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Combined inhibition of glycolysis and AMPK induces synergistic breast cancer cell killing

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Abstract

Targeting glycolysis for cancer treatment has been investigated as a therapeutic method but has not offered a feasible chemotherapeutic strategy. Our aim was to examine whether AMP-activated protein kinase (AMPK), a conditional oncogene, rescues the energetic stress and cytotoxicity induced by 2-deoxyglucose (2-DG), a glycolytic inhibitor, and the related mechanisms. Luciferin/luciferase adenosine triphosphate (ATP) determination, Western analysis, qRT-PCR analyses, MTT growth assay, clonogenic assay, and statistical analysis were performed in this study. 2-DG decreased ATP levels and subsequently activated AMPK, which contribute to intracellular ATP recovery in MCF-7 cells thus exhibiting no apparent cytotoxicity. Compound C, an AMPK inhibitor, further potentiates 2-DG-induced decrease in ATP levels and inhibits their recovery. 2-DG, via AMPK activation, stimulated cAMP response element-binding protein (CREB) phosphorylation and activity and promoted nuclear peroxisome proliferator-activated receptor gamma coactivator-1-beta (PGC-1 β) and estrogen-related receptor α (ERR α) protein expression, leading to augmented mitochondrial biogenesis and expression of fatty acid oxidation (FAO) genes including PPAR α , MCAD, CPT1C, and ACO. This metabolic adaptation elicited by AMPK counteracts the ATP-depleting and cancer cell-killing effect of 2-DG. However, 2-DG in combination with AMPK antagonists or small interfering RNA caused a dramatic increase in cytotoxicity in MCF-7 but not in MCF-10A cells. Similarly, when combined with inhibition of CREB/PGC-1 β /ERR α pathway, 2-DG saliently suppressed mitochondrial biogenesis and the expression of FAO genes, depleted ATP production, and enhanced cytotoxicity in cancer cells. Collectively, the combination of 2-DG and AMPK inhibition synergistically enhanced the

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cytotoxic potential in breast cancer cells with a relative nontoxicity to normal cells and may offer a promising, safe, and effective breast cancer therapeutic strategy.

Keywords

2-Deoxyglucose; AMPK; Glycolysis; Mitochondrial biogenesis; Metabolic adaptation

Introduction

Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer death in women accounting for nearly a third of all new cases of women's cancer [1]. The heterogeneity of breast cancers makes them a challenging solid tumor to treat, and this has intensified the search for new drug targets. Chemotherapy plays an imperative role in breast cancer treatment. Single chemotherapeutic agents such as anthracyclines, taxanes, vinorelbine, and capecitabine [2] disrupt the way cancer cells grow and divide, but they also affect normal cells, leading to various serious side effects. Other significant disadvantage of chemotherapy includes acquired and primary resistance. Thus, new systemic therapeutic strategies which are safe and effective are desperately needed.

The identification of new molecular targets and development of novel therapeutics depend on a deeper understanding of the underlying biology of breast cancer. Theoretical progresses in the past decades have enhanced our understanding on the biological significance of tumor metabolism [3]. As a result, altered energy metabolism, a biochemical fingerprint of cancer cells, has been recognized as one of the "hallmarks of cancer" [4]. This metabolic phenotype features preferential dependence on glycolysis for energy yield in an oxygen-independent manner. While glycolysis is less effective than oxidative phosphorylation in the net production of adenosine triphosphate (ATP), cancer cells acclimatize to this disadvantage by elevated glucose up-take, which in turn fosters a higher rate of glycolysis. Thus, the metabolic changes in malignant cells may be used to serve as a biochemical rationale to develop therapeutic approaches to target this metabolic aberration. Targeting tumor glycolysis is logically sound opening the door for some emerging therapeutic choices. One possibility is to inhibit glycolysis and preferentially kill the malignant cells with a mild effect or even no effect on normal cells. Indeed, targeting glycolysis for cancer treatment has been investigated previously as a therapeutic method [5]. Among all the glycolysis inhibitors assessed, 2-deoxyglucose (2-DG) has been best characterized in animal model studies and human clinical trials [6]. However, the application of 2-DG as an anticancer agent in vivo is disillusionary. Moreover, 2-DG was shown to activate pro-survival pathways in cancer cells [7], which counteract its anticancer effects. Additionally, hypoxic cancer cells exhibited chemo-resistance against 2-DG [8]. Therefore, the realization of 2-DG as a single agent for anti-glycolytic therapy has been challenged.

