa as in skeletal muscle and adipocytes, and GLUT4 might be involved in acquiring resistance to Enz. In this study, we established an Enz-resistant PCa cell line to examine changes in GR and GLUT4 expression during the process of acquiring Enz resistance. Then we examined the relationship between GR and GLUT4 and the underlying regulatory mechanism in the Enz-resistant cell line. Finally, we evaluated the effect of GLUT4 inhibition on recovery from resistance to Enz and other AR signaling inhibitors, and discuss a possible new therapeutic strategy in the treatment of CRPC.

2 | MATERIALS AND METHODS

2.1 | Reagents and cell lines

Two human PCa cell lines, androgen-dependent LNCaP cells and androgen-independent PC-3 cells, were purchased from ATCC and were maintained in RPMI-1640 and Ham's F-12 Nutrient Mixture Medium, respectively, supplemented with 10% cd-FBS to undertake the experiments. A previous report showed that LNCaP cells produce testosterone, even in cd-FBS medium.[23] In a preliminary study, prostate-specific antigen levels increased over time, even in cd-FBS medium (Figure S1). In addition, Enz and DHT + Enz treatment significantly suppressed cell proliferation to the same levels in both FBS and cd-FBS media (Figure S2). These results suggest that LNCaP cells produce testosterone even in cd-FBS medium, and that Enz suppresses the proliferation of LNCaP cells by inhibiting AR-mediated signaling, regardless of the kind of FBS medium and presence or absence of DHT treatment. The media and FBS were purchased from Thermo Fisher Scientific. All cell lines were grown

at 37°C in a humidified 5% CO_2 environment. The medium was changed every 2 days, and cultures were split once a week.

The AR signaling inhibitors Enz, Bcl (Tokyo Chemical Industry), and Abi (Chemscene), GR inhibitor RU486 (Cayman Chemical), and GLUT4 inhibitors Rit (Chemscene) and DHT (Cayman Chemical) were used. These drugs (Enz 10.0 μM, Bcl 10.0 μM, Abi 2.5 μM, RU486 20 μM, Rit 2 μM, and DHT 10 nM) were given at doses corresponding to the plasma concentrations of the patients within each treatment.

2.2 | Establishment of Enz-resistant cell line

To establish an Enz-resistant cell line, LNCaP was first maintained in RPMI medium with 10% cd-FBS. After culturing for 1 week, cells were transferred to the above medium with 10.0 μM Enz and cultured for at least 12 weeks.

2.3 | Cell viability assay

For the determination of cellular proliferation and viability, WST-1 assays were carried out in 96-well plates using a Cell Proliferation Reagent (Roche Applied Science) according to the manufacturer's protocol. Briefly, 1-7 days after the incubation of cells with DHT, Bcl, Enz, Abi, RU486, Rit, and siRNA, WST-1 reagent was added to each well and incubated for 1 hour at 37°C. Spectrophotometric absorbance of the samples was measured using a microplate reader (Varioskan Flash; Thermo Fisher Scientific) and compared against that of nontreated cells.

2.4 | cDNA construction and quantitative RT-PCR

cDNA construction was carried out using a SuperPrep Cell Lysis & RT Kit for qPCR (Toyobo) according to the manufacturer's instructions at 24 hours after treatment. The PCR reagents for GLUT1 (Hs00892681_m1), GLUT4 (Hs00168966_m1), AR (Hs00171172_m1), and GR (Hs00230813_m1) were purchased from Applied Biosystems, and custom primers were used to amplify AR-V7, GLUT3, and GLUT12 (Table S1). Quantitative RT-PCR was carried out using the PowerTrack SYBR Green Master Mix (Invitrogen) on a StepOne real-time PCR System (Applied Biosystems). The data were standardized against β-actin gene expression using β-actin control reagent (Applied Biosystems).

2.5 | Western blot analysis

At 24 or 72 hour after drug treatment, cells were lysed with RIPA buffer supplemented with protease inhibitors (Nacalai Tesque). Proteins from the LNCaP, LNEnzR, and PC3 cells were extracted and applied to SDS-PAGE. GLUT1 (ab15309), GLUT3 (ab48547), GLUT4 (ab33780), GLUT12 (ab202908), AR (ab9474),

AR-V7 (ab19839), and GR (ab3579) were used as primary Abs. Anti-β-actin Ab (Sigma) was used as an internal control. Protein bands were visualized using SuperSignal West Dura Extended Duration Substrate (Thermo Fisher Scientific) and imaged with the ChemiDoc XRS plus system (Bio-Rad). Individual bands were quantified with Image Lab 3.0 software (Bio-Rad) and normalized against the control value.

2.6 | Small interfering RNA and transfection

We used Silencer Select Pre-Designed siRNA (Invitrogen), GR (s6186), GLUT4 (s12934, Table S2), and the negative control (Silencer Select Negative Control No. 1 siRNA). For siRNA transfection, Lipofectamine RNAi MAX (Invitrogen) was used according to the manufacturer's instructions. Briefly, 1 day prior to transfection, cells were seeded without antibiotics so as to be 60%-80% confluent at the time of transfection. The siRNA-Lipofectamine complexes were prepared by mixing an adequate concentration of siRNA oligomer and Lipofectamine using Opti-MEM Medium (Gibco). The transfected cells were incubated at 37°C in a 5% CO_2 incubator for 24 hours until treatment.

2.7 | Flow cytometry

Cells were plated in 6-well plates at 1.0×10^6 cells/well for 24 hours to quantify GLUT1, GLUT3, GLUT4, and GLUT12 expression; we used immunofluorescence staining coupled with flow cytometry. Cells were detached with 0.25% trypsin (Invitrogen) and rinsed thoroughly in PBS with intermittent centrifugation. Cells were blocked for 10 minutes with PBS containing 10% normal goat serum and 0.3 M glycine. Permeabilization was skipped to evaluate subcellular GLUTs. Cells were stained for 30 minutes with anti-GLUT1 Abs (ab15309), anti-GLUT3 Abs (ab15311), anti-GLUT4 Abs (ab48547), anti-GLUT12 Abs (ab202908), and control Ab (ab91366) at a final concentration of 1 mg/mL in PBS. The cells were rinsed with On-chip T buffer (On-Chip Biotechnologies) and incubated with Alexa Fluor 488-conjugated anti-rabbit Abs (ab96879) and anti-mouse Abs (ab91366) diluted 1:500 in PBS, respectively. Fluorescence was measured at 488 nm using a flow cytometer (On-Chip Biotechnologies).

2.8 | Glucose uptake assay

The glucose uptake assay was carried out as described previously with minor modifications.[24] Briefly, cells were treated with 1 mM 2-DG for 20 minutes. The reaction was stopped by harvesting the cells and washing them three times with ice-cold PBS. After removal of an aliquot for cell counting, the cell pellet was solubilized in 10 mM Tris-HCl (pH 7.4) by sonication (Bioruptor II; BM Bio), followed by determination of the amount of 2-DG using a 2DG Uptake Measurement Kit (Cosmo Bio) according to the manufacturer's instructions. Standardization of glucose concentration was accomplished by determining the protein concentration.

2.9 | Statistical analysis

Determination of cell proliferation, IC_{50}, mRNA expression level, western blot analysis, and glucose uptake assays were repeated at least three times independently, and the results are expressed as the mean ± SE. Analyses were carried out with SPSS Statistics 21 software (IBM Japan). Data were statistically evaluated using the unpaired two-tailed Student's t test for two groups and one-way ANOVA for three groups, and values were considered statistically significant when $P < .05$.

3 | RESULTS

3.1 | Establishment of LNCaP-derived cell line with acquired resistance against Enz

We selected LNCaP cells, a high AR and low GR expression PCa cell line, and chronically treated them with 10 μM Enz for at least 12 weeks. The surviving and proliferating resistant cells were pooled, maintained, and finally termed LNEnzR cells. LNEnzR cells were morphologically identical to LNCaP cells (Figure 1A).

A WST-1 assay was performed on LNCaP and LNEnzR cells exposed to various concentrations of Enz, Abi, and Bcl. There was a clear reduction in cell viability of LNCaP and LNEnzR cells after Enz (Figure 1B), Abi (Figure 1C), and Bcl (Figure 1D) treatment, in a dose-dependent manner. Specifically, the IC_{50} values for Enz, Abi, and Bcl were 13.2 ± 6.2, 2.5 ± 1.0, and 15.5 ± 5.2 μM, respectively, for LNCaP cells, and 143.5 ± 21.3, 62.9 ± 12.5, and 75.5 ± 12.6 μM, respectively, for LNEnzR cells. The IC_{50} values obtained for Enz, Abi, and Bcl in LNEnzR cells were significantly larger than those in LNCaP cells ($P < .01$).

