Glutamate Decarboxylase 65 Signals through the Androgen Receptor to Promote Castration Resistance in Prostate Cancer

Yi Gao¹, Lu Chen¹, ZunGuo Du²,³, WenChao Gao⁴, ZhengMing Wu², XiuJuan Liu², Hai Huang¹, DanFeng Xu¹, and QingQuan Li²

Abstract

The transition from an androgen-dependent to a castration-resistant state is a critical event in the progression of prostate cancer. In this study, we compared metabolic pathways between isogenic human androgen-dependent and castration-resistant prostate cancer (CRPC) patient-derived xenograft models, and found consistent activation of the γ-aminobutyric acid (GABA) shunt in CRPC. This difference was the result of phosphorylation and activation of glutamate decarboxylase 65 (GAD65), which synthesizes GABA from glutamate by decarboxylation. Mechanistic investigation showed that GABA binds to and retains the androgen receptor (AR) in the nucleus by facilitating AR association with the nuclear zinc finger protein ZNHIT3. GAD65 knockdown decreased the growth of multiple established CRPC xenografts and markedly delayed the time to emergence of castration resistance. These data encourage exploring GAD65 as a therapeutic target for CRPC.

Introduction

Prostate cancer is the most common cancer and the second leading cause of cancer-related mortality in men (1). Androgen receptor (AR) signaling has been shown to promote prostate cancer progression. Accordingly, androgen-deprivation therapy (ADT) is a major component in the treatment of recurrent and metastatic prostate cancer. However, a considerable fraction of patients receiving ADT ultimately develop castration-resistance, mostly due to acquired mutations in the AR signaling pathway (2). These mutations typically occur after metastatic spread (3). As a result, the capability to survive ADT independent of AR pathway mutations has been hypothesized to be an early step in the evolution of some aggressive, AR-dependent castration-resistant prostate cancer (CRPC).

Metabolic reprogramming exemplified by the Warburg effect is one of the hallmarks of cancer cells, and contributes to processes of carcinogenesis and cancer progression. Identification of metabolic vulnerability of human cancers has led to the development of novel treatment strategies (4, 5). For prostate cancer, metabolic change has been an area of active inquiry with potential application in metabolic imaging and biomarker diagnostics as well as for targeted therapies (6, 7). Despite of significant genetic and clinical heterogeneity among prostate cancers, common metabolic reprogramming properties have been recognized. In comparison to normal prostate cells, citrate secretion is decreased and the Krebs cycle is reactivated in prostate cancer cells, with subsequent changes in cellular redox state and metabolic flux through interdigitated pathways (8). Another early and common metabolic alteration in prostate cancers is increased de novo lipogenesis (9, 10). Increasing evidence suggests that metabolic reprogramming could promote tumor cell growth and survival in diverse microenvironmental conditions. For example, cancer cells adapt to hypoxia by utilizing physiologic adaptation pathways that promote a switch from oxidative to glycolytic metabolism (11). Metabolic reprogramming is also critical for cancer cell survival upon distant metastasis in new environment. Specifically, upon metastasis into the liver, colorectal cancer cells undergo metabolic changes that allow using fructose (more abundant in the liver) as a major energy source (12). In this study, we characterized metabolic reprogramming that allows prostate cancer cells to survive upon androgen deprivation.

Materials and Methods

Cell culture

The PC-AD and PC-CR xenografts were mechanically dissociated and cultured in RPMI1640 (Invitrogen) medium supplemented with 10% FBS (for PC-AD cells) or 10% charcoal-stripped FBS (for PC-CR cells). After several serial passages, the serum was

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Statistical analyses
Statistical analysis was carried out with SPSS version 19.0 (SPSS Inc.). Experimental data are expressed as means ± SD and analyzed by 2-tailed unpaired t test. Association between Sirt5 expression and disease recurrence was analyzed by the Kaplan–Meier method, and curves were compared by the log-rank test. P < 0.05 was considered to indicate statistical significance throughout the study.