Our findings indicated that 2-DG activates AMP-activated protein kinase (AMPK), followed by partial restore of intracellular ATP levels. The AMPK pathway represents a key metabolic adaptation mechanism [9]. Thus, the inhibitory effect of 2-DG on cancer cell growth may be partially offset by the fact there is also 2-DG-induced AMPK activation.

Nevertheless, the mechanisms by which AMPK promotes metabolic adaptation in tumor cells are not fully understood. Here, we find that Compound C, an AMPK inhibitor, synergistically reinforced the inhibitory effect of 2-DG on ATP production and caused a dramatic increase in cytotoxicity in breast cancer MCF-7 cells but not in MCF-10A cells. Thus, we reasoned that inhibition of AMPK will antagonize metabolic adaptation and enhance the efficiency of 2-DG to deplete intracellular ATP and induced cancer cell death. Results from this study demonstrate that targeting metabolic stress (inhibition of glycolysis) and metabolic adaptation (inhibition of AMPK) is a potentially promising, safe, and effective breast cancer treatment strategy.

Materials and methods

A full description of materials and methods used, including cAMP response element-binding protein (CREB) activity assay, clonogenic assays, cell viability assay, transfection and small interfering RNA (siRNA) gene silencing, measurement of cellular ATP levels, RT-PCR, and qRT-PCR, can be found in the online-only Data Supplement.

Cell culture and treatment

Human breast cancer cell lines MCF-7, MCF-10A, MDA-MB-231, and MDA-MB-435 cells were grown at 37 °C in 5 % CO₂. Cells were cultured in Dulbecco's modified Eagle's medium/F12 media (1:1; Mediatech, Inc., Manassas, VA) supplemented with 10 % fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 mg/mL). In some experiments, cells were treated with 25 mM of 2-DG for different time (0–24 h) or with different concentration (0, 5, 10, 15, 20, 25 mM) for 8 h. AMPK inhibitor Compound C (20 μM), estrogen-related receptor α (ERRα) inhibitor 3-[4-(2,4-bis-trifluoromethylbenzyloxy)-3-methoxyphenyl]-2-cyano-*N*-(5-trifluoromethyl-1,3,4-thiadiazol-2-yl)acrylamide (XCT790, 5 μM), or DMSO were added to cells 30 min prior to 2-DG. All experiments were performed in triplicate.

Nuclear fractionation

Subcellular fractionation was performed as described previously [10]. Nuclear fractions were assessed by immunoblotting of histone H3, which was used as a loading control.

Western blotting

Protein extracts were analyzed by standard methods and antibodies to AMPK, phospho-AMPK (Thr172), CREB, phospho-CREB (Ser133), ERRα, cytochrome *c*, and H3 or β-actin as loading control. Densitometry was performed using Scion Image software (Scion Corp., Frederick, MD).

Statistical analysis

Data are presented as mean ± SD. Differences between experimental groups were determined by one-way ANOVA, followed by Student *t* test as appropriate. All results obtained from the time-course studies were analyzed with repeated-measures ANOVA. Differences were considered statistically significant at *P* < 0.05. Statistical analysis was done using SPSS for Windows (SPSS, Inc., Chicago, IL).

Results

Compound C further potentiates 2-DG-induced decrease in intracellular ATP levels and inhibits their recovery in MCF-7 breast cancer cells

To investigate the role of 2-DG and its combination with Compound C in the status of energy metabolism, we treated MCF-7 and MCF-10A cells with 25 mM 2-DG in the presence or absence of 20 μ M Compound C and test intracellular ATP levels. As indicated in Fig. 1a, ATP levels were significantly decreased in MCF-7 treated with 2-DG, with a bottom level at 8 h, and then recovered gradually. Intriguingly, Compound C can further potentiate 2-DG reduction of intracellular ATP levels and abolish ATP recovery after 8 h. As a cellular energy sensor responding to low ATP levels, AMPK activation positively regulates signaling pathways that replenish cellular ATP supplies and negatively regulates ATP-consuming biosynthetic processes [11]. Thus, we speculate that this ATP recovery represents a compensatory mechanism to maintain ATP homeostasis via AMPK activation in response to metabolic stress induced by 2-DG. In contrast, 2-DG had a modest effect on MCF-10A, a control cell line. A dose response study (Fig. 1b) shows that decreased ATP levels were clearly detected with as little as 5 mM 2-DG, with the lowest levels at 25 mM 2-DG treatment in MCF-7 cells. Thus, 25 mM 2-DG will be adopted in the future studies. Similarly, Compound C synergistically reinforced the inhibitory effect of 2-DG on intracellular ATP production in MCF-7 cells.