We examined the effect of Enz, Abi, and Bcl treatment on cell proliferation and glucose uptake in these cell lines. Treatment with DHT increased cell proliferation in LNCaP cells ($P < .01$), but not in LNEnzR cells. Treatment with Enz significantly suppressed cell proliferation in LNCaP cells ($P < .05$), but not in LNEnzR cells. These results indicate that LNEnzR cells have acquired resistance to Enz. We also examined whether LNEnzR cells showed cross-resistance to other AR signaling inhibitors, that is, Abi or Bcl. As with Enz treatment, cell proliferation was suppressed significantly after Abi or Bcl treatment in LNCaP cells ($P < .05$), but not in LNEnzR cells (Figure 2A). These results indicate that LNEnzR cells not only have resistance to Enz, but are also cross-resistant to other AR signaling inhibitors, such as Abi and Bcl.

Glucose uptake in LNCaP cells was significantly increased by DHT treatment ($P < .01$) and decreased by Enz, Abi, and Bcl treatment ($P < .05$). However, glucose uptake in LNEnzR cells was not affected by DHT, but it was significantly increased after Enz, Abi,
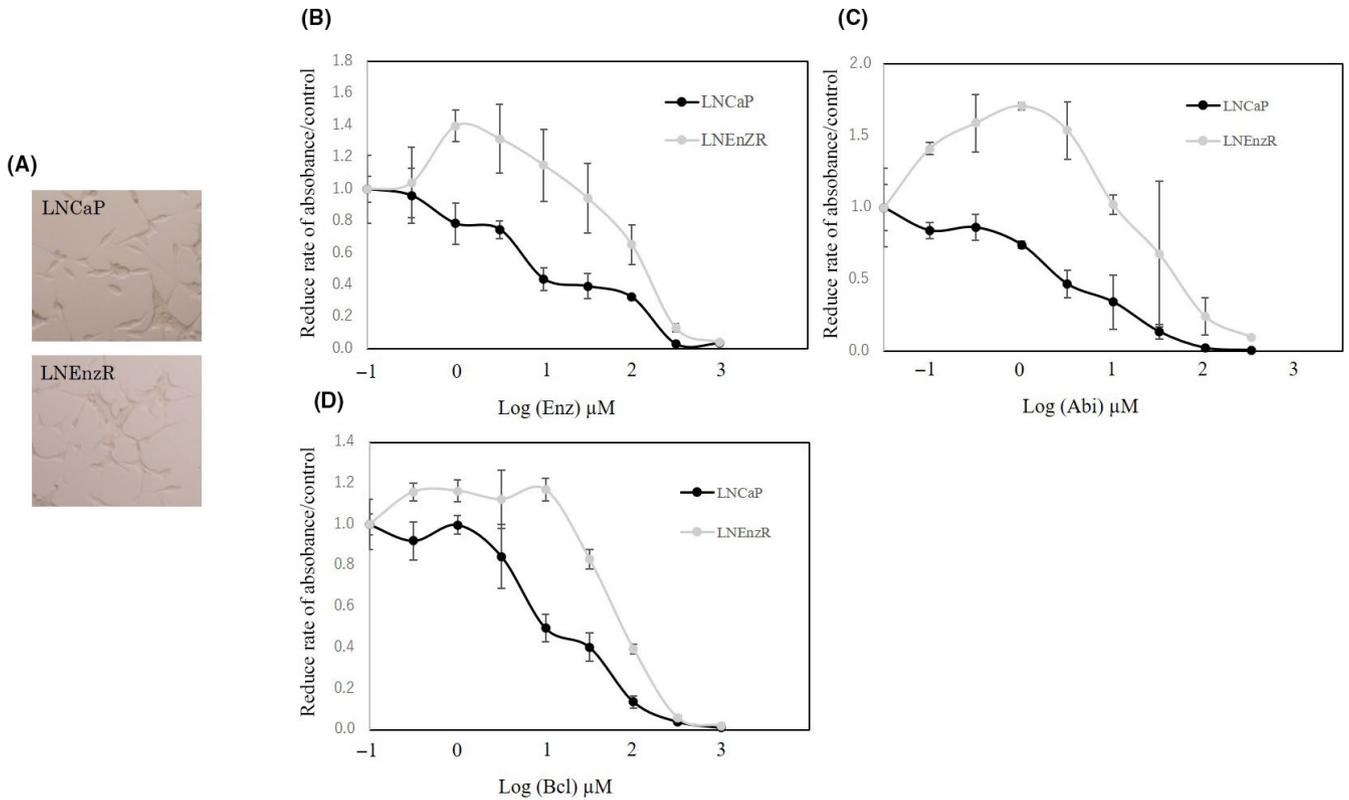
FIGURE 1 Evaluation of cell morphology and resistance to enzalutamide (Enz) and other androgen receptor signaling inhibitors in LNCaP-derived Enz-resistant LNEnzR cells. A, Morphological comparison between LNCaP cells and LNEnzR cells in a 40× field of view under an inverted microscope. B-D, Proliferation of these cells under various concentrations of Enz (B), abiraterone (Abi) (C), and bicalutamide (Bcl) (D) (WST-1 assay)
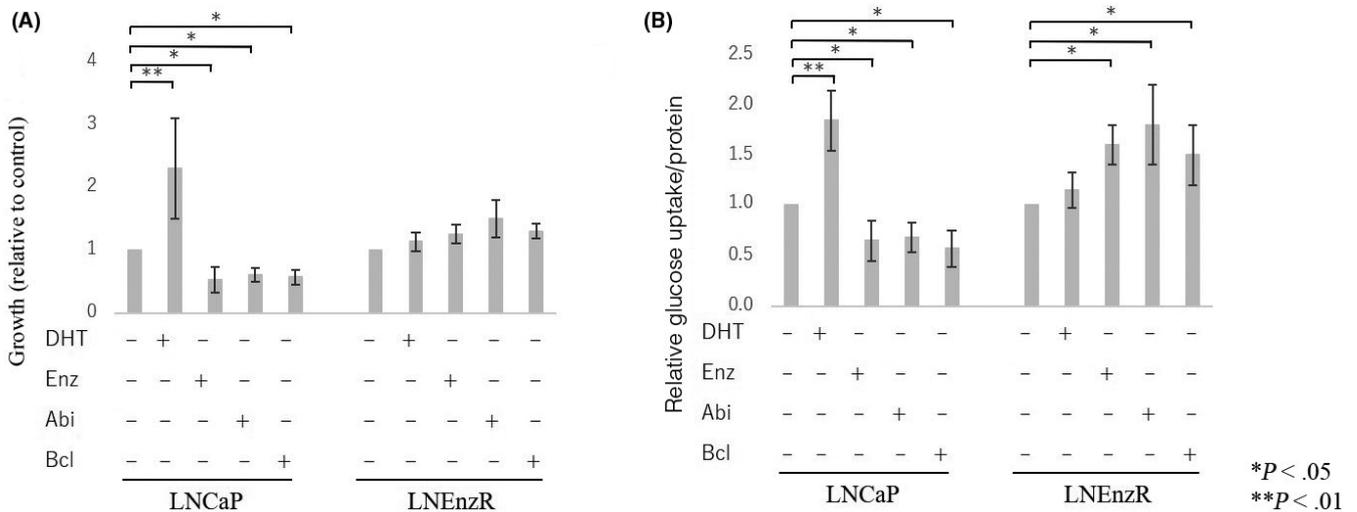

FIGURE 2 Effects of dihydrotestosterone (DHT), enzalutamide (Enz), abiraterone (Abi), and bicalutamide (Bcl) on cell proliferation and glucose uptake in LNCaP and LNEnzR cells

and Bcl treatment ($P < .05$; Figure 2B). These findings indicate that Enz, Abi, and Bcl treatment promotes glucose uptake by LNEnzR cells.

A question is how Abi, which suppressed the production of androgens by inhibiting CYP17A1, suppressed cell proliferation in LNCaP. A previous report showed that Abi inhibits AR signals by inhibiting intracellular testosterone metabolism in vitro.[25] In a preliminary study, we examined cell proliferation in LNCaP cells exposed to various concentrations of Abi. As shown in Figure S3, although Abi suppressed cell proliferation in a dose-dependent manner, Abi

treatment plus siRNA-induced AR knockdown did not show the same dose-dependent reduction in cell proliferation as Abi alone. These results indicate that both Abi and Enz have an antiproliferative effect that is related to the inhibition of the AR signal and intracellular testosterone synthesis and production in LNCaP cells, even in a cd-FBS medium.

3.2 | Alteration of AR and GR expression levels in LNEnzR cells

We compared AR and GR expression levels between LNCaP and LNEnzR cells to examine whether AR and GR expression levels changed in the process of acquiring resistance to Enz. Compared to LNCaP cells, LNEnzR cells showed statistically significant decreases in AR expression ($P < .05$) and increases in GR expression ($P < .01$) at the mRNA and protein levels (Figure 3). Chronic exposure to Enz upregulated GR expression, as described in previous reports.[6,8,18] We also examined the expression level of AR-V7 in these cells and found that AR-V7 mRNA and protein expression was not observed in either cell line (data not shown).

