Survival of Cancer Stem-Like Cells Under Metabolic Stress via CaMK2α-mediated Upregulation of Sarco/Endoplasmic Reticulum Calcium ATPase Expression

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Abstract

Purpose: Cancer cells grow in an unfavorable metabolic milieu in the tumor microenvironment and are constantly exposed to metabolic stress such as chronic nutrient depletion. Cancer stem-like cells (CSC) are intrinsically resistant to metabolic stress, thereby surviving nutrient insufficiency and driving more malignant tumor progression. In this study, we aimed to demonstrate the potential mechanisms by which CSCs avoid Ca2⁺-dependent apoptosis during glucose deprivation.

Experimental Design: We investigated cell viability and apoptosis under glucose deprivation, performed genome-wide transcriptional profiling of paired CSCs and parental cells, studied the effect of calcium/calmodulin-dependent protein kinase 2 alpha (CaMK2α) gene knockdown, and investigated the role of nuclear factor kappa B (NFκB)–mediated and glucose deprivation–induced apoptosis. We also observed the effect of combined treatment with 2-deoxy-D-glucose and thapsigargin, a specific inhibitor of SERCA, a metabolic inhibitor that mimics glucose deprivation conditions in mouse xenograft models, and thapsigargin, a specific inhibitor of sarco/endoplasmic reticulum Ca²⁺-ATPase (SERCA).

Results: We demonstrated the coordinated upregulation of SERCA in CSCs. SERCA, in turn, is transcriptionally regulated by CaMK2α via NFκB activation. Combined treatment with 2-deoxy-D-glucose and thapsigargin, a specific inhibitor of SERCA, significantly reduced tumor growth compared with that in untreated control animals or those treated with the metabolic inhibitor alone.

Conclusions: The current study provides compelling evidence that CaMK2α acts as a key antiapoptosis regulator in metabolic stress-resistant CSCs by activating NFκB. The latter induces expression of SERCA, allowing survival in glucose-deprived conditions. Importantly, our combination therapeutic strategy provides a novel approach for the clinical application of CSC treatment.

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Introduction

Metabolic stress, elicited by deprivation of key nutrients such as glucose, is a critical condition that significantly affects cell viability if prolonged. However, cancer stem–like cells (CSC) have developed molecular strategies to cope with a lack of nutrients, including metabolic and transcriptional reprogramming, to protect cellular bioenergetics (1–3). The acquisition of survival mechanisms in extreme nutrient-deficient conditions is an important characteristic of CSCs that distinguishes them from non-stem-like cancer cells (4, 5). Previously, we demonstrated that chronic metabolic stress induced the emergence of CSCs through programmed Wnt signaling (5). Notably, these CSCs exhibited resistance to glucose deprivation-induced apoptosis by altering NAD⁺ metabolism, which is mechanistically related to Ca²⁺ signaling (6).

Glucose deprivation causes endoplasmic reticulum (ER) stress by interfering with N-linked protein glycosylation. ER stress is an important adaptive cellular process that is induced by a range of stimuli and pathologic conditions, and triggers evolutionarily conserved molecular responses (7). The importance of Ca²⁺ signaling in cell death and survival has been studied in response to ER stress (8, 9). ER stress can cause Ca²⁺ release from ER stores into the cytoplasm, and the subsequent accumulation of Ca²⁺ in the mitochondrial matrix can transiently increase oxidative phosphorylation (10). However, when Ca²⁺ concentrations are elevated and sustained beyond physiologic levels, mitochondrial apoptotic signals are triggered, amplified, and executed (11). Under these conditions, unresolved ER stress may be detrimental to cell survival (12).
translated by the Ca2+-signaling homeostasis by efficiently resequestering cytoplasmic Ca2+ into the ER, once the desired physiologic response is established (14, 15). Cytosolic-free Ca2+ is involved in various processes and plays a crucial role in controlling cellular life-or-death decisions, including selection of the mode of cell death, that is, apoptosis, necrosis, or autophagy (16, 17). Ca2+-/calmodulin-dependent protein kinase (CaMK) is a multifunctional serine/threonine kinase whose activity is regulated by the Ca2+-/calmodulin complex. It is involved in many signaling cascades, including cell survival and death (18–20). CaMK2 alpha (CaMK2α) plays an important role in Ca2+-dependent apoptosis (20). As cancer cells reprogram their metabolic processes, there is a significant breach in Ca2+-dependent apoptosis (21, 22), which results in a universal increase in survival capacity. This particular characteristic may be typical of a subpopulation of tumor cells selected in unfavorable metabolic microenvironments, which links metabolic subclonal selection and malignant tumor progression. Here, we show that metabolic stress-resistant in vitro–selected CSCs (S-231 cells selected from MDA-MB-231 cells and S-MCF-7 cells selected from MCF-7 cells) avoid Ca2+-induced apoptosis during glucose deprivation. In these cells, the CaMK2αr pathway is activated and transcriptionally regulates SERCA expression via nuclear factor kappa B (NFκB) activation. This allows cells to circumvent cytoplasmic Ca2+ overload-induced apoptosis following prolonged glucose deprivation. Furthermore, we demonstrate that a combination of agents, which cause cellular bioenergetic stress and inhibition of SERCA, suppresses in vivo cancer growth, suggesting a novel clinical approach for the treatment of CSCs.