Results
GABA shunt is increased in CRPC
Biopsy specimens were obtained from 7 patients with locally advanced or metastatic prostate cancer, and were immediately implanted subcutaneously into NOG mice in the presence of Matrigel. After a minimum of 3 generations of serial passaging, we were able to establish transplantable patient-derived xenograft (PDX) lines in 5 of the 7 patients (PC-AD1, PC-AD2, PC-AD3, PC-AD4, and PC-AD7). The transplantable tumor lines retained major histopathologic characteristics of their matched patient tumor (Supplementary Table S1). Host castration resulted in a dramatic decrease in tumor volume in all established lines; however, a vestige of the regressed xenograft remained at the inoculation site. Within 7 months of continuing observation, 3 out of the 5 regressed xenografts relapsed (PC-AD1, PC-AD3, and PC-AD4; Supplementary Fig. S1A), indicating progression to an androgen-independent state. We successfully established the stable subcutaneous xenograft model from the first relapsed xenograft after 5 subcutaneous passages in castrated NOG mice (named PC-CR1, PC-CR3, and PC-CR4, respectively) next we compared the requirement for glucose or glutamine of hormone-dependent (AD) with paired castration-resistant (CR) lines. Similar to that in most proliferating cells, both PC-AD and PC-CR lines proliferated at a high rate when glucose and glutamine were abundant (Supplementary Fig. S1B), with high levels of glucose and glutamine consumption and lactate production (Supplementary Fig. S1C). Also, no apparent difference was observed in fatty acid uptake between the paired PC-AD and PC-CR xenograft cells (Supplementary Fig. S1D). TCA activity, as reflected by oxygen consumption rate (OCR), was similar between PC-AD and PC-CR cells (Supplementary Fig. S1E). Notably, steady-state level of succinyl-CoA, but not other TCA cycle metabolites (αKG, succinate, or malate), was reproducibly diminished in all PC-CR lines tested as compared with their androgen-dependent counterparts (Fig. 1A). In PC-AD cells, virtually all intracellular αKG, succinyl-CoA, and succinate were rapidly labeled after addition of [1-13C]glucose or [U-13C]glutamine. In contrast, a substantial fraction of succinyl-CoA failed to become labeled with [1-13C]glucose or [U-13C]glutamine in PC-CR cells (Fig. 1B). Quantification of metabolite fluxes revealed significantly lower glucose and glutamine fluxes through succinyl-CoA in PC-CR cells (Fig. 1C) despite of similar flux of glucose- or glutamine-derived carbons through αKG in PC-CR versus PC-AD (Supplementary Fig. S1F).

The y-aminobutyric acid (GABA) shunt bypasses the αKG dehydrogenase complex (KGDHC) and succinyl-coenzyme A ligase (SUCL) for the conversion of αKG into succinate, and thus decreases the formation of succinyl-CoA (14). PC-CR cells had substantial glutamate or glucose-dependent GABA and succinic semialdehyde (SSA) production (Fig. 1D). Also, these cells preserved larger intracellular pools of GABA and SSA than their

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parental hormone-naive cells (Fig. 1E). Glutamic acid decarboxylase (GAD), GABA-transaminase (GABA-T), and succinic semi-aldehyde dehydrogenase (SSADH) are key enzymes in the GABA shunt. To assess the role of the GABA shunt in CRPC growth, we impaired the enzyme activity individually using RNA interference (Supplementary Fig. S1G). The growth of CRPC was markedly decreased by silencing the GAD65 isoform (Supplementary Fig. S1H–S1I), but not by knockdown of GAD67, GABA-T, or SSADH (Supplementary Fig. S1J). Consistent with this finding, 13C-tracing experiments showed increased glutamate (and total glutamate) and decreased GABA (and total GABA) upon GAD65 knockdown in PC-CR4 cells (Fig. 1F), suggesting an increased GABA shunt activity in CRPC.

GAD65 is required for cell proliferation in CRPC

Silencing GAD65 produced more profound inhibition on the growth in PC-CR4 cells than in matched PC-AD4 cells (Fig. 2A; Supplementary Figs. S1H–S1I), but not by knockdown of GAD67, GABA-T, or SSADH (Supplementary Fig. S1J). Consistent with this finding, 13C-tracing experiments showed increased glutamate (and total glutamate) and decreased GABA (and total GABA) upon GAD65 knockdown in PC-CR4 cells (Fig. 1F), suggesting an increased GABA shunt activity in CRPC.