Next, we examined the changes in AR expression levels in these cell lines due to AR stimulation by DHT and AR signal inhibition by Enz (Figure 4A,B,E). Androgen receptor mRNA expression levels were significantly reduced by DHT, Enz, and DHT + Enz treatment in LNCaP cells ($P < .05$). Androgen receptor/protein levels were significantly increased by DHT treatment and reduced by Enz and DHT + Enz treatment in LNCaP cells ($P < .05$). However, significant changes in AR mRNA and protein expression levels in response to the administration of these drugs were not observed in LNEnzR cells. These results indicate that LNEnzR cells lost the AR reactivity that was observed in LNCaP cells after DHT and Enz treatment. We also examined the changes in GR expression levels in these cell lines due to DHT and Enz treatment. In LNCaP cells, the GR expression level was not significantly changed by DHT, Enz, or DHT + Enz treatment, whereas in LNEnzR cells, it was significantly increased by Enz and DHT + Enz ($P < .01$), but not by DHT treatment (Figure 4C,D,E).

These results suggest that in LNEnzR cells, which have high GR expression levels, Enz and Abi treatment increases GR expression levels.

3.3 | Alterations of GLUT1, GLUT3, GLUT4, and GLUT12 expression levels in LNEnzR cells

As a previous study reported that upregulation of GLUT1, 3, 4, and 12 has been observed in PCa,[16] we compared the expression levels between LNCaP and LNEnzR cells. No statistically significant difference in GLUT1, GLUT3, or GLUT12 mRNA and protein levels was observed between the two cell lines; however, significantly higher levels of GLUT4 expression were observed in LNEnzR cells compared to LNCaP cells ($P < .01$; Figure 5A,B,C). To evaluate the expression of activated GLUT1, GLUT3, GLUT4, and GLUT12 proteins on the cell surface, we carried out flow cytometry using LNCaP and LNEnzR cells. Although there were no significant differences among GLUT1, GLUT3, and GLUT12 expression levels at the cell surface in either cell line, GLUT4 expression levels at the cell surface were significantly higher in LNEnzR cells than in LNCaP cells ($P < .05$; Figure 5D-G). These results indicate that chronic Enz treatment not only induces increased expression, but also activates GLUT4 expression.

To evaluate the effect of androgen stimulation and inhibition on GLUT1 and GLUT4 expression, both of which are related to AR signaling-dependent cell proliferation,[16,20] we examined changes in GLUT1 and GLUT4 expression levels after DHT and Enz treatment in LNCaP and LNEnzR cell lines (Figure 6). In androgen-dependent LNCaP cells, AR stimulation by DHT significantly increased GLUT1 and GLUT4 expression levels ($P < .01$), but Enz and DHT + Enz suppressed these expression levels ($P < .05$). Conversely, in LNEnzR cells, which lose the AR reactivity observed in LNCaP cells and have high GR expression, DHT treatment did not change GLUT1 or GLUT4 expression levels, but Enz and DHT + Enz did increase GLUT4 expression levels ($P < .01$). These findings showed that in LNEnzR cells, which originally had
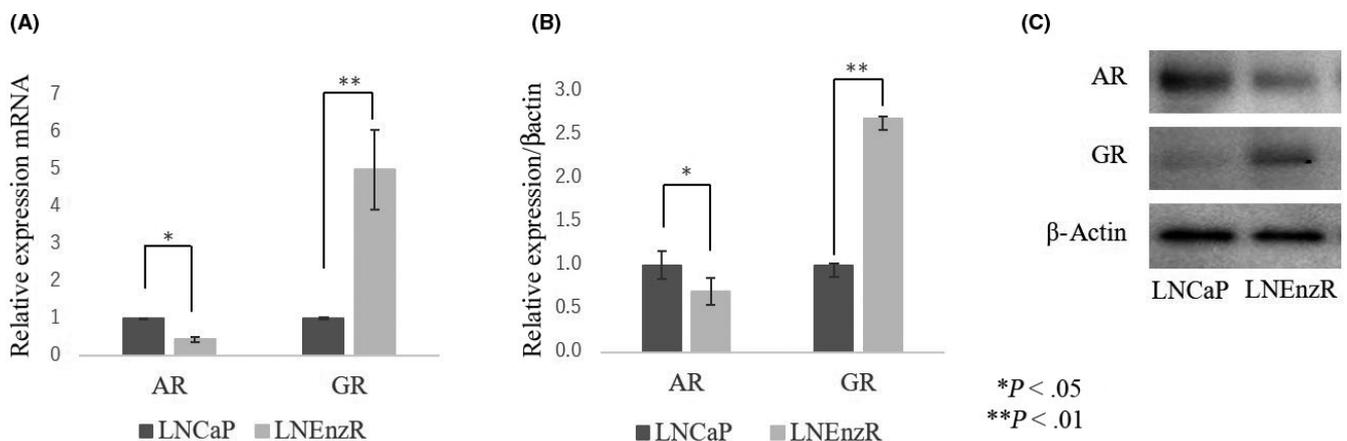

FIGURE 3 Expression analysis of androgen receptor (AR) and glucocorticoid receptor (GR) in LNCaP and LNEnzR cells. The mRNA and protein expression levels of AR and GR were analyzed by quantitative RT-PCR (A) and western blotting (B, C), respectively
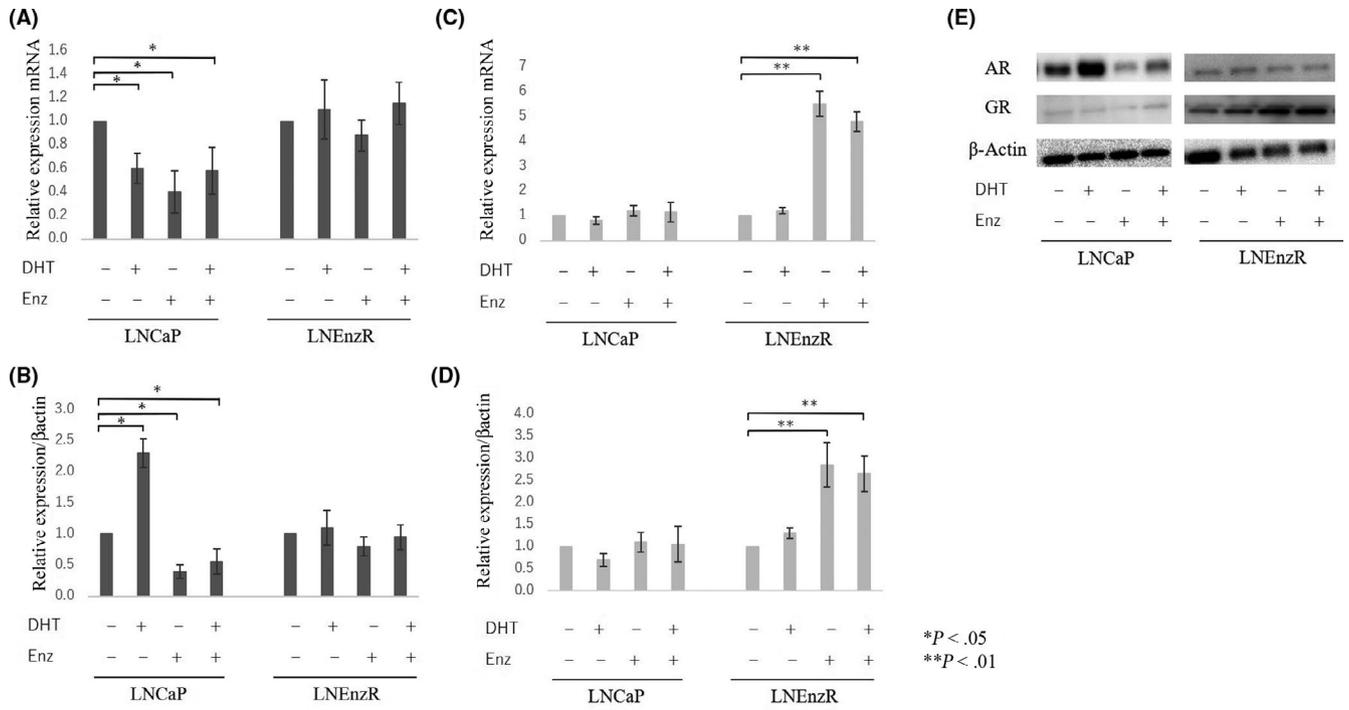
FIGURE 4 Effects of dihydrotestosterone (DHT) and/or enzalutamide (Enz) treatment on androgen receptor (AR) and glucocorticoid receptor (GR) expression in LNCaP and LNEnzR cells. The mRNA (A, C) and protein levels (B, D, E) for AR (A, B, E) and GR (C-E) were analyzed by quantitative RT-PCR and western blotting, respectively
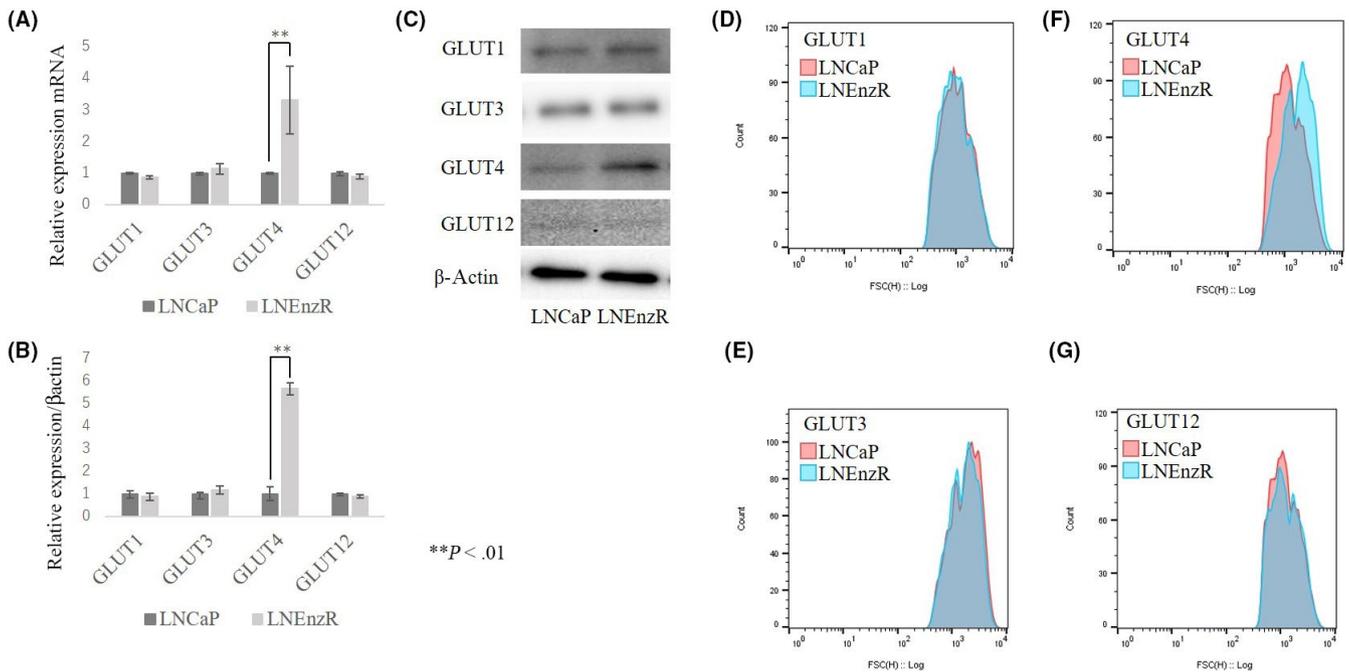

