Caloric restriction (CR) is a dietary intervention known to enhance cardiovascular health. The glucose analog 2-deoxy-D-glucose (2-DG) mimics CR effects in several animal models. However, whether 2-DG is beneficial to the heart remains obscure. Here, we tested the ability of 2-DG to reduce cardiomyocyte death triggered by doxorubicin (DOX, 1 μM), an antitumor drug that can cause heart failure. Treatment of neonatal rat cardiomyocytes with 0.5 mM 2-DG dramatically suppressed DOX cytotoxicity as indicated by a decreased number of cells that stained positive for propidium iodide and reduced apoptotic markers. 2-DG decreased intracellular ATP levels by 17.9%, but it prevented DOX-induced severe depletion of ATP, which may contribute to 2-DG-mediated cytoprotection. Also, 2-DG increased the activity of AMP-activated protein kinase (AMPK). Blocking AMPK signaling with compound C or small interfering RNA-mediated knockdown of the catalytic subunit markedly attenuated the protective effects of 2-DG. Conversely, AMPK activation by pharmacological or genetic approach reduced DOX cardiotoxicity but did not produce additive effects when used together with 2-DG. In addition, 2-DG induced autophagy, a cellular degradation pathway whose activation could be either protective or detrimental depending on the context. Paradoxically, despite its ability to activate autophagy, 2-DG prevented DOX-induced detrimental autophagy. Together, these results suggest that the CR mimetic 2-DG can antagonize DOX-induced cardiomyocyte death, which is mediated through multiple mechanisms, including the preservation of ATP content, the activation of AMPK, and the inhibition of autophagy.

Caloric restriction (CR) is the practice of reduced calorie intake in the absence of malnutrition. CR was first reported to extend life span in rats 93 years ago (1). This result has since been reproduced in diverse model systems, including yeast, nematode worms, flies, fish, mice, rats, dogs, and monkeys (2–5). Not surprisingly, enhancing cardiovascular health and preventing tumorigenesis or inhibiting tumor growth appear to be the major mechanisms by which CR retards aging and extends life span (6–11). Indeed, CR was shown to slow aging in Rhesus monkeys by delaying the onset of age-associated pathologies, including cancer and cardiovascular disease (5). Despite the extraordinary ability of CR to prolong life, it is difficult to implement and sustain a CR regimen in humans. Thus, increasing research interest has focused on identifying agents that can mimic CR health-promoting effects without the need to reduce calorie intake. Several compounds have shown potential as a CR mimetic to prolong life in animals (18) despite evidence that 2-DG can significantly extend the life span in Caenorhabditis elegans (19). Regardless of this discrepancy, it remains formally possible that 2-DG may produce beneficial effects on the heart under more acute conditions such as ischemia/reperfusion and chemotherapy-induced cardiac damage.

The glucose analog 2-DG can enter cells and be phosphorylated but not further metabolized, thus competitively blocking glucose utilization presumably mimicking CR effects at the cellular level. In support of this notion, 2-DG has been shown to produce beneficial effects reminiscent of CR in several animal models (12, 14). Specifically, 2-DG can decrease insulin levels and body temperature, two changes that typically occur in animals subjected to CR (15). It can also decrease resting blood pressure and heart rate and markedly enhance cardiovascular adaptation to stress (16). In addition, 2-DG was shown to substantially reduce ischemic brain injury (17). However, long-term use of high dose 2-DG in rats can cause cardiac injury, casting doubt on the potential of 2-DG being used as a CR mimetic to prolong life in animals (18) despite evidence that 2-DG can significantly extend the life span in Caenorhabditis elegans (19). Regardless of this discrepancy, it remains formally possible that 2-DG may produce beneficial effects on the heart under more acute conditions such as ischemia/reperfusion and chemotherapy-induced cardiac damage.