Materials and Methods

Cell culture

Human breast cancer cell lines MDA-MB-231 and MCF-7 were obtained in May 2007 from the ATCC and grown in RPMI1640 medium with 5% FBS. The identities of all cell lines were validated by short tandem repeat (STR) DNA fingerprinting using the AmpFISTR Identifier kit, according to the manufacturer’s instructions (Applied Biosystems; 4322288) at the Characterized Cell Line Core Facility. The STR profiles were compared with known ATCC fingerprints (ATCC.org) and to the Cell Line Integrated Molecular Authentication (CLIMA) database version 0.1.200808 (http://bioinformatics.itg.iti.gr/); Nucleic Acids Research 37: D925-D932 PMCID: PMC2686526). Mycoplasma contamination was invariably checked with the Lookout Mycoplasma PCR Detection Kit (Sigma-Aldrich; MP0035).

Cell viability assay

Cell viability was measured using the MIT (Roche; 11465007001) and crystal violet assay. Cells were seeded in 96-well plates at 6 × 10^4 cells/well and incubated overnight to 80% confluence. Cells were exposed to glucose-deprived conditions or CaMK2αr inhibitor treatment; incubated for 0, 12, 24, 36, 48, 60, and 72 hours; and cell viability was measured using the MIT reagent at 550 nm, according to the manufacturer’s protocol. Briefly, MIT reagent (10 μL) was added to every well (final concentration, 0.5 mg/mL) and the plate was incubated at 37°C. After 4 hours, 100 μL of solubilization solution (10% SDS) was added to each well and the plate was incubated for 24 hours. After the solubilization, the formazan dye is quantitated using a scanning multiwell spectrophotometer (ELISA reader).

Microarray experiments and data analysis

Gene expression data from the cancer cell lines were generated by hybridizing labeled RNAs to Human-6 v2 Expression BeadChips (Illumina). Total RNA was isolated from cells harvested after glucose deprivation for the indicated time using the mirVana miRNA Isolation Kit (Ambion Inc.; AM1560). According to the manufacturer’s protocol. Biotin-labeled cRNA was prepared using the Illumina Total Prep RNA Amplification Kit (Ambion Inc.). Total RNA (500 ng) was used for the synthesis of cDNA followed by amplification and biotin labeling. Biotinylated cRNA (1.5 μg) per sample was hybridized to an Illumina Human-6 BeadChip v2 microarray and the signal was developed using Amersham fluorolink streptavidin-Cy3 (GE Healthcare Bio-Sciences). All statistical analysis was performed using R 2.3.0 and BRB ArrayTools Version 3.5 (https://bhr.nci.nih.gov/BRB-ArrayTools/) with quantile normalization. Further protocol and data analysis details are described in our previous article (5).

Intracellular calcium concentration measurements

The intracellular-free Ca2+ concentration was titrated using calcium calibration buffer KIT#1 (Life Technologies; C3008MP), according to the manufacturer’s protocol. Fluorescence in the presence of different Ca2+ and ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) standard solutions was measured using a fluorophore with the cell suspension. Measurements were performed by mixing 3 μL of purified GCaMP (~100 μmol/L) with 100 μL of different ratios of calcium buffer and 39 μmol/L free-calcium buffer in 96-well transparent plates (Greiner BIO-one) and measuring the fluorescence at 485 nm excitation and 510 nm emission, with 5 nm slits and gain = 90 V.

Total RNA extraction and quantitative real-time reverse transcription RT-PCR

Total RNA was prepared from tumor cells by extraction with the RNeasy Mini Kit (Qiagen; 74106) and the One-Step RT-PCR Kit.
(Qiagen; 204243) according to the manufacturer's protocols. All data were normalized to the expression level of α-tubulin. Primers for SERCA1, SERCA2, and SERCA3 are listed in Supplementary Table S1.