FIGURE 5 Expression analysis of glucose transporter (GLUT)1, GLUT3, GLUT4, and GLUT12 in LNCaP cells and LNEnzR cells. A-C, Analysis of mRNA (A) and protein levels (B, C) for GLUT1, GLUT3, GLUT4, and GLUT12 by quantitative RT-PCR and western blotting in both cells. D-G, Expression of GLUT1 (D), GLUT3 (E), GLUT4 (F), and GLUT12 (G) on the surface of both cell lines was analyzed by flow cytometry
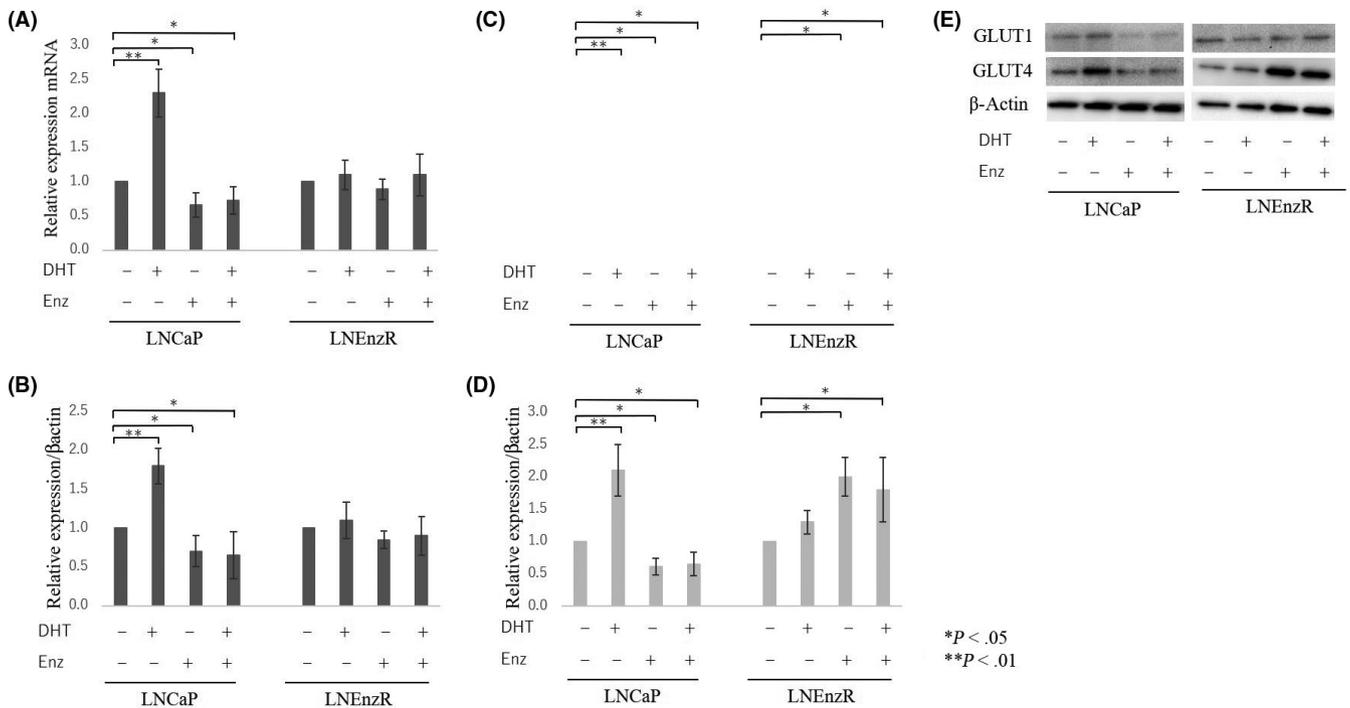
FIGURE 6 Effects of dihydrotestosterone (DHT) and/or enzalutamide (Enz) treatment on glucose transporter (GLUT)1 and GLUT4 expression in LNCaP and LNEnzR cells. mRNA (A, C) and protein levels (B, D, E) of GLUT1 (A, B, E) and GLUT4 (C-E) were analyzed by quantitative RT-PCR and western blotting in both cell lines

high GLUT4 expression levels, Enz treatment promotes further increases in GLUT4 expression.

3.4 | Effects of GR and GLUT4 inhibition on cell proliferation in PC3 cells with negative AR expression, but positive GR expression

As shown above, both GR and GLUT4 were upregulated in LNEnzR cells. To assess the correlation between GR and GLUT4 in GR-positive PCa cells, we examined the change in GR and GLUT4 expression levels by GR and GLUT4 inhibition in PC3 cells. As it was possible that the expression of GLUT4 would be influenced by AR signals, we first utilized PC3 cells, which are characterized by negative AR but positive GR expression. Both siRNA-induced GR knockdown and treatment with the GR inhibitor RU486 significantly decreased GR and GLUT4 expression at the mRNA and protein levels, whereas siRNA-induced GLUT4 knockdown and treatment with the GLUT4 inhibitor Rit decreased only GLUT4 expression without changing GR expression (Figure 7A-C). These results indicate that GR regulated GLUT4 expression, but GR expression was not influenced by GLUT4 inhibition.

We used WST-1 assays to evaluate the effect of GR and GLUT4 inhibition on cell proliferation. The siRNA-induced GR and GLUT4 knockdown and treatment with a GLUT4 inhibitor significantly reduced cell proliferation at 3, 5, and 7 days after treatment compared to controls ($P < .01$). Treatment with GR inhibitor significantly reduced cell proliferation at 5 ($P < .05$) and 7 days ($P < .01$) after treatment compared to the control (Figure 7D). We also undertook

glucose uptake assays to evaluate the changes in glucose uptake by GR and GLUT4 inhibition. The siRNA-induced GR and GLUT4 knockdown, as well as treatment with GR and GLUT4 inhibitors, significantly reduced cellular glucose uptake compared to controls (Figure 7E; $P < .05$). These results indicate that GR regulated GLUT4 expression independently of AR signals, and that GR and GLUT4 inhibition reduced cell proliferation and glucose uptake.