The anthracycline antibiotic doxorubicin (DOX) is a highly effective antitumor agent with a remarkably wide spectrum of activity in human cancers. However, dose-dependent cardiotoxicity of DOX that culminates in congestive heart failure has limited its use in cancer patients (20–22). The exact mechanisms of DOX-induced cardiotoxicity remain incompletely understood, but most studies suggest oxidative stress as a common

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2 Supported by Juvenile Diabetes Research Foundation Research Grant 1-2007-741 and Career Development Grant 1-09-CD-09 from the American Diabetes Association. To whom correspondence should be addressed. Tel.: 605-312-6304; Fax: 605-312-6071; E-mail: Qiangrong.Liang@sanfordhealth.org.
3 The abbreviations used are: CR, caloric restriction; 2-DG, 2-deoxy-D-glucose; mTOR, mammalian target of rapamycin; AMPK, AMP-activated protein kinase; NRC, neonatal rat cardiomyocytes; DOX, doxorubicin; 3-MA, 3-methyladenine; BFA, bafilomycin A1; PARP, poly(ADP-ribose) polymerase; AICAR, 5-aminoimidazole-4-carboxamide riboside; PI, propidium iodide; ACC, acetyl-CoA Carboxylase; eEF2k, elongation factor-2 kinase; AV, autophagic vacuoles; ANOVA, analysis of variance; m.o.i., multiplicity of infection; Cpd C, compound C.
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Vergent mechanism responsible for its pathogenesis. In animal studies, several compounds with antioxidant properties have been shown to reduce DOX cardiotoxicity to some degree (23–25). However, clinical trials have failed to reproduce these results in humans (26), suggesting that mechanisms other than oxidative stress might also contribute to DOX-induced heart failure. Thus, a comprehensive therapy that targets multiple mechanisms, including oxidative stress, may be more efficacious to attenuate DOX cardiotoxicity. In this respect, CR triggers drastic changes in multiple signaling pathways that modulate diverse fundamental physiological processes (3, 27) to induce a general attenuation of oxidative damage (28), inflammation (29), and apoptosis (30). Remarkably, CR has demonstrated an ability to eliminate the mortality and cardiac dysfunction in DOX-treated rats (31). Given the anticancer effect of CR, this result suggests that CR could be incorporated into a DOX-containing therapeutic regimen to increase the anticancer efficacy and at the same time to reduce cardiotoxicity in cancer patients. It is exactly in this context that a CR mimetic may be more feasible because cancer patients, at least those who are diagnosed late, may already suffer from malnutrition and cannot tolerate CR well.

Expectedly, 2-DG has been shown to mimic the antitumor effects of CR. Indeed, inhibition of glycolysis with 2-DG was toxic to various tumor cells (32–34). 2-DG can also enhance the anticancer efficacy of chemotherapy or radiotherapy and at the same time protect normal cells (35–40). In addition, chemotherapeutic regimens with 2-DG as a component have entered phase I/II clinical trials (36, 41). Importantly, 2-DG has been shown to enhance the antitumor activity of DOX in cell culture (42) and in tumor-bearing mice (43). Given the potential cardiovascular benefits of 2-DG (15, 16), these data collectively suggest a viable chemotherapy strategy that uses 2-DG together with other anticancer drugs that have cardiotoxicity such as DOX. It would have a significant clinical impact if the combined use of 2-DG and DOX is proven to selectively destroy tumors while concurrently limiting cardiac damage. As an important step toward this end, we examined the ability of 2-DG to impact DOX-induced cardiotoxicity in cultured cardiomyocytes and explored the potential underlying mechanisms. Our results clearly established the proof-of-principle that the caloric restriction mimetic 2-DG can be used together with DOX to reduce cardiomyocyte injury.

**EXPERIMENTAL PROCEDURES**

**Cardiomyocyte Culture**—Neonatal rat cardiomyocytes (NRC) were cultured as described previously (44). Briefly, NRC were isolated from 0- to 2-day-old Harlan Sprague-Dawley rat neonates using trypsin and DNase. NRC were cultured on gelatinized 60-mm tissue culture dishes, grown for 24 h, and then transfected with siRNA using Lipofectamine RNAiMAX and Opti-MEM in serum-free and antibiotic-free medium, according to the manufacturer’s instructions. Control shRNA adenovirus (AdshBCN1) was created as described previously (44). Control shRNA adenovirus (AdshCon) was generated by cloning a small interfering RNA (siRNA) sequence not having any predicted sequence matches to coding regions in mouse or rat genomes (46).

Unless otherwise indicated, cardiomyocytes were infected with each adenovirus at a multiplicity of infection (m.o.i.) of 100 plaque-forming units for 2 hours (h). Following infection, cardiomyocytes were cultured in DMEM containing 2% bovine serum for an additional 24 h in the case of AdAMPK-CA and AdGFP-LC3, and 48 h in the case of AdshBCN1 and AdshCon before treatment with 2-DG and other drugs.