Phosphorylation at S6 activates GAD65

To clarify whether activation of the GABA shunt in CRPC is due to increased GAD65 enzyme activity, we expressed flag-tagged GAD65 in PC-AD and PC-CR cells (Supplementary Fig. S2A). GAD65 activity after immunoprecipitation was significantly higher in PC-CR cells than in PC-AD cells (Fig. 3A). Phosphorylation status plays an important role in regulation of GAD activity (16). Four putative phosphorylation sites (serine 3, serine

Figure 1. CRPC cells exhibit increased GABA shunt activity. A, Intracellular metabolite levels of the paired PC-AD and PC-CR cells. B, Fraction of each metabolite labeled by 13C derived from [U-13C]glucose (left) or [U-13C]glutamine (right) over time (0–12 hours) in the paired PC-AD3 and PC-CR3 cells. C, [U-13C]glucose (left) and [U-13C]glutamine (right) flux through the succinyl-CoA pool in the paired PC-AD and PC-CR cells. D, The paired PC-AD and PC-CR cells were incubated with medium containing [U-13C]glucose (left) or [U-13C]glutamine (right) for 4 hours, and the fraction of GABA and SSA containing glucose- or glutamine-derived carbons was determined. E, Intracellular GABA (left) and SSA (right) levels of the paired PC-AD and PC-CR cells. F, Relative metabolite abundance in PC-CR4 cells grown in [U-13C]glucose (left) or [U-13C]glutamine (right) upon GAD65 knockdown. Data are presented as the total metabolite pool and the 13C-labeled metabolite pool. All experiments were independently repeated three times in triplicate. Values shown are mean ± SD. A two-tailed unpaired t test was used to compare experimental groups. **, P < 0.01; a.u., arbitrary units.
Figure 2. GAD65 is required for androgen-independent proliferation of CRPC. 

A and B, Colony formation of the paired androgen-dependent and castration-resistant PC cells (A, PC-AD4 vs. PC-CR4; B, LNCaP vs. abl) with GAD65 knockdown. C, Left two panels show tumor growth and size of xenograft mice injected with parental or GAD65 knockdown CWR22Rv1 or PC-AD4 cells. Middle panels show the dissected tumors in representative mice. Right two panels show representative images and quantification of IHC staining of Ki-67 of tumors. D, Effect of GAD65 knockdown on cell proliferation rates of PC-CR4 or abl cells expressing GAD65 WT or K396R. Right, the presence of ectopically expressed and endogenous GAD65 was verified by Western blotting. E, Left two panels, tumor growth and size of xenograft mice injected with parental or GAD65 knockdown CWR22Rv1 cells with rescue expression of Flag-GAD65 WT or enzyme-deficient K396R mutant. Right panel shows the dissected tumors in representative mice. All experiments were independently repeated three times in triplicate. Values shown are mean ± SD. A two-tailed unpaired t test was used to compare experimental groups. **, P < 0.05. Bars, 50 μm.
6, threonine 95, and serine 417) have been identified in GAD65 by mass spectrometry (17). We mutated each of the 4 putative phosphorylation sites individually to alanine (A), and examined their phosphorylation. GAD65 phosphorylation was decreased by mutation at T95, and more so with S6 (by approximately 90%), but not at S3 and S417 (Fig. 3B), indicating that S6 is the major phosphorylation site in GAD65. To test the effect of S6 phosphorylation, we also generated the GAD65S6E mutant, in which S6 was substituted with glutamate to mimic constitutive phosphorylation, and overexpressed flag-tagged mutant or WT