Small-interfering RNA transfection for CaMK2α knockdown
CSCs (S-231 and S-MCF-7) were transfected with CaMK2α siRNA or control scrambled siRNA. The sequence of the CaMK2α siRNA was designed using si-Designed software (Bioneer) and the siRNA duplex was purchased from Bioneer. The sense and anti-sense sequences of the siRNA were as follows: 5'-UGAUCGAAGCCAUAAAGCAA(dTdT)-3' (forward) and 5'-UUGCUUAUGGCUUCGAUCA(dTdT)-3' (reverse).

Evaluation of cell death using TUNEL assay
For the measurement of apoptotic death, cells were fixed with 4% paraformaldehyde for 48 hours and stained using a TUNEL Kit (Promega; G3250). Apoptotic cells (fluorescent green) and total cells were counted under a fluorescence microscope, and the data were recorded. Images were collected using a confocal microscope (LSM Meta 700, Carl Zeiss) and were analyzed using the Zeiss LSM Image Browser software program, version 4.2.0121.

Subcellular fractionation and immunoblot analysis
Antibodies against caspase-3, caspase-7, caspase-9, Bcl-2, Bcl-xL, pNFκB, NFκB, Histone H2B, β-actin (all Santa Cruz Biotechnology), pCaMK2α, CaMK2α, pIP3R, IP3R, pIkKα, IkKα, and SERCA2 (all Abcam) were used. Nuclear fractions were prepared using the NE-PER Nuclear and Cytoplasmic Extraction reagents (Thermo Scientific; 78833) in accordance with the manufacturer's instructions. Protein bands were quantified using ImageJ software (NIH, Bethesda, MD).

Flow cytometry for cell-cycle analysis
Cells were treated with glucose-deprived RPMI1640 medium with 10% FBS for 40 hours, harvested by trypsinization, and fixed...
with 70% ethanol. Cells were stained for total DNA using a solution containing 40 μg/mL propidium iodide (PI) and 100 μg/μL RNase I in phosphate-buffered saline (PBS) for 30 minutes at 37°C. Cell-cycle distribution was then analyzed using a FACSCalibur Flow Cytometer (BD Biosciences). The proportions of cells in the G0–G1, S, and G2–M phases were analyzed using FlowJo v8 software (Tree Star). This experiment was repeated three times, and the results were averaged.

**Electrophoretic mobility shift assay**

The DNA binding of NFκB to the SERCA2 promoter (23) was investigated using an electrophoretic mobility shift assay (EMSA) with 32P-labeled oligonucleotides and nuclear extracts from parental cells and CSCs. Nuclear extracts were isolated using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce; 78833). The DNA-binding activity of NFκB against the SERCA2 promoter was confirmed using a 32P-labeled oligonucleotide. Labeled and unlabeled oligonucleotides specific for NFκB (forward 5'-GGGGGTTCCC-3', reverse 5'-GGGAACCCCCC-3') and mutant NFκB (forward 5'-GGGGCgTCCC-3', reverse 5'-GGGAAgCgCCC-3') were synthesized by Bioneer. Nuclear extracts–DNA interactions were carried out in a 30-μL reaction mixture containing 5 μg of nuclear protein extract, 50 mmol/L HEPES (pH 7.8), 300 mmol/L KCl, 1% Igepal, 30% glycerol, 1 mmol/L DTT, 0.02 μg of poly (dI-dC), 0.1 μg of ssDNA, and 20,000 CPM of 32P-radiolabeled oligonucleotide. For the supershift assay, 0.5 μg of anti-NFκB antibodies (Santa Cruz Biotechnology) were added to the mixture of nuclear extracts-DNA and incubated for one additional hour at 4°C. After incubation for 30 minutes at room temperature, the nuclear extracts–DNA complexes were resolved by 5% non-denaturing polyacrylamide gel (pre-run for 30 minutes) electrophoresis at 4°C in a buffer containing 50 mmol/L Tris, pH 7.9, 40 mmol/L glycine, and 1 mmol/L EDTA, run at 150 V. The gel was placed on Whatman paper, covered with plastic wrap, and dried on a gel dryer at 80°C for approximately 120 minutes. The gel was scanned using an Image Reader FLA-7000 scanner (GE Healthcare Bio-Sciences) and quantified using MultiGauge V3.1 software (Fuji photo film Co, Ltd.).

**Dual luciferase reporter assay**

Promoter activity was evaluated using the Dual-Luciferase Reporter Assay (Promega; E1960), according to the manufacturer’s protocol. Regions of NFκB-binding sites were amplified by PCR from human genomic DNA (NFκB primers: Forward 5'-GGGGGTTCCTCCC-3', Reverse 5'-GGGAACCCCCC-3'). The PCR products were cloned into the pGL4.70 promoter Vector (Promega) using T4 DNA ligase (Thermo Scientific; EL0011). All insertions were confirmed by sequencing. Cells were co-transfected with a plasmid containing the 3XκB-Luc reporter, as well as with a Renilla luciferase in pGL4.70 (as the control). Luciferase detection was carried out 48 hours after reporter transfection. Expression was estimated as the relative Firefly luciferase activity normalized to the activity of transfection control Renilla luciferase.
**Immunofluorescence analysis**

CSCs were incubated with a CaMK2α inhibitor (KN-62) in 35-mm glass-bottom microwell (14 mm) dishes. Images were observed under a confocal microscope (LSM Meta 700) and were analyzed using the Zeiss LSM Image Browser software, version 4.2.0121.