**Gene Silencing with siRNA**—The silencer select siRNAs targeting rat beclin 1 mRNA (AdshBCN1) was kindly provided by Dr. Tolkovsky (45). Adenovirus expressing the short hairpin RNA (shRNA) targeting rat beclin 1 mRNA (AdshBCN1) was created as described previously (44). Control shRNA adenovirus (AdshCon) was generated by cloning a small interfering RNA (siRNA) sequence not having any predicted sequence matches to coding regions in mouse or rat genomes (46).

Reagents and Antibodies—2-DG, DOX, 3-methyladenine (3-MA), compound C (Cpd C), and propidium iodide (PI) were obtained from Sigma. Rapamycin and bafilomycin A₁ (BFA) were obtained from LC laboratories. 5-Aminoimidazole-4-carboxamide riboside (AICAR) was purchased from Toronto Research Chemicals Inc. The following primary antibodies were purchased from Cell Signaling Technology: cleaved caspase 3; poly(ADP-ribose) polymerase (PARP); AMP-activated protein kinase α (AMPKa), AMPKα1, AMPKα2, phospho-AMPKα (Thr-172), AMPKβ1/2, phospho-AMPKβ1 (Ser-108); acetyl-CoA carboxylase (ACC), phospho-ACC (Ser-79); Akt, phospho-Akt (Ser-473), phospho-Akt (Thr-308); FoxO1, phospho-FoxO1 (Thr-24); FoxO3/4 (Thr-32); glycogen synthase kinase 3β (GSK3β), phospho-GSK3β (Ser-9); mammalian target of rapamycin (mTOR), phospho-mTOR (Ser-2448); p70 S6 kinase (p70S6K), phospho-p70S6K (Thr-389), S6 ribosomal protein (S6), phospho-S6 (Ser-235/236); microtubule-associated protein light chain 3 (LC3); autophagy-related 5 (Atg5), Atg7, Atg12; β-actin; elongation factor-2 kinase (eEF2k), and phospho-eEF2k (Ser-366). We purchased primary antibodies against GATA4, Bcl2 and Beclin 1 from Santa Cruz Biotechnology.

**Measurement of ATP**—The level of intracellular ATP was measured by using a colorimetric/fluorometric assay kit (Abcam, San Francisco). Briefly, cells were lysed in the ATP assay buffer and centrifuged at 15,000 × g for 2 min to pellet insoluble materials. The supernatant was added to a 96-well plate and followed by the addition of 50 μl of the Reaction Mix to each well. The absorbance was read at 550 nm using a Thermomax microplate reader from Molecular Devices. The ATP content was calculated based on a standard curve generated at the same time.

**Replication-deficient Adenoviruses and Infection**—Constitutively active AMPKα2 (AdAMPK-CA) recombinant adenovirus was obtained from Eton BioScience (San Diego). Adenovirus encoding GFP-LC3 (AdGFP-LC3) was kindly provided by Dr. Tolkovsky (45). Adenovirus expressing the short hairpin RNA (shRNA) targeting rat beclin 1 mRNA (AdshBCN1) was created as described previously (44). Control shRNA adenovirus (AdshCon) was generated by cloning a small interfering RNA (siRNA) sequence not having any predicted sequence matches to coding regions in mouse or rat genomes (46).
The concentrations of siRNAs were chosen based on dose-response studies. The media were replaced 24 h later with fresh 2% serum containing DMEM, and the cells were then treated with 2-DG or other drugs.

**Western Blot Analysis**—Whole cell lysates were prepared in lysis buffer as described previously (47). The lysates (40–50 μg) were resolved by SDS-PAGE. Proteins were transferred to PVDF membrane (GE Healthcare), which was incubated with the primary antibody followed by horseradish peroxidase-conjugated secondary antibody. Specific proteins were detected by chemiluminescent detection using an ECL Advanced Western blotting kit (GE Healthcare). Protein abundance on Western blots was quantified by densitometry with the Quantity One program from Bio-Rad.