2-DG increases AMPK phosphorylation in cultured MCF-7 and MCF-10A cells

AMPK plays a key role as a master regulator of cellular energy homeostasis. To test whether 2-DG can phosphorylate AMPK, MCF-7 and MCF-10A cells were treated with 2-DG for up to 24 h. As shown in Fig. 2a, 2-DG significantly increased the phosphorylation of Thr172 of AMPK, which is thought to correlate with enzyme activity [12], in a time-dependent manner with as early as 4-h treatment in MCF-7 cells. However, the phosphorylation of AMPK in MCF-10A increased transiently, with a peak level at 8 h, and then recovered to the normal levels. In addition, the activating effects of 2-DG on AMPK in MCF-10A were not as strong as in MCF-7 cells. This phenomena may be explained by the weak decrease in ATP levels induced by 2-DG in MCF-10A (Fig. 1). As shown in Fig. 2b, AMPK phosphorylation increased in a 2-DG dose-dependent manner. Figure 2c, d shows that in the presence of the potent AMPK inhibitor Compound C, 2-DG treatment failed to activate AMPK in both cell lines.

Cytotoxic and anti-proliferative effects of 2-DG and Compound C in cancer and non-cancerous cells

The cytotoxicity of 2-DG alone and its combination with Compound C or AMPK RNA interference in MCF-7 cancer cells and non-cancerous MCF-10A cells was monitored by the MTT assay at the indicated times (Fig. 3a, b). 2-DG alone did not exhibit apparent cytotoxicity in both cell lines. However, both 2-DG + Compound C and 2-DG + AMPK siRNA caused a dramatic increase in cytotoxicity in MCF-7 cancer cells but not in MCF-10A cells. One of the main characteristics of cancer cells is their altered metabolism when compared to normal cells [13]. Cancer cells generally exhibit augmented glycolysis for ATP generation (the Warburg effect) because of mitochondrial respiration injury and

hypoxia [14]. As such, sustaining a high rate of glycolytic activity is indispensable for cancer cells to survive and grow. Here, we demonstrate that introduction of metabolic stress by inhibiting glycolysis (2-DG) and suppression of metabolic adaption via inhibiting AMPK (Compound C/AMPK siRNA) result in significant intracellular ATP depletion (Fig. 1) and cell death (Fig. 3) in cancerous cells.

It is reasonable to speculate that cancer cells that are more dependent on glucose flux or glycolysis are likely more sensitive to this combined treatment. To test the hypothesis, we employed two cancerous cell lines, i.e., MDA-MB-231 cells, whose GLUT1 mRNA level and the glucose uptake rate are higher than those in MCF-7 cells [15], and MDA-MB-435 cells that have been shown to have higher rates of glycolysis compared to MCF-7 cells [16]. MDA-MB-435 cells, although the tissue of origin of which has been a matter of debate [17], will still be used due to its higher rates of glycolysis [16]. We used a clonogenic assay to monitor the antiproliferative effects of 2-DG and Compound C. Figure 3c shows the calculated survival fractions of MCF-10A, MCF-7, MDA-MB-231, and MDA-MB-435 cells. 2-DG + Compound C significantly decreased the survival fraction in MCF-7, MDA-MB-231, and MDA-MB-435 cells as compared to MCF-10A cells. Notably, the combination treatment exhibited more potent inhibitory effects on the survival fraction in MDA-MB-231 and MDA-MB-435 cells than MCF-7 cells (Fig. 3c).