**IHC**

IHC staining was performed using a standard protocol. Tumor tissues were fixed in 10% formaldehyde and were embedded in paraffin. Tissue sections (5 μm) were dewaxed, and antigen retrieval was carried out in citrate buffer (pH 6), with an electric pressure cooker set at 120°C for 8 minutes. Sections were incubated for 10 minutes in 3% hydrogen peroxide to quench endogenous tissue peroxidase. Primary mAbs against CaMK2α (Abcam), Bcl-2 (Abcam), and SERCA2 were diluted with PBS at a ratio of 1:100 and incubated overnight at 4°C. All tissue sections were counterstained with hematoxylin, dehydrated, and mounted.

**Image analysis**

MetaMorph 4.6 software (Universal Imaging Co.) was used for computerized quantification of immunostained target proteins.

**In vivo mouse xenograft study**

Cancer cells were cultured and subcutaneously injected into the mammary fat pads of 5- to 6-week-old BALB/c nude mice (1.0 × 10^6 cells/mouse). The tumor size was measured every other day using calipers. The tumor volume was estimated using the following formula: \( V = \frac{L \times S^2}{2} \), where \( L \) = longest diameter and \( S \) = shortest diameter. When the tumor size reached approximately 100–200 mm³, tumor-bearing mice were randomly grouped (\( n = 8–9 \)/group) and 2DG (500 mg/kg), thapsigargin...
Samples of (15 mg/kg), or combinations of 2DG + thapsigargin, 2DG + metformin (250 mg/kg), 2DG + metformin + thapsigargin were injected intraperitoneally or via oral gavage, once every 3 days. Animals were maintained under specific pathogen-free (SPF) conditions. All experiments were approved by the Animal Experiment Committee of Yonsei University.

Statistical analysis
Statistical analysis was performed using Prism 6.0 software (GraphPad Software, Inc.), Excel (Microsoft Corp.), and R version 2.17. One-way ANOVA was performed for the multigroup analysis, and two-tailed Student t test was performed for the two-group analysis.

Results
Metabolic stress–resistant CSCs exhibit increased antiapoptotic capabilities upon prolonged glucose deprivation
Previously, we demonstrated that adaptation to chronic metabolic stress led to the positive selection of subclones with cancer stem–like characteristics (5). To evaluate the acquired capability of stress-resistant CSCs against metabolic stress conditions, we assessed cell viability in parental cells and CSCs (derived from both MDA-MB-231 and MCF-7 cells) after exposure to glucose deprivation for 0, 24, 36, 48, or 60 hours. Both parental cells (P-231 and P-MCF-7) and CSCs (S-231 and S-MCF-7) exhibited increased apoptotic cell death as the period of glucose deprivation increased (Fig. 1A). However, a significant number of CSCs survived even after 60 hours of glucose deprivation, whereas most parental cells did not (Fig. 1A–D). A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay confirmed these results (Fig. 1B).

Given that caspase activation is an indicator of apoptosis, we measured caspase-3, -7, and -9 cleavage (Fig. 1C). As MCF-7 cells do not express caspase-3, we measured caspase-9 cleavage instead in these cells. Bcl-2, an antiapoptotic protein, was also elevated in CSCs compared with that in parental cells following long-term exposure to glucose deprivation (Fig. 1C). The TUNEL assay also revealed more DNA fragmentation, indicating increased apoptosis, in parental cells compared with
that in CSCs (Fig. 1D). Cell-cycle analysis indicated that the number of cells in the sub-G0–G1 phase was greater in the parental cells than in the CSCs after long-term exposure to glucose deprivation (40 hours, P-231 29.3% vs. S-231 4.4%; P-MCF-7 21.3% vs. S-MCF-7 7.5%), whereas this difference was insignificant after short-term exposure (12 hours, P-231 1.2% vs. S-231 1.1%; P-MCF-7 0.2% vs. S-MCF-7 0.2%; Fig. 1E). Taken together, these data indicated that CSCs exhibit significant resistance to apoptosis upon prolonged glucose deprivation compared with their parental lineages.