3.5 | Effects of GR and GLUT4 inhibition on cell proliferation and glucose uptake in LNEnzR cells

To evaluate whether GR and GLUT4 inhibition influenced the proliferation and glucose uptake of LNCaP cells, which have low GR expression levels, and LNEnzR cells, which have high GR expression levels, we carried out siRNA-induced GR and GLUT4 knockdown and treatment with GR and GLUT4 inhibitor on these cell lines, and measured cell proliferation by a WST-1 assay and glucose uptake by a glucose uptake assay. The siRNA-induced GR knockdown and treatment with the GR inhibitor RU486 did not reduce cell proliferation or glucose uptake in LNCaP cells, whereas siRNA-induced GLUT4 knockdown and treatment with the GLUT4 inhibitor Rit significantly reduced cell proliferation and glucose uptake in this cell line (Figure 8). These results indicated that GR inhibition did not reduce cell proliferation or glucose uptake in LNCaP cells, because LNCaP cells have low GR expression, but GLUT4 inhibition reduced cell proliferation by inhibiting glucose uptake in this cell line. In contrast, siRNA-induced GR and GLUT4 knockdown, and treatment with the
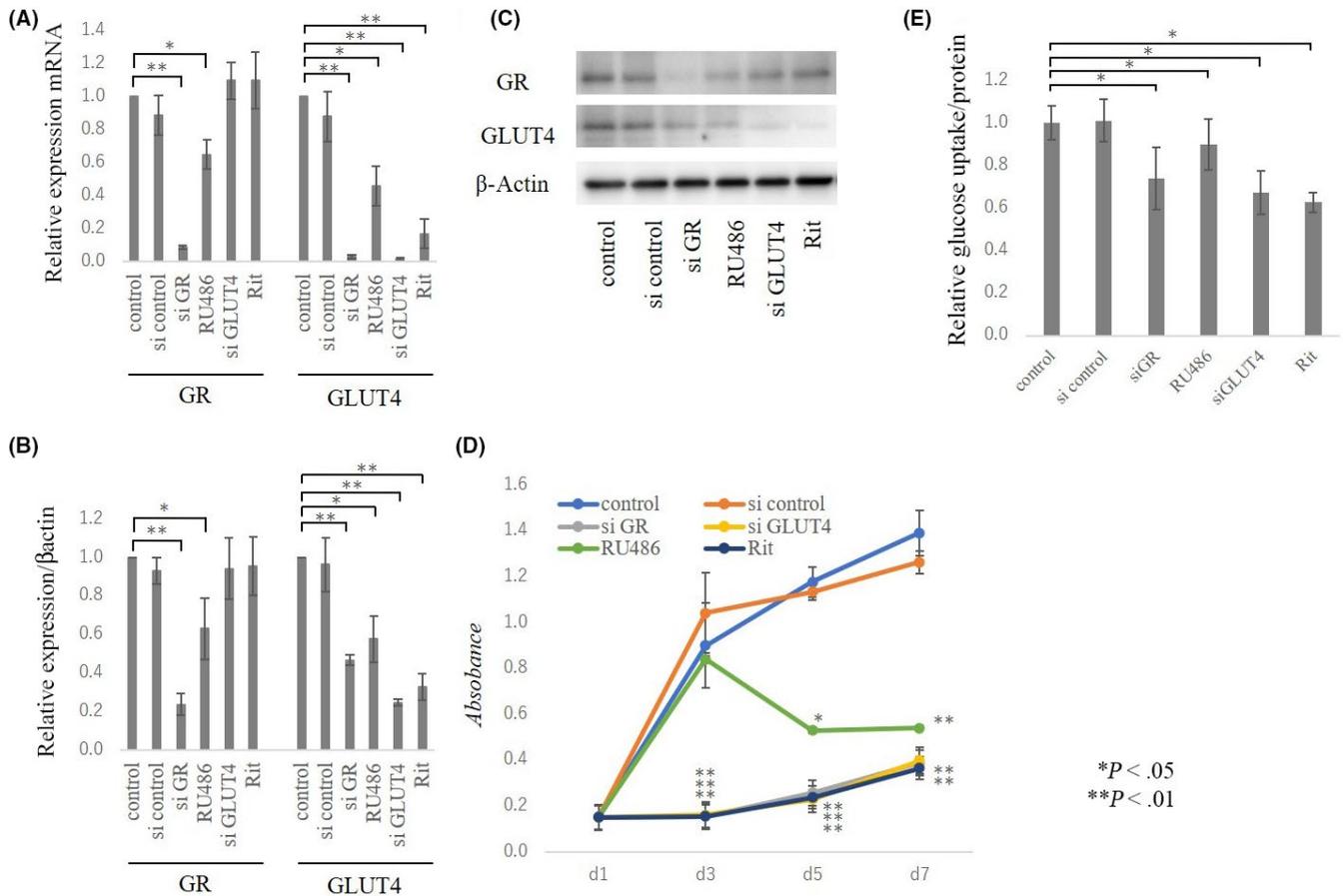
FIGURE 7 Effects of glucocorticoid receptor (GR) and glucose transporter (GLUT)4 inhibition on GR and GLUT4 expression, cell proliferation, and glucose uptake in PC3, the androgen receptor-negative and GR-positive prostate cancer cell line. A-C, GR and GLUT4 mRNA (A) and protein expression (B, C) levels under siRNA control (si control), siRNA-induced GR (siGR), or GLUT4 (siGLUT4) knockdown, and treatment with the GR inhibitor RU486 or the GLUT4 inhibitor ritonavir (Rit) in PC3 cells. D, E, WST-1 assay (D) and glucose uptake assay (E) were carried out in PC3 cells after treatment with these drugs

GLUT4 inhibitor Rit, reduced cell proliferation and glucose uptake (P < .05); however, treatment with the GR inhibitor RU486 significantly promoted cell proliferation and glucose uptake in LNEnzR cells, which have high levels of GR expression (P < .01, Figure 8). As described above, RU486 significantly decreased the proliferation of PC3 cells. Completely opposite results were observed in LNEnzR cells, which are AR positive and have high levels of GR expression, and PC3 cells, which are AR negative and GR positive.

We evaluated whether the changes in cell proliferation by GR and GLUT4 inhibition resulted from changes in GR and GLUT4 expression. We examined the changes not only of GLUT4, but also of GLUT1 expression, because it is possible that GR and GLUT4 inhibition could induce changes in GLUT1 expression. In LNCaP cells, siRNA-induced GR knockdown significantly decreased GR expression (P < .05), but use of the GR inhibitor RU486 did not change GR expression at the mRNA and protein levels. Neither siRNA-induced GR knockdown nor the GR inhibitor influenced GLUT1 or GLUT4 expression levels in this cell line. Both siRNA-induced GLUT4 knockdown and treatment with the GLUT4 inhibitor Rit significantly decreased GLUT4 expression without changing GR or GLUT1 expression levels in this cell line (P < .01; Figure 9A,B,E). These results indicate that GR and GLUT4 do

not regulate each other in AR signaling-dependent or GR signaling-independent LNCaP cells. In contrast, in LNEnzR cells, siRNA-induced GR knockdown decreased both GR (P < .01) and GLUT4 expression (P < .05) at the mRNA and protein levels without changing the GLUT1 expression level. In addition, GLUT4 knockdown and the GLUT4 inhibitor Rit significantly decreased GLUT4 expression levels without changing GR or GLUT1 expression levels (P < .01). These results indicate that GR regulated GLUT4 expression, and that, like PC3 cells, GR expression was not influenced by GLUT4 inhibition in LNEnzR cells. The GR inhibitor RU486 significantly increased GR and GLUT4 expression levels (P < .01; Figure 9C,D,E). As described above, GR inhibitor significantly decreased both GR and GLUT4 expression at the mRNA and protein levels in PC3 cells. Thus, completely opposite results were observed between LNEnzR and PC3 cells.