2-DG activates nuclear CREB, PGC-1 β , and ERR α in cultured MCF-7 cells

Our findings indicate that in breast cancer cells, 2-DG activates AMPK, followed by partial restore of intracellular ATP. Thus, we reasoned that the inhibitory effect on cancer cell growth produced by 2-DG may be partially offset by the fact that there is also 2-DG-induced AMPK activation and subsequent energy compensation. To further delineate the molecular mechanisms underlying 2-DG-mediated metabolic adaptation, we measured nuclear CREB phosphorylation and protein expression of peroxisome proliferator-activated receptor gamma coactivator-1-beta (PGC-1 β) and ERR α , which are key regulators of energy metabolism [18, 19]. As shown in Fig. 4a, the phosphorylation of Ser133 of CREB in MCF-7 cells increased in a time-dependent manner in response to 2-DG treatment. Treatment with 2-DG did not change the levels of CREB in MCF-7 cells. The increase in CREB phosphorylation was accompanied by gradual augmentation of protein expression of PGC-1 β and ERR α . The increased CREB phosphorylation coincided with an augmented CREB DNA-binding activity, as assessed by CREB (pS133) transcription factor assay kit (Fig. 4c). As shown in Fig. 4b, d, PGC-1 β , ERR α , and CREB phosphorylation and CREB activity increased in a 2-DG dose-dependent manner. The activation of CREB was also observed in MDA-MB-231 and MDA-MB-435 cells stimulated with 2-DG (data not shown).

PGC-1 β is required for AMPK-mediated ERR α activation, ATP restoration, and cancer cell survival in response to 2-DG

AMPK, PGC-1 β , and ERR α are all critical regulators of mitochondrial biogenesis and function as well as energy metabolism [20, 21]. To gain deeper insight into their regulatory relationships and AMPK signaling cascades involving metabolic adaptation, we treated MCF-7 cells with 2-DG (25 mM) for 24 h in the presence or absence of Compound C and

transfection with siRNA-targeting AMPK and PGC-1 β . Cellular proteins or nuclear fractions were extracted and subjected to Western blot analysis for phosphorylated AMPK, AMPK, ERR α , and PGC-1 β . 2-DG treatment robustly elevated the levels of AMPK phosphorylation (p-AMPK) and protein expression of PGC-1 β and ERR α , but total AMPK protein levels remained unchanged (Fig. 5a). Inhibition of AMPK activity with Compound C or RNA interference attenuated 2-DG-stimulated increases in PGC-1 β and ERR α protein expression. The expression of ERR α and PGC-1 β correlates very well in MCF-7 breast cancer cells. In contrast, PGC-1 α expression was almost undetectable in MCF-7 cells, and although it is lowly expressed in MDA-MB-231 cells, its expression was not changed by treatment with 2-DG (data not shown). Importantly, interfering RNA (siRNA)-mediated knockdown of PGC-1 β significantly diminished 2-DG-stimulated increases in ERR α protein expression without affecting AMPK phosphorylation, suggesting that PGC-1 β functions as a downstream target of AMPK and upstream of ERR α .

Because PGC-1 β mediated AMPK-induced ERR α activation, we next evaluated whether AMPK and PGC-1 β influenced expression of fatty acid oxidation (FAO) genes such as PPAR α , MCAD, CPT1C, and ACO, which may be directly regulated by ERR α [22]. As indicated in Fig. 5b, the inhibition of AMPK and PGC-1 β by genetic or pharmaceutical approach attenuated 2-DG-stimulated increases in PPAR α , MCAD, CPT1C, and ACO gene expression. To further substantiate these genes are metabolic target genes downstream of ERR α activated by 2-DG in MCF-7 cells, we employed XCT790, a selective ERR α antagonist/inverse agonist. As shown in Fig. 5c, 2-DG dramatically increased mRNA expression of PPAR α , MCAD, CPT1C, and ACO. The stimulatory effect of 2-DG on these genes was blocked by XCT790, with PPAR α showing less susceptible to XCT790 versus MCAD, CPT1C, and ACO. This may be explained by the fact that PPAR α can be alternatively activated by other upstream proteins such as PGC-1 [23]. Furthermore, XCT790 alone suppressed basal levels of PPAR α , MCAD, CPT1C, and ACO. These results demonstrated that FAO genes such as PPAR α , MCAD, CPT1C, and ACO are downstream targets of ERR α activated by 2-DG via AMPK/PGC-1 β pathway.

To determine if AMPK/PGC-1 β pathway is involved in metabolic adaptation elicited by 2-DG and counteraction of cancer cell-killing effect of 2-DG, we measured intracellular ATP production and cell viability. Treatment of MCF-7 cells with 2-DG induced an insignificant decrease in the intracellular ATP levels and cell viability. When combined with AMPK or PGC-1 β inhibition by genetic or pharmaceutical approach, 2-DG significantly depleted intracellular ATP production and enhanced cytotoxicity in breast cancer cells (Fig. 5d, e).