CSCs undergo distinct transcriptional reprogramming

Considering that gene expression is largely influenced by signals from the environment, we speculated that CSCs would achieve a stem-like phenotype by transcriptional reprogramming. We performed genome-wide transcriptional profiling of paired P-231 and S-231 samples. A large number of genes were significantly differentially expressed between P-231 and S-231 cells, suggesting that multiple biological processes were reprogrammed in CSCs. Calcium signaling was identified as one of the most significantly over-represented pathways in CSCs by gene set analysis (Fig. 2A, left). Considering the distinctive gene expression patterns observed in the presence and absence of glucose across P- and S-cells, the most notable differentially expressed gene was SERCA2 (ATP2A2), which is involved in calcium signaling (Fig. 2A, right). Furthermore, the SERCA2 protein level was remarkably increased in CSCs compared with that in parental cells during glucose deprivation (Fig. 2B). Cytosolic-free calcium levels were lower in CSCs than in parental cells following short- or long-term glucose deprivation (Fig. 2C). In contrast, no significant differences in SERCA and cytosolic-free calcium levels were observed between parental cells and CSCs at baseline (complete medium; Fig. 2B and C).

Figure 4.

(Continued.) C, NFκB-binding sites and consensus sequences in the SERCA2 promoter. D and E, EMSA was performed using nuclear extracts prepared from MDA-MB-231 (D) and MCF-7 (E) cells. F and G, the NFκB-luciferase reporter system was used to compare NFκB transcriptional activity between P- and S- cells (F, MDA-MB231; G, MCF-7 cells). Relative luciferase activity was presented as a readout of NFκB activity. Values of samples were normalized to Renilla luciferase. Data represent the average of at least three separate independent experiments, with the SD indicated; *, P < 0.05; **, P < 0.01 versus control or parental.
Collectively, these results suggested that CSCs survive long-term glucose deprivation by transcriptional reprogramming involving intracellular calcium homeostasis regulatory pathways.

Survival of CSCs upon prolonged glucose deprivation depends on CaMK2a

The release of Ca\(^{2+}\) from the ER into the cytosol is an early and crucial event in apoptosis (24, 25). CaMK2a is regulated by Ca\(^{2+}\) and plays an important role against apoptosis (26, 27). During glucose deprivation, CSCs exhibited increased levels of phosphorylated CaMK2a (pCaMK2a) as well as antiapoptosis-related Bcl-2 and Bcl-2, whereas the expression of the channel proteins itself did not differ, but phosphorylated IP3R (pIP3R) was significantly downregulated (Fig. 3A). Interestingly, knockdown of CAMK2A (encoding CaMK2a) in CSCs using short interfering RNAs (siRNAs) S-231 si-CaMK2a and S-MCF-7 si-CaMK2a, Fig. 3B) led to reduced levels of antiapoptosis-related protein levels (pIkK, pNFκB, Bcl-2, and Bcl-2) and SERCA2, but higher levels of pIP3R (Fig. 3C). Furthermore, failure to restore basal intracellular Ca\(^{2+}\) levels in CSCs (Fig. 3D) was accompanied by increased apoptosis after long-term glucose deprivation (Fig. 3E and F). On CaMK2a knockdown in P-MCF-7 for long-term glucose deprivation, all cells died and hence the release of Ca\(^{2+}\) from the ER into the cytosol could not be measured (Fig. 3D).

A TUNEL assay revealed that siRNA-mediated knockdown of CAMK2A led to more DNA fragmentation compared with that in CSCs without CAMK2A knockdown after long-term glucose deprivation (Fig. 3E). Knockdown of CAMK2A in S-231 and S-MCF-7 cells increased sub-G\(_{1}\)-G\(_{0}\) phase cells following long-term glucose deprivation (Fig. 3F). These results suggested that CaMK2a-mediated antiapoptosis might be related to restoration of cytosolic Ca\(^{2+}\) levels to the basal state, providing protection in CSCs against prolonged glucose deprivation–induced cell death.

NFκB transcriptionally activates SERCA2 expression during glucose-deprived conditions

Long-term exposure of cancer cells to glucose-deprived conditions causes severe ER stress, which induces mitochondrial Ca\(^{2+}\) overload and eventually ER stress–mediated apoptosis (7, 28). NFκB, a transcriptional activator, acts as an inducible regulator of antiapoptotic gene expression, thereby controlling the prosurvival response (29). Moreover, research has shown that NFκB signaling induces Bcl-2, which affects the ER Ca\(^{2+}\) store by upregulating SERCA2 expression (30, 31).