3.6 | Inhibition of GR and GLUT4 improves resistance to antiandrogens in LNEnzR cells

To evaluate whether the inhibition of GR and GLUT4 can increase drug resistance to AR signaling inhibitors in LNEnzR cells, we used
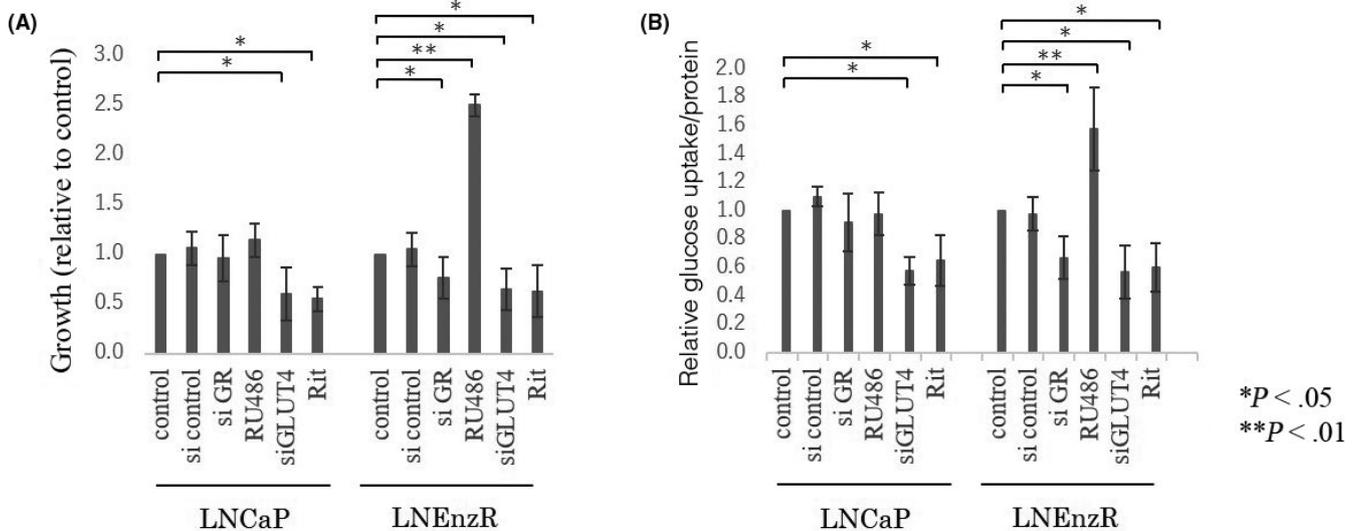
FIGURE 8 Effects of glucocorticoid receptor (GR) and glucose transporter (GLUT)4 inhibition on cell proliferation and glucose uptake in LNCaP and LNEnzR cells. Cell proliferation (A) and glucose uptake (B) under siRNA control (si control), siRNA-induced GR (siGR) or GLUT4 (siGLUT4) knockdown and treatment with the GR inhibitor RU486 or the GLUT4 inhibitor ritonavir (Rit)
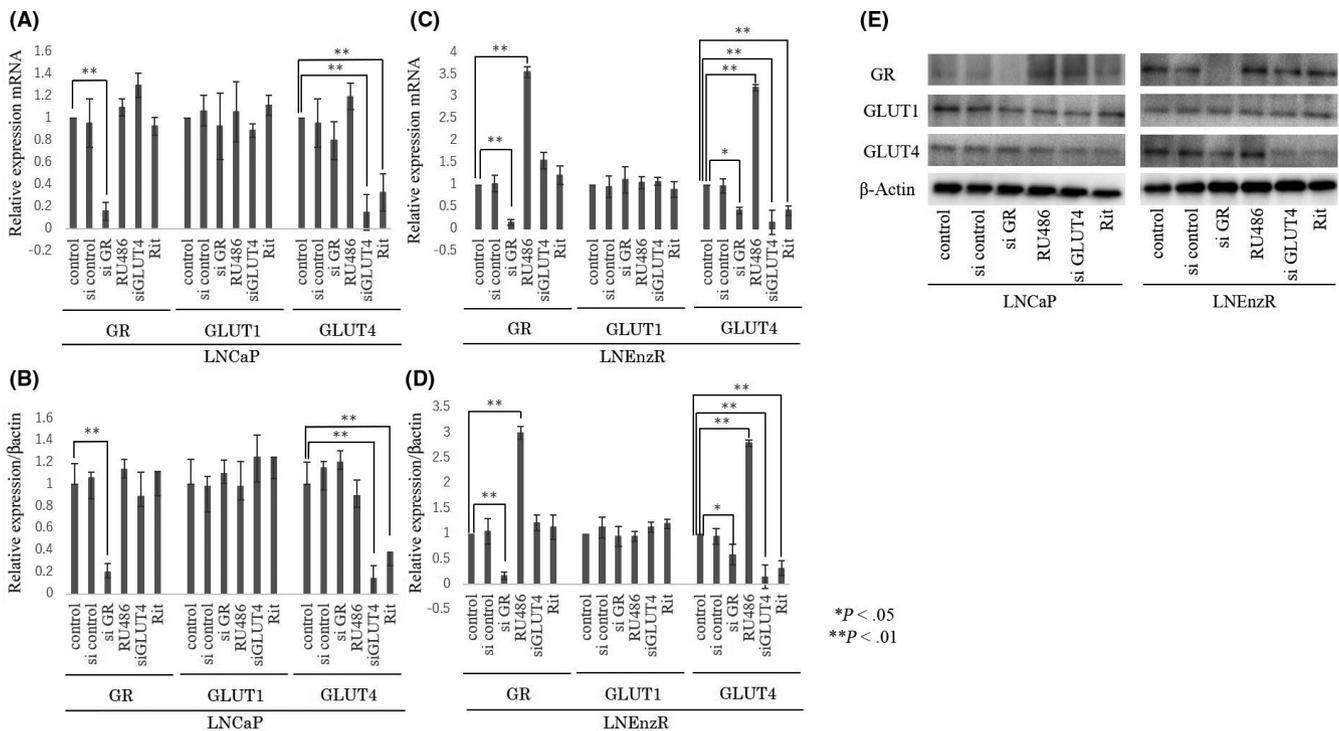

FIGURE 9 Effects of glucocorticoid receptor (GR) and glucose transporter (GLUT)4 inhibition on GR and GLUT expression in LNCaP (A, B) and LNEnzR (C, D) cells. mRNA (A, C) and protein (B-E) expression levels for GR, GLUT1, and GLUT4 under siRNA-induced GR (siGR) or GLUT4 (siGLUT4) knockdown and treatment with the GR inhibitor RU486 or GLUT4 inhibitor ritonavir (Rit) were analyzed by quantitative RT-PCR and western blotting, respectively

WST-1 assays to examine the effect of Enz or Abi treatment on cell proliferation when GR and GLUT4 are inhibited. Although treatment with Enz or Abi alone did not reduce cell proliferation, treatment with Enz or Abi combined with GR or GLUT4 inhibition, such as by siRNA-induced GR or GLUT4 knockdown or by a GLUT4 inhibitor,

significantly reduced cell proliferation ($P < .01$). These reductions were significantly larger than GR or GLUT4 inhibition alone ($P < .05$; Figure 10). These results suggest that GR-mediated GLUT4 upregulation contributes to resistance to Enz in PCa cells, and that GR and GLUT4 inhibition can improve the resistance.
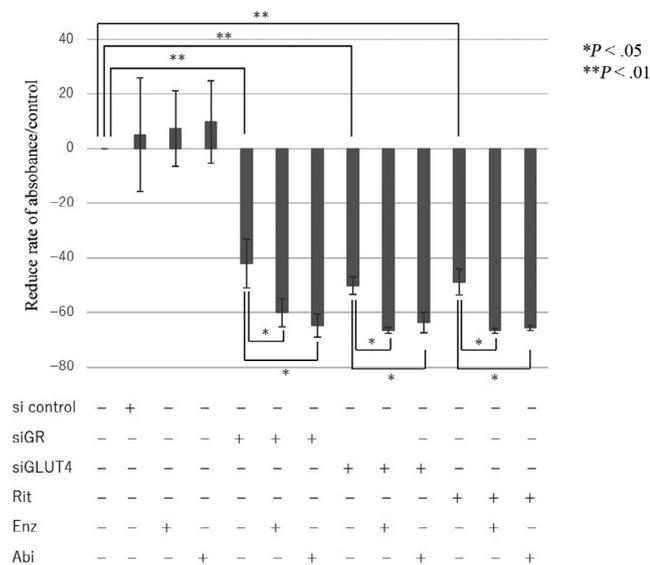
FIGURE 10 Effects of glucocorticoid receptor (GR) and glucose transporter (GLUT)4 inhibition on drug resistance to enzalutamide (Enz) and abiraterone (Abi) in LNEnzR cells. WST-1 assay was used to evaluate the effect of Enz or Abi treatment with or without siRNA-induced GR (siGR) or GLUT4 (siGLUT4) knockdown or the GLUT4 inhibitor ritonavir (Rit) on cell proliferation

4 | DISCUSSION

In this study, for the first time, we showed that GR-mediated GLUT4 upregulation was involved in Enz resistance and cross-resistance in PCa treatment, and that GLUT4 inhibition improved resistance. Although it has been reported that GR is upregulated as a bypass for AR signal inhibition by Enz, no reports have shown that GLUT4 is regulated by GR or can contribute to acquiring resistance to Enz and other AR signaling inhibitors.