CREB phosphorylation is required for AMPK-mediated PGC-1 β activation and mitochondria biogenesis in response to 2-DG

Next, we sought to elucidate the mechanism by which AMPK activated PGC-1 β upon 2-DG challenge. PGC-1 has been shown previously to induce mitochondrial DNA replication and biogenesis in muscle cells, adipocytes, and cardiomyocytes [24]. Here, we used cytochrome c, a well-conserved electron transport protein and part of the respiratory chain localized to the mitochondrial intermembrane space [25], as a mitochondrial biogenesis marker. As indicated in Fig. 6a, 2-DG treatment significantly increased CREB phosphorylation at S133

and cytochrome *c* expression, and these effects were blocked by AMPK inhibition with Compound C or AMPK siRNA in MCF-7 cells. These results suggested that AMPK mediated 2-DG-induced CREB activation and mitochondria biogenesis. To investigate further the role of CREB in AMPK-mediated PGC-1 β activation and mitochondrial biogenesis, MCF-7 cells were transfected with CREB Dominant-Negative pCMV-CREB133 Vector (S133A) or empty vector (EV) and then treated with 2-DG for 24 h. As shown in Fig. 6b, S133A instead of EV transfection eliminated endogenous p-CREB. S133A transfection dramatically ablated 2-DG-induced expression of PGC-1 β and cytochrome *c* but had no effect on p-AMPK, implying that CREB phosphorylation is required for AMPK-mediated PGC-1 β activation and mitochondrial biogenesis.

Finally, we asked whether the induction of the CREB phosphorylation by AMPK is required for AMPK-mediated FAO gene expression, intracellular ATP recovery, and cancer cell survival. Figure 6c, d illustrates that inhibition of CREB with S133A greatly attenuated 2-DG-induced mRNA expression of PPAR α , MCAD, CPT1C, and ACO. Importantly, 2-DG in combination with S133A significantly depleted intracellular ATP production and enhanced cytotoxicity in breast cancer cells (Fig. 6e, f). Taken together, these data strongly suggest that phosphorylation of CREB by AMPK initiates a cascade of protein and gene expression that controls many mitochondrial target genes and respiration and genes of fatty acid oxidative metabolism in breast cancer cells, which play important roles in AMPK-mediated metabolic adaptation.

Discussion

In the present study, we have presented evidence that AMPK regulates survival pathways via nuclear CREB/PGC-1 β /ERR α signaling and elicits metabolic adaptation in response to metabolic stress induced by 2-DG in breast cancer cells. We also have demonstrated that 2-DG in combination with AMPK inhibition by genetic or pharmaceutical approach significantly depleted intracellular ATP production and enhanced cytotoxicity in breast cancer cells but not in normal breast cells. Our data support the hypothesis that inhibition of AMPK antagonizes metabolic adaptation and underpin the efficiency of 2-DG to induced cancer cell death (Fig. 6g). Thus, our results reveal a novel metabolic adaption mechanisms by which cancer cells survive metabolic stress associated with glycolysis inhibition and elucidate a safe and effective therapeutical strategy to preferentially kill the malignant cells that are dependent on glycolytic pathway for ATP generation with a minor effect on normal cells.

Previous studies have suggested that supraphysiological activation of AMPK might impede tumor growth [26]. Use of the AMPK activator biguanide metformin is associated with a decreased incidence of cancer in patients with diabetes [27], but it has been indistinguishable whether this requires AMPK. Recently, Shackelford et al. show, unexpectedly, that biguanides are more efficient in the treatment of mouse tumors lacking a functional AMPK pathway [28]. More importantly, AMPK has recently been shown to facilitate cancer cell survival in the presence of extrinsic and intrinsic stressors such as bioenergetic and oncogene stress [29]. In addition, pharmacologic stimulation of AMPK promotes β -oxidation of fatty acids as a substitutive source of energy and rescues viability of

cancer cells. The deficiency of LKB1/AMPK activation rendered cancer cells more susceptible to cell death elicited by glucose deprivation [30]. Thus, whether AMPK should be targeted for activation or inhibition during cancer treatment is controversial and entails illumination.