Therefore, we investigated the role of NFκB in CSCs during time-dependent Ca\(^{2+}\)–mediated and glucose deprivation–induced apoptosis. Basal levels of antiapoptosis-related proteins (pCaMK2a, pIkK, pNFκB, Bcl-2, and cleaved caspase-3, -7, and -9), pIP3R, and SERCA2 did not significantly differ between CSCs and parental cells following short-term glucose deprivation (Fig. 4A). However, pCaMK2a, pIkK, pNFκB, Bcl-2, and SERCA2 increased prominently, whereas levels of pIP3R and cleaved caspase-3, -7, and -9 were reduced, in CSCs after long-term glucose deprivation (Fig. 4A).

SERCA is one of the main Ca\(^{2+}\) transporters and removes Ca\(^{2+}\) from the cytoplasm; therefore, we measured the levels of SERCA isoforms. None of the SERCA isoforms were induced in P-231 cells; however, SERCA isoform levels increased in S-231 cells as the length of glucose deprivation increased. Among the three SERCA isoforms, SERCA2 mRNA showed the most significant induction (Fig. 4B), which was confirmed in S-MCF-7 cells in comparison with P-MCF-7 cells.

Given the role of NFκB as a transcriptional regulator of various antiapoptotic genes during stress conditions, we hypothesized that SERCA2 would also be a transcriptional target of NFκB. To investigate potential NFκB binding to the SERCA2 promoter region, an EMSA was carried out in the presence of an anti-NFκB antibody with a \(^{32}\) P-labeled oligonucleotide containing NFκB-binding sites found in the SERCA2 promoter (Fig. 4C). Supershift analysis showed significant differences between CSCs and parental cells after short-term glucose deprivation, which were even more pronounced following long-term deprivation (Fig. 4D and E).

We next examined NFκB transcriptional activation using an NFκB-luciferase reporter assay system, which contains an NFκB-binding site from the SERCA2 promoter. There was a greater than 2- to 4-fold increase in relative luciferase activity in both S-231 and S-MCF7 cells, particularly after long-term glucose deprivation, compared with that seen in their parental cells (Fig. 4F and G, respectively).

Taken together, these results demonstrated that NFκB induces the transcription of SERCA2, as well as other antiapoptosis–related genes, in CSCs.

NFκB activation and the survival of stress-resistant CSCs are suppressed by inhibition of CaMK2a activity

Compared with parental cells, CSCs showed increased capacity for restoration of intracellular-free Ca\(^{2+}\) levels after long-term glucose deprivation (as shown in Fig. 2C). However, CSCs failed to restore cytotoxic Ca\(^{2+}\) levels after genetic silencing of CaMK2a (Fig. 3D), suggesting a potential link between CaMK2a activity and the intracellular Ca\(^{2+}\)–regulating mechanism. As shown in Fig. 4, NFκB induces SERCA2 expression and NFκB activation is induced by CaMK2a (32, 33). Thus, we hypothesized that inhibition of CaMK2a activity would suppress NFκB activation, SERCA2 expression, and CSCs survival.

We assessed expression levels of NFκB p65 in the nucleus, a barometer of NFκB activation, and the expression of SERCA2 after treatment with a CaMK2a inhibitor (KN-62) during glucose deprivation. KN-62 treatment downregulated expression levels of nuclear NFκB p65 compared with that seen in untreated cells (Fig. 5A and B). Furthermore, levels of SERCA2 and antiapoptosis–related proteins in CSCs were remarkably reduced after treatment with the CaMK2a inhibitor during long-term glucose deprivation (Fig. 5B), indicating that CaMK2a plays a critical role in NFκB’s entry into the nucleus. Consequently, CSCs treated with KN-62 exhibited reduced survival in response to long-term glucose deprivation (Fig. 5C).

Collectively, CaMK2a activity is required to translocate NFκB into the nucleus, where it activates the transcription of SERCA2 and the expression of antiapoptotic genes such as BCL2, thereby promoting the survival of CSCs during prolonged glucose deprivation.

Combined treatment with a SERCA inhibitor and glucose deprivation–mimetic suppresses tumor progression in mouse xenograft tumor models

To investigate the antitumor effect of a SERCA inhibitor in vivo, we developed mouse xenograft tumor models using parental cells
and CSCs. Xenograft tumors with parental cancer cells exhibited significantly reduced tumor volumes and weights compared with those comprising CSCs (Fig. 6A and B). Levels of antiapoptosis proteins (pCaMK2α, pNFκB, Bcl-2), and calcium restoration–related proteins (pIP3R and SERCA2) in tumors comprising CSCs were significantly increased compared with those in tumors comprising parental cells (Fig. 6C). Consistent with the in vitro results, IHC analysis revealed much higher levels of CaMK2α, Bcl-2, and SERCA2 in CSCs (S-231 and S-MCF-7) derived tumor tissues than in parental cell–derived tumors (Fig. 6D and E).