Previous reports have shown that the expression of GR changes during PCa treatment.[6,8,26] The expression levels of GR in untreated PCa cells is lower than in normal prostate cells, and in the process of acquiring resistance to second-generation antiandrogens (Enz and Abi), GR expression increases inversely with AR expression.[8] In addition, the upregulation of GR enhances the expression of *KLK3* and *FKBP5*, which are androgen-responsive genes, and *SGK1*, which is a glucocorticoid-responsive gene, and might contribute to the acquisition of resistance to AR signaling inhibitors.[6] *FKBP5* and *SGK1* are involved in the expression and activation of GLUT1 and GLUT4 through the PIK3 pathway and AMPK pathway.[27-30] In this study, we showed that GR and GLUT4 were upregulated in LNEnzR cells, but not GLUT1. As activation of the PIK3 and AMPK pathways due to upregulation of FKBP5 and SGK1 influences both GLUT1 and GLUT4 expression,[29-31] the upregulation of GLUT4 expression without alteration of GLUT1 expression might not be mediated only by these pathways when acquiring Enz resistance. Previous reports have shown that the functions of GLUT1 and GLUT4 in PCa cells depend on whether the cells are androgen dependent or independent,[20,32] and also that the expression of GLUT1 is lower and the expression of GLUT4 is higher in androgen-independent PCa cells than in androgen-dependent PCa

cells.[20] Their findings suggest that GLUT4 could play a more significant role than GLUT1 in androgen-independent PCa cells, such as Enz-resistant cells. In this study, we also found that GLUT4, which was regulated by GR, was involved in Enz resistance and cross-resistance in LNEnzR cells. Therefore, GLUT4 might be regulated by GR through a different pathway from GLUT1 regulation in Enz resistance and cross-resistance in PCa cells; however, the detailed mechanism and pathway by which GR mediates GLUT4 to confer resistance remains unknown, and further study is needed.

In our study, although treatment with Enz or Abi alone did not reduce cell proliferation, Enz or Abi combined with GR or GLUT4 inhibition significantly reduced cell proliferation. These reductions were significantly larger than those for GR or GLUT4 inhibition alone. These findings showed that GR and GLUT4 inhibition can improve the resistance of LNEnzR cells to Enz or Abi. Here, we hypothesize how GR or GLUT4 inhibition facilitated the recovery of sensitivity to Enz or Abi in LNEnzR cells. Briefly, although AR expression levels were low in LNEnzR cells, expression still occurred and was not changed by GR or GLUT4 inhibition (Figure S4). Consequently, Enz could still have potential to inhibit cell proliferation through AR in LNEnzR cells. However, this potential could be masked by Enz treatment, because Enz markedly increased cell proliferation in LNEnzR cells, which in turn showed higher levels of GR expression, increasing GLUT4 expression and activating glucose uptake. However, treatment with Enz combined with GR or GLUT4 inhibition improved resistance to Enz, possibly because GR and GLUT4 inhibition could unmask the potential of Enz to inhibit cell proliferation through AR in LNEnzR cells. Further studies are required to better clarify these mechanisms.

In our study, there was discrepancy in the effects on cell proliferation for LNEnzR cells between siRNA-induced GR knockdown and treatment with the GR inhibitor RU486; siRNA-induced GR knockdown suppressed cell proliferation, but the GR inhibitor promoted it. Several reasons should be considered to explain this discrepancy. First, RU486 treatment of LNEnzR cells, like Enz, induced GR upregulation as it has antiandrogenic effects. Second, GR inhibitors can possibly induce iatrogenic tumor proliferation because of receptor-to-receptor interactions caused by structural similarities between AR and GR, [33-35] the suppression of tumor immunity,[33] and activation of the *p53* gene by therapeutic modification.[36,37] Third, RU486 does not have sufficient inhibitory activity.[38] As siRNA-induced GR knockdown could suppress cell proliferation in LNEnzR cells, sufficient inhibition of GR with a highly selective GR inhibitor, which is in development,[39] might be able to suppress cell proliferation in LNEnzR cells. Fourth, the effects of RU486 on cell proliferation depend on the AR and GR expression levels, which were different among the three cell lines, PC3, LNCaP, and LNEnzR; RU486 treatment reduced cell proliferation in PC3 cells, did not change cell proliferation in LNCaP cells, and promoted cell proliferation in LNEnzR cells. These differences could have been caused by the different expression levels of AR and GR in these cells.

Our study could have clinical significance for the treatment of patients with CRPC. First, GR expression in PCa cells might be

increased in some CRPC patients with resistance to Enz and other AR signaling inhibitors, and GLUT4 inhibition might mitigate resistance to them in CRPC patients with increased GR expression. Second, although GR is a classic therapeutic target for CRPC, the therapeutic effect of the GR inhibitor RU486 was limited,[40] as described above. As GLUT4 inhibition did not affect GR expression, a GLUT4 inhibitor could provide good therapeutic effects without the iatrogenic cancer cell proliferation induced by GR inhibitors in some CRPC patients. Third, in our study, the expression of GLUT4 increased in the process of acquiring resistance to Enz and AR signaling inhibitors, and its inhibition mitigated the resistance. Therefore, the GLUT4 expression level, regardless of the GR expression level, could be a predictor for the therapeutic effect not only of Enz, but also of other AR signaling inhibitors. In general, glucose is the primary substrate for energy metabolism in tissues, and a continuous supply of glucose is required for cells to function. Thus, glucose utilization measured using FDG-PET has become an established method for quantifying local functional activity in brain, heart, and most cancers. 2-Deoxy-D-glucose is a glucose analog that utilizes the GLUTs, including GLUT4, for entry into the cell. The kinetics of 2-DG are similar to those of FDG. As PCa cells have low expression levels of GLUT1, FDG-PET is not well suited for the detection of PCa. However, FDG-PET could potentially be used to evaluate the expression of GLUT4,[41] which could then be used to predict the therapeutic effect of AR signaling inhibitors in PCa in actual clinical settings.

Glucose transporter 4 is an insulin-sensitive GLUT that facilitates insulin-stimulated glucose uptake in adipose tissue, skeletal muscle, and cardiac tissues.[42,43] As GLUT4 expression is low in the central nervous system and most organs except the heart, GLUT4 inhibitors might lead to fewer fatal complications than GLUT1 inhibitors, as GLUT1 is expressed in many important organs. In fact, the GLUT4 inhibitor Rit is actually used for patients with AIDS due to HIV infection, and it rarely induces fatal complications.[44,45] Additionally, a clinical trial using GLUT4-selective inhibitors for patients with melanoma has been reported.[46] Combination therapy with AR signaling inhibitors, including Enz and a GLUT4 inhibitor, could be a new therapeutic strategy for patients with CRPC.

There are several limitations to our study. First, we did not consider the effects of AR signals in the process of acquiring resistance to AR signaling inhibitors in our cell lines. In patients with CRPC, AR and GR interact with each other to control tumor survival and proliferation;[8] however, as AR expression is low in the cell line used in this study, it is unclear whether GR or GLUT inhibition is effective under the presence of AR expression and AR-GR interaction. Second, the assessment of GLUT4 expression in patients with PCa is difficult because the expression can be changed by the effects of insulin, insulin-like proliferation factors, and testosterone. Third, we did not show how glucose uptake was involved in Enz resistance or how GR regulates GLUT4. Finally, this is an in vitro study; detailed mechanisms will be clarified by in vivo studies or clinical samples compatible with Enz-resistant conditions. Further study will be needed to establish a method for evaluating the expression of GR and GLUT4 in vivo.

In conclusion, GR-mediated GLUT4 upregulation by chronic Enz treatment could be involved in Enz resistance as well as cross-resistance to other AR signaling inhibitors. The inhibition of GLUT4 suppressed the proliferation of Enz-resistant PCa cells without changing GR expression, and recovered Enz resistance and cross-resistance. Our study could provide a new therapeutic strategy for Enz-resistant CRPC patients in the future.