Figure 3.
GAD65 enzymatic activity in CRPC is enhanced by phosphorylation at residue serine 6. A, Flag-tagged GAD65 was ectopically expressed in the paired PC-AD and PC-CR cells, after which, enzyme activity of exogenous GAD65 was determined. B, Analysis of phosphorylation of individual GAD65 mutants. The indicated plasmids were transfected into PC-CR3 cells, and proteins were immunoprecipitated for phosphorylation analysis. C, The indicated Flag-tagged GAD65 proteins were overexpressed in HEK293T cells. WT and mutant GAD65 proteins were purified by Flag beads and eluted with Flag peptide, followed by determination of their enzyme activity. D, The indicated Flag-tagged GAD65 proteins were overexpressed in HEK293T cells, with or without combined OA and Vd treatment, for 48 hours. WT and mutant GAD65 proteins were purified by Flag beads and eluted with Flag peptide, followed by determination of their enzyme activity (top) and their serine phosphorylation (bottom). E, GAD65S6 phosphorylation in the paired androgen-dependent versus castration-resistant PC cells. F, FACS analysis of phosphorylated GAD65S6 in serial passages (P) of PC4 from AD (PC-AD4) to CR (PC-CR4). G, IHC analysis of GAD65S6 phosphorylation in multiple prostate cancer metastases. Tumor tissues from LNCaP, PC-AD3, and PC-AD4 xenografts were also included for comparison. H, Phosphorylated GAD65S6 IHC of high-expression prostate cancer metastases (M), showing clear staining in M13, M12, and M10 and negligible staining in PC-AD3. I, IHC evaluation of GAD65S6 phosphorylation in 136 patients with prostate cancer with localized, hormonally treated, and castration-resistant prostate cancer. J, BPH was negative, whereas untreated tumors showed rare immunoreactivity for phosphorylated GAD65S6. GAD65S6 phosphorylation was significantly upregulated in NHT samples and also in CRPC. The data in C and E show mean percentage of cells staining positive for phosphorylated GAD65S6 among all patients. All experiments were independently repeated three times in triplicate. Values shown are mean ± SD. A two-tailed unpaired t test was used to compare experimental groups. **, P < 0.05. Bars, 50 μm. BPH, benign prostate hyperplasia; NHT, neoadjuvant hormone therapy.
GAD65 in HEK293T cells (Supplementary Fig. S2B). GAD65 enzyme activity was significantly increased by substitution with glutamate (GAD65<sup>E64</sup>) but not with alanine (GAD65<sup>A64</sup>; Fig. 3C). Consistent with an stimulatory effect of phosphorylation on GAD65 activity, inhibition of phosphatase by okadaic acid (OA) and vanadate (Vd) treatment significantly increased GAD65 activity in cells overexpressing WT GAD65 (Fig. 3D), but not in cells expressing either GAD65<sup>S6A</sup> or GAD65<sup>S6E</sup> mutant (Fig. 3D). Taken together, these data suggest that phosphorylation at S6 activates GAD65.

**GAD65<sup>S6</sup> phosphorylation is elevated in CRPC**

Next, we generated an antibody specifically recognizing the S6-phosphorylated GAD65. The specificity of the anti-phospho-GAD65<sup>S6</sup> antibody was verified by recognition of the S6-phosphorylated peptide but not the unphosphorylated control peptide (Supplementary Fig. S2C). Phosphorylation of GAD65 could readily be detected by this antibody; the signal was diminished by GAD65 knockdown (Supplementary Fig. S2D) and completely blocked by preincubation with the antigen peptide (Supplementary Fig. S2E), confirming the specificity of the anti-phospho-GAD65<sup>S6</sup> antibody. GAD65<sup>S6</sup> phosphorylation was negligible or absent in hormone-sensitive PC-AD and LNCaP cells but present in the paired castration-resistant PC-CP and ab1b cells (Fig. 3E). Consistent with the key role of PKCe in GAD65 phosphorylation (16), we found higher levels of the active PKCe in castration-resistant cells (Supplementary Fig. S2F). The activity of PK3K, an upstream activator of PKCe (18) and an essential regulator of CRPC (19), was also increased in hormone-resistant cells (Supplementary Fig. S2G), suggesting major contribution of the PI3K/PKC<sub>e</sub> signaling to GAD65<sup>S6</sup> phosphorylation in CRPC.

Furthermore, we evaluated the kinetics of GAD65<sup>S6</sup> phosphorylation in serial passages of PC-CP4 tumors in castrated mice. Percentage of the cells with GAD65<sup>S6</sup> phosphorylation increased with passing of the PC-CP4 line, from ~37% after the first passage to ~80% by passage 5 (Fig. 3F). Such an increase was accompanied with slight increase in AR expression, moderate increases in PSA/TMPRSS2 expression (Supplementary Fig. S2H), and decreased tumor doubling time (9 days in P5 vs. 15 days in P1), suggesting a growth advantage of phosphorylated over unphosphorylated GAD65<sup>S6</sup> cells in castrated mice.

To determine whether GAD65<sup>S6</sup> phosphorylation can be detected in clinical CRPC, the anti-phospho-GAD65<sup>S6</sup> antibody was characterized by its suitability for IHC. We found that this antibody could detect strong signals that were specifically blocked by the phospho-S6 antigen peptide in paraffin-embedded normal colon tissues (Supplementary Fig. S2I). In soft-tissue and bone metastases obtained from 16 men who died from CRPC, majority (12/16) exhibited high level of GAD65<sup>S6</sup> phosphorylation (>30% phosphorylated S6-positive cells; Fig. 3G and H). We also stained tissue microarrays containing samples from individuals with benign prostatic hyperplasia (BPH), hormone-naive prostate cancer, prostate cancer treated with neoadjuvant hormone ablation and CRPC. We detected high GAD65<sup>S6</sup> phosphorylation in 1.5%, 12.3%, 56.7%, and 83.1% of these samples, respectively. The mean percentage of cells positive for GAD65<sup>S6</sup> phosphorylation among all samples increased from 2.7% in benign prostatic hyperplasia to 5.3% in hormone-naive disease, 40.5% in men treated with neoadjuvant ADT and 76.2% in CRPC (<i>P</i> < 0.01; Fig. 3I and J). These data demonstrate that GAD65<sup>S6</sup> phosphorylation is rare in untreated androgen-dependent prostate cancer, increases with androgen deprivation, and is highest in CRPC.