After confirming the in vivo tumor expression of SERCA2, we treated mice with thapsigargin, a SERCA inhibitor, together with various metabolic stress inducers (Fig. 6F–H). Thapsigargin treatment alone did not result in significant suppression of tumor growth in mouse xenografts (Fig. 6F). However, when we induced energetic stress on the mouse xenograft tumors by injecting the metabolic inhibitor 2-deoxy-D-glucose (2DG), mimicking in vivo glucose deprivation, or metformin, which suppresses mitochondrial bioenergetics, the combined treatment group (2DG plus metformin or/and thapsigargin) exhibited significantly smaller tumor volumes compared with those of groups receiving control treatment, 2DG, or thapsigargin alone (Fig. 6F). Intriguingly, in the parental group, the 2DG+metformin treatment alone resulted in significantly smaller tumor volumes compared with those in the control group. Among CSCs, however, 2DG+metformin treatment had no significant effect on tumor size. Taken together, these results indicated that parental cells are more vulnerable to metabolic stress than CSCs. Notably, a synergistic effect of 2DG, metformin, and thapsigargin caused the greatest tumor shrinkage among the combination treatments in both P-231 and S-231 cells (Fig. 6F and G). No evidence of systemic toxicity or treatment-related death was observed in any group. There was also no significant effect on the body weight of mice treated with 2DG, metformin, or thapsigargin alone, or in combination (Fig. 6H). Antiapoptotic and calcium restoration–related proteins were prominently downregulated, whereas

Figure 5.
CaMK2α activity is crucial for NFκB nuclear translocation, SERCA2 expression, and cell survival. A, Immunofluorescence visualization of NFκB in the nucleus of CSCs after treatment with KN-62, a CaMK2α inhibitor, during long-term (40 hours) glucose deprivation conditions; scale bar, 12.5 μm. Immunofluorescence staining was performed in triplicate on different days and representative images are displayed. B, Immunoblot analysis of anti-apoptosis– and calcium restoration–related proteins in CSCs after long-term (40 hours) exposure to glucose deprivation. C, Cell viability assay after short- (12 hours) and long-term (40 hours) exposure to glucose deprivation in CSCs treated with KN-62; *P < 0.05 versus control, **P < 0.01 versus control.
calcium release–related proteins were upregulated in the P-cell xenograft model. In contrast, antiapoptotic- and calcium restoration–related proteins were significantly upregulated in CSCs, whereas calcium release–related proteins were downregulated (Fig. 6I and J). These results may offer a new therapeutic opportunity to treat cancers, especially those comprising CSCs.

Discussion

The metabolic reprogramming of cancer cells has received significant attention recently and is considered an emerging hallmark of cancer (34). A high level of glycolysis, known as the Warburg effect, to satisfy the elevated needs of tumor cells for biosynthetic precursors, can also render cells exquisitely sensitive to glucose deprivation (34, 35). Conversely, more malignant cancer cells, such as highly metastatic subclones, have acquired a universal survival capacity and are better equipped than the less malignant cells to cope with nutrient deprivation (35, 36).

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To the best of our knowledge, the study is the first to demonstrate that CaMK2α plays a critical role in antiapoptosis by inducing SERCA in CSCs under glucose-deprived conditions. Induction of SERCA, which restores excess cytosolic Ca\(^{2+}\) to the ER, is mainly responsible for resistance to glucose deprivation–triggered apoptosis. In addition, increased Bcl-2 expression, induced by CaMK2α, acts with SERCA and contributes to the blocking of Ca\(^{2+}\)-dependent apoptosis. All these effects are fine-tuned by CaMK2α, which regulates SERCA and BCL2 expression via NFκB activation, thereby maintaining cytoplasmic Ca\(^{2+}\) homeostasis under glucose-deprived conditions.

The binding of inhibitor of kappa B (IκB) to NFκB masks the nuclear localization signals of NFκB. Phosphorylation of IκB by IκB kinase (IKK) results in IκB degradation. When IκB is degraded, the nuclear localization domain on NFκB is revealed and NFκB moves to the nucleus. Multiple kinases are reported to phosphorylate and activate IKK, one of which appears to be CaMK2α (27, 37).
Glucose deprivation induces an increase in cytosolic-free Ca$^{2+}$ via ER stress (7, 38). The ER is the major storage site for Ca$^{2+}$ in the cell, and mitochondria also play an important role in Ca$^{2+}$ homeostasis. Mitochondria can take up cytosolic Ca$^{2+}$ rapidly, acting as a buffer when cytosolic-free Ca$^{2+}$ is elevated. In the mitochondria, Ca$^{2+}$ enhances substrate uptake, NADH production, and the activities of ATP synthase, pyruvate, isocitrate, and α-ketoglutarate dehydrogenase in the tricarboxylic acid cycle, thus promoting mitochondrial metabolism and ATP production (39, 40). Intriguingly, this temporary augmentation of respiration through mitochondrial reflexive buffering of cytosolic Ca$^{2+}$ explains the homeostasis mechanisms of cellular bioenergetics in acute and early glucose starvation. However, prolonged cytosolic-free Ca$^{2+}$ overload is potentially lethal, as it decreases mitochondrial respiration, leading to a decline in membrane potential, mitochondrial swelling, cytochrome c release, and finally, apoptotic cell death (24, 28, 41). Thus, enhanced restoration of Ca$^{2+}$ to the ER may explain how CSCs survive sustained glucose deprivation–induced apoptosis.