Given AMPK activation is readily invertible when circumstances become advantageous for cancer cell proliferation, it is reasonable to conceive that the energy-sensing function of AMPK plays a conditional oncogenic role, which may endow a survival advantage under selection pressure, contributing to cancer cell development and the escalation of progressive cell populations [29]. Thus, a logical implication is that hindering instead of activating AMPK may provide a therapeutic approach for incapacitating cancer cell metabolic adaption, preventing metastasis, and eliminating residual disease, in which circumstances eradicating surviving and quiescent cancer cells become more desirable than constraining cell proliferation. Thus, highly specific AMPK inhibitors, expected to interrupt AMPK-dependent bioenergetic homeostasis, will provide a potential approach for cancer therapy. In the current study, we found that AMPK activation by 2-DG, via CREB/PGC-1 β /ERR α pathway, promotes mitochondria biogenesis and FAO gene expression, leading to intracellular ATP recovery and counteraction of cancer cell-killing effect of 2-DG. AMPK inhibition synergistically enhances the efficiency of 2-DG to induce cancer cell death.

Mitochondria are mainly responsible for providing cells with energy in the form of ATP, which is produced by oxidative phosphorylation consuming the energy released from electron transfer on the mitochondrial inner membrane. Studies over the last decade have revealed that the nuclear receptors and the PGC-1 family are key regulators of mitochondrial biogenesis and function, and fatty acid β -oxidation [31]. This family includes PGC-1 α and PGC-1 β , and PGC-related coactivator [32]. In contrast to PGC-1 α , factors and pathways regulating the expression of PGC-1 β are poorly known. Nonetheless, it has recently been demonstrated that PGC-1 β expression is up-regulated by c-Myc in renal carcinoma cells [33], implying that PGC-1 β expression can be regulated by oncogenic signals. Together, these results implicate that PGC-1 β may be a critical regulator of mitochondrial biogenesis; however, the mechanism still remains unclear. In the present study, we demonstrate that CREB phosphorylation is required for AMPK-mediated PGC-1 β activation and mitochondrial biogenesis. Importantly, interfering RNA (siRNA)-mediated knockdown of PGC-1 β significantly diminished 2-DG-stimulated increases in ERR α protein expression, implicating that PGC-1 β functions as an upstream of ERR α (Fig. 5a). ERR α is a member of the nuclear hormone receptor superfamily of transcription factors and represents a novel target for the future development of breast cancer treatments [34]. Also, ERR α plays a role in energy homeostasis and will probably be targeted for the treatment of metabolic disorders. Here, we have defined the AMPK/CREB/PGC-1 β pathway that influences and modulates ERR α transcriptional activity in breast cancer cells and delineated the processes downstream of the receptor, i.e., activation of FAO genes such as PPAR α , MCAD, CPT1C, and ACO contributing to metabolic adaptation, which was exploited by breast cancer cells to survive metabolic stress. Inhibition of AMPK/CREB/PGC-1 β pathway with genetic or pharmaceutical approaches suppressed the expression of ERR α and its downstream targets, saliently enhancing 2-DG-induced intracellular ATP depletion and cytotoxicity in breast cancer cells.

The concept of co-inhibition of glycolysis and AMPK could be developed into a safe and effective therapeutical strategy to preferentially kill the malignant cells including but not limited to breast cancer cells that are dependent on glycolytic pathway for ATP generation. Our study provides a strong theoretical basis for subsequent pre-clinical and clinical trials and a promising novel therapeutic regimen for clinical practice. To deliver 2-DG and AMPK inhibitor to breast tumor in vivo or in clinical practice for high efficacy but low toxicity, three aspects need to be taken into account. First, before treatment, the susceptibility of the animal or patient's tumor to 2-DG-mediated combination therapy needed to be determined by evaluating hypoxic state, hypoxia-inducible factor 1 α expression, energy state (ATP concentration), glucose utilization level, and the expression of glucose transporters of the tumor [8, 35–37]. Secondly, the animal or patient should be maintained on a low-glucose or low-carbohydrate diet during the combination therapy. A low-carbohydrate diet reduces glucose availability for the body, rendering the cells in hypoxic areas of solid tumors more ravenous for glucose and thus making those cells even more vulnerable to 2-DG. Additionally, consumption of a low-carbohydrate diet generates ketone bodies by forcing the body to metabolize fat for energy. Ketone bodies are favored by the brain versus glucose for ATP production, thus diminishing the brain's reliance on glucose and protecting it from 2-DG. Finally, the 2-DG should be administered contemporaneously with the administration of the AMPK inhibitors orally, intravenously, or even intratumorally. An assay or test to detect ATP levels in the tumor should be performed to adjust dosage regimens.