ORCID
Seiji Hoshi https://orcid.org/0000-0003-1545-1827

REFERENCES
1. Khalaf DJ, Annala M, Taavitsainen S, et al. Optimal sequencing of enzalutamide and abiraterone acetate plus prednisone in metastatic castration-resistant prostate cancer: a multicentre, randomised, open-label, phase 2, crossover trial. *Lancet Oncol.* 2019;20:1730-1739.
2. Pal SK, Patel J, He M, et al. Identification of mechanisms of resistance to treatment with abiraterone acetate or enzalutamide in patients with castration-resistant prostate cancer (CRPC). *Cancer.* 2018;124:1216-1224.
3. Tucci M, Zichi C, Buttigliero C, Vignani F, Scagliotti GV, Di Maio M. Enzalutamide-resistant castration-resistant prostate cancer: challenges and solutions. *Onco Targets Ther.* 2018;11:7353-7368.
4. Chen CD, Welsbie DS, Tran C, et al. Molecular determinants of resistance to antiandrogen therapy. *Nat Med.* 2004;10:33-39.
5. Kregel S, Chen JL, Tom W, et al. Acquired resistance to the second-generation androgen receptor antagonist enzalutamide in castration-resistant prostate cancer. *Oncotarget.* 2016;7:26259-26274.
6. Arora VK, Schenkein E, Murali R, et al. Glucocorticoid receptor confers resistance to antiandrogens by bypassing androgen receptor blockade. *Cell.* 2013;155:1309-1322.
7. Chen X, Chen F, Ren Y, et al. Glucocorticoid receptor upregulation increases radioresistance and triggers androgen independence of prostate cancer. *Prostate.* 2019;79:1386-1398.
8. Puhr M, Hoefer J, Eigentler A, et al. The Glucocorticoid receptor is a key player for prostate cancer cell survival and a target for improved antiandrogen therapy. *Clin Cancer Res.* 2018;24:927-938.
9. Sahu B, Laakso M, Pihlajamaa P, et al. FoxA1 specifies unique androgen and glucocorticoid receptor binding events in prostate cancer cells. *Can Res.* 2013;73:1570-1580.
10. Pavlova NN, Thompson CB. The Emerging hallmarks of cancer metabolism. *Cell Metab.* 2016;23:27-47.
11. Keenan MM, Chi JT. Alternative fuels for cancer cells. *Cancer J.* 2015;21:49-55.
12. Galbraith L, Leung HY, Ahmad I. Lipid pathway deregulation in advanced prostate cancer. *Pharmacol Res.* 2018;131:177-184.

13. Giunchi F, Fiorentino M, Loda M. The Metabolic landscape of prostate cancer. *Eur urol oncol*. 2019;2:28-36.

14. Stoykova GE, Schlaepfer IR. Lipid metabolism and endocrine resistance in prostate cancer, and new opportunities for therapy. *Int J Mol Sci*. 2019;20(11):2626.

15. Strmiska V, Michalek P, Eckschlager T, et al. Prostate cancer-specific hallmarks of amino acids metabolism: towards a paradigm of precision medicine. *Biochim Biophys Acta*. 2019;1871:248-258.

16. Gonzalez-Menendez P, Hevia D, Mayo JC, Sainz RM. The dark side of glucose transporters in prostate cancer: Are they a new feature to characterize carcinomas? *Int J Cancer*. 2018;142:2414-2424.

17. Wade CA, Kyprianou N. Profiling prostate cancer therapeutic resistance. *Int J Mol Sci*. 2018;19(3):904.

18. Prekovic S, van den Broeck T, Linder S, et al. Molecular underpinnings of enzalutamide resistance. *Endocr Relat Cancer*. 2018;25:R545-R557.

19. Macheda ML, Rogers S, Best JD. Molecular and cellular regulation of glucose transporter (GLUT) proteins in cancer. *J Cell Physiol*. 2005;202:654-662.

20. Gonzalez-Menendez P, Hevia D, Rodriguez-Garcia A, Mayo JC, Sainz RM. Regulation of GLUT transporters by flavonoids in androgen-sensitive and–insensitive prostate cancer cells. *Endocrinology*. 2014;155:3238-3250.

21. Weinstein SP, Paquin T, Pritsker A, Haber RS. Glucocorticoid-induced insulin resistance: dexamethasone inhibits the activation of glucose transport in rat skeletal muscle by both insulin- and non-insulin-related stimuli. *Diabetes*. 1995;44:441-445.

22. Kim HI, Moon SH, Lee WC, et al. Inhibition of cell growth by cellular differentiation into adipocyte-like cells in dexamethasone sensitive cancer cell lines. *Animal cells and systems*. 2018;22:178-188.

23. Sedelaar M, Isaacs JT. Tissue culture media supplemented with 10% fetal calf serum contains a castrate level of testosterone. *Prostate*. 2009;69:1724-1729.

24. Wang X, Wang L, Zhu L, et al. PAQR3 modulates insulin signaling by shunting phosphoinositide 3-kinase p110α to the Golgi apparatus. *Diabetes*. 2013;62:444-456.

25. Li R, Evaul K, Sharma KK, et al. Abiraterone inhibits 3β-hydroxysteroid dehydrogenase: a rationale for increasing drug exposure in castration-resistant prostate cancer. *Clin Cancer Res*. 2012;18:3571-3579.

26. Kassi E, Moutsatsou P. Glucocorticoid receptor signaling and prostate cancer. *Cancer Lett*. 2011;302:1-10.

27. Jeyaraj S, Boehmer C, Lang F, Palmada M. Role of SGK1 kinase in regulating glucose transport via glucose transporter GLUT4. *Biochem Biophys Res Comm*. 2007;356:629-635.

28. Palmada M, Boehmer C, Akel A, et al. SGK1 kinase upregulates GLUT1 activity and plasma membrane expression. *Diabetes*. 2006;55:421-427.

29. Lang F, Görlach A, Vallon V. Targeting SGK1 in diabetes. *Expert opinion on therapeutic targets*. 2009;13:1303-1311.

30. Balsevich G, Häusl AS, Meyer CW, et al. Stress-responsive FKBP51 regulates AKT2-AS160 signaling and metabolic function. *Nat Commun*. 2017;8:1725.

31. Sidibeh CO, Pereira MJ, Abalo XM, et al. FKBP5 expression in human adipose tissue: potential role in glucose and lipid metabolism, adipogenesis and type 2 diabetes. *Endocrine*. 2018;62:116-128.

32. González Menéndez P, Hevia D, Alonso R, Gonzalez-Pola I, Mayo J, Sainz R. GLUT1/GLUT4 balance is a marker of androgen-insensitivity in prostate cancer. *Eur J Cancer*. 2016;61:S41.

33. Ayroldi E, Cannarile L, Delfino DV, Riccardi C. A dual role for glucocorticoid-induced leucine zipper in glucocorticoid function: tumor growth promotion or suppression? *Cell Death Dis*. 2018;9:463.

34. Montgomery B, Cheng HH, Drechsler J, Mostaghel EA. Glucocorticoids and prostate cancer treatment: friend or foe? *Asian journal of andrology*. 2014;16:354-358.

35. Pufall MA. Glucocorticoids and cancer. *Adv Exp Med Biol*. 2015;872:315-333.

36. Sengupta S, Vonesch JL, Waltzinger C, Zheng H, Wasylyk B. Negative cross-talk between p53 and the glucocorticoid receptor and its role in neuroblastoma cells. *EMBO J*. 2000;19:6051-6064.

37. Li H, Qian W, Weng X, et al. Glucocorticoid receptor and sequential P53 activation by dexamethasone mediates apoptosis and cell cycle arrest of osteoblastic MC3T3-E1 cells. *PLoS One*. 2012;7:e37030.

38. Clark RD. Glucocorticoid receptor antagonists. *Curr Top Med Chem*. 2008;8:813-838.

39. Kach J, Long TM, Selman P, et al. Selective glucocorticoid receptor modulators (SGRMs) delay castrate-resistant prostate cancer growth. *Mol Cancer Ther*. 2017;16:1680-1692.

40. Taplin ME, Manola J, Oh WK, et al. A phase II study of mifepristone (RU-486) in castration-resistant prostate cancer, with a correlative assessment of androgen-related hormones. *BJU Int*. 2008;101:1084-1089.

41. Van den Abbeele AD, Gatsonis C, de Vries DJ, et al. ACRIN 6665/RTOG 0132 phase II trial of neoadjuvant imatinib mesylate for operable malignant gastrointestinal stromal tumor: monitoring with 18F-FDG PET and correlation with genotype and GLUT4 expression. *J Nucl Med*. 2012;53:567-574.

42. Fukumoto H, Kayano T, Buse JB, et al. Cloning and characterization of the major insulin-responsive glucose transporter expressed in human skeletal muscle and other insulin-responsive tissues. *J Biol chem*. 1989;264:7776-7779.

43. Charron MJ, Brosius FC 3rd, Alper SL, Lodish HF. A glucose transport protein expressed predominantly in insulin-responsive tissues. *Proc Natl Acad Sci USA*. 1989;86:2535-2539.

44. Croxtall JD, Perry CM. Lopinavir/Ritonavir: a review of its use in the management of HIV-1 infection. *Drugs*. 2010;70:1885-1915.

45. Murata H, Hruz PW, Mueckler M. The mechanism of insulin resistance caused by HIV protease inhibitor therapy. *J Biol Chem*. 2000;275:20251-20254.

46. Wei C, Bajpai R, Sharma H, et al. Development of GLUT4-selective antagonists for multiple myeloma therapy. *Eur J Med Chem*. 2017;139:573-586.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.