Some studies have indicated that Bcl-2 might affect mitochondrial Ca$^{2+}$ homeostasis (42, 43), which was first demonstrated in neural cells. In this model, overexpression of BCL2 allows mitochondria to take up more Ca$^{2+}$ without causing mitochondrial respiratory disorder, indicating that Bcl-2 can protect mitochondria from an excess of Ca$^{2+}$ (44). In this study, we showed that despite Ca$^{2+}$ overburden in CSCs after long-term glucose deprivation, apoptotic cell death was not triggered, because CSCs increased their capacity to restore calcium levels by upregulating SERCA levels. CaMK2α has been previously reported to modulate cellular Ca$^{2+}$ homeostasis, either directly or by upregulating the expression of SERCA (45). Pharmacologic inhibition or genetic knockdown of CaMK2α in CSCs caused increased cytoplasmic-free Ca$^{2+}$ and reduced SERCA expression after long-term glucose deprivation, as well as reduced antiapoptotic capacity. Thus, the activation of CaMK2α provides a selective advantage to CSCs during long-term glucose deprivation by regulating cytoplasmic-free Ca$^{2+}$ levels via SERCA, whose transcription is also induced by CaMK2α-mediated NFκB activation.

Previously, we showed that dual inhibition of the tumor energy pathway by 2DG and metformin was effective against a broad spectrum of cancer cells (46). Glucose deprivation can directly affect cellular bioenergetics by suppressing ATP production. It is well appreciated that decreased ATP levels leads to cell death; thus,
glucose starvation or 2DG treatment can result in synergistic effects when combined with SERCA inhibition, which also receives inputs from multiple upstream signals (47–50) owing to glucose deprivation–induced cellular stress, other than ER stress. Together, these multiple independent and partially interdependent cellular processes would exert synergistic effects in inhibiting tumor growth in metabolic stress caused by glucose deprivation. In this study, we recapitulated that 2DG alone did not have significant antitumor effects. Consistent with this result, the combined treatment of thapsigargin with 2DG, but not 2DG alone, which mimics glucose deprivation, resulted in significant antitumor effects, suppressing tumor growth in mouse xenograft models. Taken together, these results suggested that CaMK2α-mediated induction of SERCA is a central mechanism by which CSCs survive lethal metabolic stress. Clinically, these observations have significant implications for the development of novel combinatorial strategies that target the selective vulnerabilities of highly malignant cells.

Figure 6. (Continued.) I, Immunoblot analysis of antiapoptotic- and calcium-related proteins in tumor tissues derived from parental and CSCs. J, IHC analysis for Bcl-2 and SERCA2 proteins in tumor tissues according to the indicated treatments. Each assay was performed in triplicate and representative images are displayed. *, *P < 0.05 versus control, **, *P < 0.01 versus control.
Disclosure of Potential Conflicts of Interest

G.B. Mills has ownership interests (including patents) at Catena Pharmaceuticals, ImmunoMet, Myriad Genetics, PTV Ventures and Spindler Ventures, reports receiving speakers bureau honoraria from Allosterix, AstraZeneca, ImmunoMet, ISIS Pharmaceuticals, Lilly, MedImmune, Novartis, Pfizer, Symphogen, and Tarveda, is a consultant/advisory board member for Adventist HealthCare, AstraZeneca, Catena Pharmaceuticals, Critical Outcome Technologies, ImmunoMet, ISIS Pharmaceuticals, Lilly, MedImmune, Novartis, Precision Medicine, Provista Diagnostics, Signalchem Lifesciences, Symphogen, Takeda/Millennium Pharmaceuticals, Tarveda, and Tau Therapeutics, and reports receiving commercial research grants from Adelson Medical Research Foundation, AstraZeneca, Breast Cancer Research Foundation, Critical Outcome Technologies, Illumina, Karus, Komen Research Foundation, Nanosting, Pfizer, Takeda/Millennium Pharmaceuticals. No potential conflicts of interest were disclosed by the other authors.

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