**GAD65 maintains AR nuclear accumulation in CRPC via GABA**

To decipher the role of GAD65 in bioenergetics and anabolic biosynthesis of CRPC, we performed a set of metabolic assays using CRPC cells with GAD65 knockdown. In comparison with the empty vector control, GAD65 knockdown did not affect intracellular ATP level, glucose uptake, glycolytic rate, lactate production, oxygen consumption rate, glutamine uptake, and glutaminolysis rate (Supplementary Fig. S3A–S3G). GAD65 knockdown also did not affect oxidative pentose phosphate pathway (PPP) flux (Supplementary Fig. S3H) and the overall lipid and RNA biosynthesis (Supplementary Fig. S3I–S3J).

Because GAD65 knockdown reduced cell proliferation and tumor growth, we hypothesized that other mechanisms beyond metabolic defects might be responsible for the proliferative disadvantage in cells conferred by GAD65 knockdown. Further analyses showed that knockdown of GAD65 selectively inhibited transcriptional activity of AR and reduced the mRNA levels of AR target genes (PSA and TMPRSS2) in CRPC cells (Fig. 4A and B). Such effects were reversed by ectopic expression of shRNA-resistant GAD65<sup>WT</sup> or the GAD65<sup>S6E</sup> mutant, but not the GAD65<sup>S6A</sup> mutant (Fig. 4C and D). GAD65 knockdown did not significantly change the levels of AR (Supplementary Fig. S4A), but substantially reduced the levels of nuclear AR in ab1b and PC-CP3 cells (Fig. 4E), which was confirmed by immunofluorescence (Fig. 4F). No similar phenomena were detected in the matched androgen-sensitive cells (Fig. 4E and F).

We further explored the molecular mechanism by which GAD65 regulates AR nuclear localization. GAD65 did not form a protein complex with AR. We speculated that GAD65 may indirectly regulate AR nuclear localization by controlling intracellular levels of GABA shunt intermediates. Addition of GABA up to 250 μmol/L in the culture media restored intracellular GABA levels in GAD65 knockdown cells (Supplementary Fig. S4B). Interestingly, the decrease in AR nuclear localization caused by GAD65 knockdown in CRPC cells was also reversed by GABA treatment, but not by succinate or SSA treatment (Fig. 4G and H). We also demonstrated the ability of GABA to restore AR transcriptional activity in GAD65 knockdown cells by quantifying ARE reporter activity (Fig. 4I), as well as the expression of AR target genes (Fig. 4J). Consequently, addition of GABA, but not succinate or SSA, significantly reversed the reduced cell proliferation due to GAD65 knockdown (Fig. 4K). Based on these results, we hypothesized that GAD65-mediated conversion of glutamate into GABA functions in a pathway that is used to promote AR nuclear accumulation in CRPC.

**GABA enhances AR binding with ZNHIT3**

To distinguish whether GAD65 facilitates AR nuclear accumulation through either nuclear import or nuclear retention, we analyzed the nuclear localization of AR in GAD65 knockdown ab1b cells after treatment with leptomycin B (LMB), an inhibitor of protein nuclear export by CRM1/exportin 1 (20). As shown in Fig. 5A, AR was primarily localized in the nucleus in the presence of LMB, strongly suggesting that the cytoplasmic localization of AR after GAD65 knockdown is not due to defects in nuclear import but due to a combination of deficient nuclear retention and effective nuclear export. Coimmunoprecipitation

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of AR, followed by a mass spectrometry analysis suggested binding of AR to zinc finger HIT domain-containing protein 3 (ZNHIT3) in PC-CR4 cells (Supplementary Table S2), as well as in PC-CR3 and abl cells (Fig. 5B). Such an interaction was significantly disrupted by treating cells with a GAD65 shRNA (Fig. 5C).