In summary, our findings indicate that increased mitochondria biogenesis and FAO via AMPK/CREB/PGC-1 β /ERR α pathway rescue the energetic stress and cytotoxicity in breast cancer cells induced by 2-DG. We report in this study that the combination of an anti-glycolytic agent 2-DG and AMPK pathway inhibition synergistically enhanced the cytotoxic potential in breast cancer cells with a relative nontoxicity to normal cells. The present studies improve understanding of AMPK regulation and its roles in metabolic adaptation of tumor cells, which is important for implementation of AMPK-targeted drugs into clinical management.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Time- and dose-dependent effect of 2-DG or combination of 2-DG and Compound C on intracellular levels of ATP in MCF-7 human breast cancer cells and MCF-10A cells. **a** MCF-7 or MCF-10A cells were treated with 25 mM of 2-DG for different time (0–24 h) in the presence or absence of 20 μ M Compound C (Comp C). **b** Cells were exposed to 2-DG at 0, 5, 10, 15, 20, or 25 mM in the presence or absence of 20 μ M Comp C for 8 h. Intracellular ATP levels were monitored using a luciferase-based assay. All experiments were repeated thrice. In each experiment, three replicates at each concentration per time point were analyzed. The results of a representative experiment are presented. *Points* mean for ATP concentration expressed as a percent of values observed in controls to facilitate visual interpretation of the data, *bars* SD

Fig. 2.

2-DG activates AMPK in cultured MCF-7 and MCF-10A cells. MCF-7 and MCF-10A cells were treated with **a** 2-DG 25 mM for the times indicated and **b** various concentrations of 2-DG for 8 h. The cells were lysed, 100 µg of the cell lysates underwent SDS-PAGE followed by Western blotting using anti-p-AMPK-T172 to detect AMPK phosphorylation, and then were visualized by the enzyme-linked chemiluminescence system. Total AMPK and β-actin were used as loading controls. The *bar graphs above* are densitometry analyses of the phosphorylated AMPK. Data presented are mean ± SD from three independent experiments,

with nontreated controls set as 1. $*P < 0.05$ between 2-DG-treated groups and nontreated controls. **c, d** Compound C can block 2-DG-mediated AMPK phosphorylation. Cells were pretreated with 20 μ M Compound C for 30 min before 2-DG treatment. Cell lysates were collected after 2-DG treatment, and phosphorylation of AMPK was assessed with anti-p-AMPK-T172 using total AMPK and β -actin as loading controls

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Fig. 3.

The cytotoxic effect of 2-DG in the presence or absence of Compound C in MCF-7 and MCF-10A cells. MCF-7 (a) and MCF-10A (b) cells seeded in 96-well plates were treated with 25 mM 2-DG in the presence or absence of 20 μ M Compound C or siRNAs-targeting AMPK. The corresponding treatments were re-introduced every 48 h after the medium had been refreshed. Cytotoxicity was monitored by MTT assay, and absorbance was read at 560 nm. The experiments were performed in triplicates and repeated at least three times. Data are presented as the mean $\pm$ SD. * P < 0.05 between 2-DG-treated groups and 2-DG + AMPK siRNA or 2-DG + Comp C. c Antiproliferative effects of 2-DG in the presence and absence of Compound C in MCF-7, MCF-10A, MDA-MB-231, and MDA-MB-435 cells. Cells were seeded at 300 cells per dish in 6 cm cell culture dishes and treated with different concentrations of 2-DG in the presence of 20 μ M Compound C or its vehicle DMSO. The corresponding treatments were re-introduced every 48 h after the medium had been refreshed. After 12 days, the number of colonies formed was counted. The cell survival fractions were calculated. Data shown represent the mean $\pm$ SD. * P < 0.05 versus MCF-10A, # P < 0.05 versus MCF-7 under the same treatment conditions (n = 3)

Fig. 4. 2-DG stimulates CREB phosphorylation and activates nuclear PGC-1β and ERRα expression in cultured MCF-7 cells. MCF-7 cells were treated with **a** 2-DG 25 mM for the times indicated and **b** various concentrations of 2-DG for 8 h. Nuclear fractions were prepared as described in “Materials and methods” section and underwent SDS-PAGE followed by Western blotting using anti-CREB, anti-phospho-CREB (Ser133), anti-PGC-1β, and anti-ERRα antibodies and then were visualized by the enzyme-linked chemiluminescence system. Histone H3 was used as loading controls. The *bar graphs above* are densitometry analyses of the phosphorylated CREB and expression of PGC-1β and ERRα. **c, d** Nuclear fractions were extracted and subjected to CREB activity assays with a CREB (pS133) transcription factor assay kit. Data presented are mean ± SD from three independent experiments, with non-treated controls set as 1. *P < 0.05 between 2-DG-treated groups and non-treated controls

Fig. 5.