Next, we performed cell-free pull-down assays using purified recombinant AR incubated with purified ZNHIT3, in the presence of increasing amounts of GABA. Although ZNHIT3 displayed negligible interaction with AR, GABA (50 – 250 μmol/L) increased AR-bound ZNHIT3 in a concentration-dependent manner (Fig. 5D). More importantly, we found that purified AR pretreated with increasing concentrations of GABA displayed increasing binding ability to ZNHIT3, whereas GABA pretreated ZNHIT3 had no effect (Fig. 5E), suggesting that GABA directly affects the AR–ZNHIT3 complex. In line with this, a radiometric metabolite–protein interaction analysis using 14C-labeled metabolites revealed that labeled GABA but not succinate, or SSA, was retained on purified recombinant AR (Supplementary Fig. S4C). To determine the selectivity of GABA binding to AR, we generated an AR mutant with substitutions at L704, Q711, M745, R752, and F764. These residues were predicted to be critical for GABA binding to AR by molecular docking (Fig. 5F). The results demonstrated that the L704A/Q711A/M745A/R752A/F764A (5A) mutant was resistant to GABA binding (Supplementary Fig. S4D); the association between the 5A mutant and ZNHIT3 was no longer induced by GABA (Fig. 5G).

To clarify whether ZNHIT3 facilitates AR nuclear retention, we performed fluorescence recovery after photobleaching (FRAP) GFP-AR in abl cells expressing scramble shRNA or ZNHIT3 shRNA (Fig. 5H). After photobleaching of GFP-AR in the nucleus, no significant differences were observed in the recovery of nuclear GFP-AR among control and ZNHIT3 knockdown cells. In contrast, after photobleaching of cytoplasmic AR, reduction of nuclear GFP-AR was significantly less in control cells compared with ZNHIT3 shRNA-expressing cells, thus indicating an important role of ZNHIT3 in AR nuclear retention.

ZNHIT3 binding in the hinge region facilitates AR nuclear retention

In the next experiment, GST-fusion constructs of different AR truncation mutants lacking the NTD, hinge, or DBD domains...
**GABA binding to AR facilitates its association with ZNHIT3 and promotes AR nuclear retention.** 
A, Subcellular localization of AR in abl cells with stable knockdown of GAD65 was determined in the presence or absence of LMB (25 μM; 4 hours). B, Coimmunoprecipitation of AR and ZNHIT3 in PC-CR3 and abl cells.

C, Coimmunoprecipitation of AR and ZNHIT3 in PC-CR3 and abl cells with stable knockdown of GAD65. D, Effect of GABA on AR-ZNHIT3 binding in cell-free, in vitro assays using purified recombinant AR and ZNHIT3. E, Effect of pretreatment of AR (left) or ZNHIT3 (right) with increasing concentrations of GABA on AR-ZNHIT3 binding in cell-free, in vitro assays. F, Schematic representation of molecular docking study of GABA based on the crystal structure of AR. GABA is docked in a pocket surrounded by residues, L704, Q711, M745, R752, and F764. G, Effect of GABA on the interaction of Flag-AR WT and L704A/Q711A/M745A/R752A/F764A (5A) mutant with ZNHIT3 in cell-free, in vitro assays. H, Abi cells with ZNHIT3 knockdown were transfected with GFP-AR and were subjected to FRAP analysis. The intensity of nuclear fluorescence was monitored for ~750 seconds and plotted against time. All experiments were independently repeated three times in triplicate. Values shown are mean ± SD. A two-tailed unpaired t test was used to compare experimental groups. ***, P < 0.001. Bars, 10 μm.

*Figure 5.*

**Inhibition of the GABA shunt by GAD65 knockdown impedes the development and growth of CRPC**

A retroviral vector, in which GAD65 shRNA was expressed under the control of the doxycycline (Dox)-inducible activator rtTA (LV-tet-on-shGAD65), was constructed and used to infect PC-CR3, abl, and CWR22Rv1 cells. Dox treatment induced knockdown of endogenous GAD65 in these cells (Supplementary Fig. S5A). After palpable tumor formation, castrated mice bearing infected PC-CR3, abl, or CWR22Rv1 xenografts were treated twice weekly with PBS or Dox for 5 weeks. Dox administration significantly slowed the growth of all 3 tumor models, with the most prominent effect in CWR22Rv1 xenograft (Fig. 7A). Dox-treated tumors were pale, nonadherent to underlying muscle, and noninvasive histologically, whereas control tumors grossly invaded underlying muscle (Fig. 7B). Metastasis to lymph nodes was less frequent in Dox-treated mice (1 of 5 mice) versus the control (5 of 5; Fig. 7C). Prolonged administration of Dox led to long-term growth suppression (Supplementary Fig. S5B) and increased the duration of survival by >100% in mice bearing abl tumors. Dox-treated tumors had large areas of cell loss, reduced proliferation (Ki-67 staining), and fewer blood vessels (CD31 staining) compared with the PBS control (Fig. 7D).

In the last set of experiments, androgen-sensitive PC-AD3 cells were transfected with the Dox-inducible vector for GAD65 shRNA expression, and then implanted subcutaneously into castrated mice. Treatment of the mice with Dox significantly delayed time to castration resistance (Fig. 7E). At week 6 after implantation, progression to castration-resistance (defined as serum PSA at >8 ng/mL) was apparent in 8 of 10 mice and none of the 10 Dox-treated mice (Fig. 7F). Of note, AR immunostaining in xenografts treated with Dox was predominantly localized to the cytoplasm of tumor cells, whereas the PBS control tended to have higher level of AR in the nucleus (Supplementary Fig. S5C). These
Figure 6.
GABA increases the accessibility of AR hinge region for ZNHIT3. A, Plasmid construction of full-length AR and AR deletion mutant fusion proteins. AR contains four domains: NTD, DBD, H, and LDB. The domain deletion mutants Δhinge (ΔH), ΔDBD, and ΔNTD were created. B, Purified recombinant His-ZNHIT3 protein (1 μg) was incubated with glutathione-Sepharose beads coupled with WT or domain deletion mutant GST AR proteins (10 μg) in the presence of GABA (250 μmol/L). Subsequently, proteins bound to the beads were analyzed by PAGE-Western blotting analysis using an anti-His antibody (top). Total input WT GST/C1 AR and mutant GST/C1 AR detected by anti-GST antibodies are shown (bottom). C, Schematic presentation of AR deletion mutants used for identification of amino acid sequence required for ZNHIT3 association. D, Purified WT or deletion mutant GST AR proteins (10 μg) were immobilized on glutathione-Sepharose beads. The beads were then incubated with purified recombinant His-ZNHIT3 protein (1 μg) in the presence of 250 μmol/L GABA. Proteins bound to the beads were detected by Western blotting with an anti-His antibody (top). Total input WT GST/C1 AR and mutant GST/C1 AR detected by anti-GST antibodies are shown (bottom). E, HEK293 cells transfected with GFP-AR or GFP-ARΔ635-652 were treated with 10 nmol/L DHT for 4 hours, washed to remove DHT, and then treated with GABA (250 μmol/L) for the indicated times. Nuclear fractions from the cells and whole cell lysates were subjected to Western blotting analysis. F, Specificity of antibody against amino acid 633-653 of AR was determined by probing the indicated Flag-tagged proteins (top). Total input of the indicated Flag-tagged AR proteins was detected by an anti-Flag antibody (bottom). G, The GST-hinge-LBD fusion protein was adsorbed to microtiter wells and incubated with a dilution series of anti-AR (633-653) antibody in the presence or absence of 250 μmol/L GABA. The binding of the anti-AR (633-653) antibody was measured by ELISA. All experiments were independently repeated three times in triplicate. Values shown are mean ± SD. A two-tailed unpaired t test was used to compare experimental groups.
Knockdown of GAD65 inhibits growth of established tumor and blocks progression to castration resistance in vivo. PC-CR3, abl, and CWR22Rv1 cells infected with LV-tet-on-shGAD65 were transplanted subcutaneously into castrated NOG mice. A, In vivo castration-resistant growth inhibition of PC-CR3, abl, and CWR22Rv1 tumors upon treatment with PBS or Dox (8 mg/kg, twice weekly), beginning when subcutaneous tumors were palpable in castrated mice. Right, dissected tumors in representative mice. B and C, Histologic analyses of PC-CR3 tumors treated with PBS or Dox, showing decrease in tumor-muscle invasion (B) and metastases to axillary lymph nodes (C). D, Quantification of changes between PBS- and Dox-treated PC-CR3 tumors in the following parameters: hypocellular regions by hematoxylin and eosin (H&E) staining (top), CD31 staining (middle), and Ki-67 staining (bottom). Quantification was determined by counting 5 different fields per tumor, followed by averaging the values for the three tumors. E and F, PC-AD3 cells infected with LV-tet-on-shGAD65 were transplanted subcutaneously into castrated SCID mice. PC-AD3 tumor emergence in castrated mice was compared between PBS and Dox (8 mg/kg, twice weekly) treatment group (E). Intact, mice bearing PC-AD3 tumors without castration. Serum PSA was measured to determine relapse of PC-AD3 tumors 6 weeks after implantation (F). A PSA value of 8 ng/mL, marked by the dotted line, was considered indicative of tumor relapse. Values shown are mean ± SD. A two-tailed unpaired t-test was used to compare experimental groups. **, P < 0.05. Bars, 50 μm.
findings suggest that GAD65 inhibition maintain the sensitivity of AR in androgen-sensitive tumors in castrated hosts and inhibit the development of castration resistance.