PGC-1β is responsible for AMPK-induced ERRα activation in response to 2-DG. MCF-7 cells were pre-treated with AMPK inhibitor Compound C (20 μM), ERRα inhibitor XCT790 (5 μM), or DMSO for 30 min, followed by 2-DG (25 mM) treatment for 24 h. In some experiments, MCF-7 cells were transfected with human-specific AMPK siRNA, PGC-1β siRNA, or scrambled siRNA for 24 h using BioT transfection reagent, followed by 2-DG treatment for another 24 h. Cellular proteins or nuclear fractions were extracted and subjected to Western blot analysis for phosphorylated AMPK, AMPK, ERRα, and PGC-1β (a). PPARα, MCAD, CPT1C, and ACO mRNA levels were assayed using real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR, b, c). Intracellular ATP levels in MCF-7 cells with indicated treatments (Control, 2-DG, 2-DG + Compound C, 2-DG + scrambled siRNA, 2-DG + AMPK siRNA, or 2-DG + PGC-1β siRNA) were monitored using a luciferase-based assay (d). Cell viability of MCF-7 cells with indicated treatments for 48 h was detected by MTT assay (e). Data are presented as the mean ± SD for three independent experiments. *P < 0.05 versus control, #*P* < 0.05 versus 2-DG-treated groups

Fig. 6. CREB phosphorylation mediated AMPK-induced PGC-1 activation in response to 2-DG. **a** MCF-7 cells were pre-treated with AMPK inhibitor Compound C (20 μ M) for 30 min, followed by 2-DG (25 mM) treatment for 24 h. In some experiments, MCF-7 cells were transfected with human-specific AMPK siRNA or scrambled siRNA for 24 h, followed by 2-DG treatment for another 24 h. Cellular proteins were extracted and subjected to Western blot analysis for phosphorylated CREB, CREB, and cytochrome *c*. **b** MCF-7 cells transfected with Dominant-Negative pCMVCREB133 Vector (S133A) or empty vector (EV) were treated with 25 mM 2-DG for 24 h, were indicated, and then analyzed by Western blot for p-AMPK, total AMPK, cytochrome *c*, nuclear p-CREB, CREB, and PGC-1 β . β -Actin and histone H3 were used as loading controls. The bar graphs above show densitometry analyses of the corresponding Western blot bands. Data presented are mean $\pm$ SD from three independent experiments, with nontreated controls set as 1. * P < 0.05 versus control, # P < 0.05 versus 2-DG-treated groups. **c, d** PPAR α , MCAD, CPT1C, and ACO mRNA levels were assayed using reverse transcription polymerase chain reaction (RT-PCR) or real-time quantitative RT-PCR (qRT-PCR). **e** Intracellular ATP levels in MCF-7 cells with indicated treatments were monitored using a luciferase-based assay. **f** Cell viability of MCF-7 cells with indicated treatments for 48 h was detected by MTT assay. Data are presented as the mean $\pm$ SD for three independent experiments. * P < 0.05 versus control, # P

< 0.05 versus 2-DG-treated groups. **g** Proposed mechanism by which AMPK inhibition enhances 2-deoxy-D-glucose-mediated breast cancer cell death. In breast cancer cells, 2-DG inhibits glycolysis and decreases ATP levels, leading to activation of AMP-activated protein kinase and cAMP response element-binding protein (CREB). In response to activated CREB, activation of the PGC-1 β regulates genes involved in mitochondrial respiration and biogenesis, whereas activation of its downstream targets, ERR α and PPAR α , regulates fatty acid uptake and mitochondrial fatty acid oxidation (FAO) enzymes. The up-regulation of mitochondrial biogenesis and metabolic target genes augments intracellular ATP levels, which partially offset the inhibitory effect of 2-DG on intracellular ATP production and cancer cell growth, leading to cancer cell survival. Thus, the AMPK pathway represents a key metabolic adaptation mechanism in cancer cells. Inhibition of AMPK with Compound C or RNA interference antagonizes metabolic adaptation in response to metabolic stress and potentiates the efficiency of 2-DG to induce cancer cell death