Discussion

In efforts to develop more effective therapies, it is critical to improve the understanding of molecular mechanisms underlying CRPC development. In this study, we approached this question by identifying "metabolic vulnerabilities" specifically required for CRPC development. Distinct metabolic states are required for tumor growth under different environments (11–12). We found increased GABA shunt activity due to GAD65 activation in CRPC. The GAD65 activation was due to S6 phosphorylation, and mediated by the PI3K–PKCe signaling axis. This finding is consistent with a previous study showing a critical role of the PI3K–PKCe signaling axis in the development and maintenance of CRPC (19). Specifically, we detected GAD65 S6 phosphorylation in multiple CRPC cell lines and in a majority of metastatic and castration-resistant prostate cancer tissues. Interestingly, GAD65 S6 phosphorylation increased with passing of castration-resistant tumors in the xenograft models, suggesting that phospho-S6-positive cells have a growth advantage over phospho-S6-negative cells. We also provided convincing evidence showing that activated GAD65 promotes CRPC proliferation via facilitating AR nuclear accumulation. It is well recognized that AR is activated by androgen binding and translocates to nucleus to activate transcription of AR-target genes. However, in CRPC cells, AR remains in the nucleus even in the absence of androgens, transactivating androgen-responsive genes and leading to uncontrolled growth (22). Blockade of AR nuclear localization is a putative treatment strategy for CRPC. The results from this study showed that GAD65 selectively promotes AR nuclear retention in CRPC by controlling intracellular levels of its product GABA. GABA is the major inhibitory neurotransmitter in the mammalian brain but recent studies have suggested that GABA is also implicated in many other biological functions (23, 24), as well as in the development of a variety of cancers (25). Uptake of GABA and its subsequent catabolism via the GABA shunt increase NADH levels in tumor microenvironment, which in turn confer a proliferative advantage to tumor cells (26). Also, GABA acting via its receptors ensures a beneficial and important function on tumor cell proliferation and a "GABAergic Mr. Hyde" has been described in different types of tumors where components of the GABAergic signaling are overexpressed (25). Our findings link the GABA shunt to AR nuclear accumulation in CRPC and clarify a signaling function of GABA that is independent of its role in cell metabolism or GABA receptor-mediated effects. GABA serves as a protopanaxinhibitor by binding to AR in its ligand binding domain. GABA induces a conformational switch in AR that increases hinge accessibility to ZNHT3. ZNHT3 is a nuclear zinc finger protein previously implicated in transcriptional regulation and in small nucleolar ribonucleoprotein particle assembly and thus possibly to preribosomal RNA processing (27, 28). ZNHT3 inhibits nuclear export and promotes nuclear retention of AR. These results suggest that metabolic intermediates may function as signaling molecules to allow crosstalk between metabolic and cell signaling pathways. For example, we previously reported that TCA cycle intermediate succinate binds to and activates the ROS scavenging enzyme TrxR2 to maintain redox homeostasis (29), whereas others have found that acetocetate specifically enhances binding of BRAF V600E to MEK1 in V600E-positive cancer cells to promote activation of MEK–ERK signaling (30). On the other hand, increasing evidence suggests that posttranslational modifications, including tyrosine phosphorylation (31, 32) and lysine acylation (33, 34) of metabolic enzymes, are common and important to link cell signaling pathways to metabolic pathways in cancer cells. These findings together represent a realm of crosstalk with "back and forth" signal flows between metabolic and cell signaling networks that "acutely" regulate cell metabolism and proliferation, which, unfortunately, are "hijacked" by cancer cells.

Finally, the findings from this study encourage exploring the GABA shunt, GAD65 in particular, as a molecular target in the treatment of CRPC. Future studies, however, must address the specificity issue due to the critical functional role of GABA in the central nervous system (14).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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