**Cell Death Assay**—Cardiomyocyte death was measured by staining with PI (1 μg/ml) that was added directly to the culture media. PI enters dead cells through disrupted membranes to bind to DNA. Cardiomyocytes were photographed under both phase contrast and fluorescent conditions. The PI-positive cells (stained red) were expressed as a percentage of the total number of cells (200–250 counted under phase contrast).

**Apoptosis Assays**—DOX-induced apoptosis was determined by a DNA ladder assay and the cleavage of caspase 3 and PARP. For the DNA laddering assay, the cells were harvested, and DNA was extracted. A semiquantitative PCR-based DNA laddering kit from Maxim Biotech, Inc. (San Francisco), was used to assess the degree of apoptosis. Cleaved caspase 3 and PARP were determined by Western blot analysis.

**Autophagy Assay**—When autophagy was induced, microtubule-associated protein light chain 3 (LC3), an 18-kDa mammalian homolog of autophagy-related protein 8 (Atg8), was processed from LC3-I to LC3-II and conjugated to the nascent autophagosome membrane. LC3-II remained associated with autophagic vacuoles (AVs) until degraded by lysosomal proteases (48). Therefore, autophagy can be measured by the amount of LC3-II formed. For detection of LC3-II, cells lysates were prepared, and 30 μg of total proteins were subjected to Western blot analysis using a polyclonal anti-LC3 antibody. To determine whether the accumulation of LC3-II was caused by an enhanced autophagosome formation rather than an impaired degradation, we measured the difference in LC3-II protein levels in the absence and presence of the lysosomal inhibitor BFA (50 nm). This difference is referred to as autophagic flux that reflects the number of AVs that are delivered to and degraded in the lysosome (49). Additionally, autophagy has been routinely visualized by fluorescence imaging of the GFP-LC3 fusion protein that is transfected into cells (50). We infected cardiomyocytes with AdGFP-LC3 and performed the indicated experiments 24 h later. GFP-LC3 images were captured at ×600 using an FV1000 Olympus confocal microscope. For population analysis, cells were inspected at ×200 magnification, and the positive cells were expressed as the percentage of cells that have >25 GFP-LC3 dots (representing AVs) over the number of total GFP-expressing cells examined. At least 200 cells were scored in each of five or more independent experiments. BFA was also used in GFP-LC3 related experiments to assess autophagic flux.

Statistical Analysis—Quantitative data were presented as the means ± S.E. Differences between experimental groups were examined by one- or two-way analysis of variance (ANOVA) followed by the Bonferroni post test using Prism software (GraphPad). For all analyses, p < 0.05 were considered statistically significant.

**RESULTS**

**2-DG Protected against DOX-induced Cardiomyocyte Death**—Numerous studies have suggested cardiomyocyte death, including both apoptosis and necrosis as one of the most prominent features of DOX-induced cardiac toxicity (51). Here, we evaluated DOX-induced cardiomyocyte death by using PI staining, which estimates the number of dead cells regardless of the cause of death, and apoptosis, which was determined by DNA laddering and cleavage of caspase 3 and PARP. 2-DG reproduced the beneficial effects of CR in several animal models (12, 14) and was able to attenuate DOX induced apoptosis in the mouse small intestinal epithelium (52). To test if 2-DG can affect DOX-induced cardiomyocyte death, we pretreated NRC cells (cultured in DMEM with 2% bovine serum) with 500 μM 2-DG (an effective dose determined in pilot studies) for 24 h. DOX (1 μM) was then added, and the cells were cultured for another 16 h. As expected, DOX treatment led to increased PI-positive cells (Fig. 1, A and B), enhanced DNA laddering (Fig. 1C), and increased cleavage of caspase 3 (cCasp3) and PARP (Fig. 1D). Although 500 μM 2-DG did not have any effect at base line, it substantially attenuated DOX-induced cardiomyocyte death as indicated by reduced PI-positive cells (from 36.8 ± 5.4 to 14.8 ± 2.7, n = 4, p < 0.01, Fig. 1, A and B), attenuated DNA laddering (Fig. 1C), and decreased cleavage of caspase 3 and PARP (Fig. 1D). These results confirmed the ability of 2-DG to reduce DOX-induced cardiotoxicity.

**2-DG Attenuated DOX-induced ATP Depletion**—2-DG mimics CR by competitively inhibiting glucose usage, thereby affecting cellular energy metabolism. We assessed the impact of 500 μM 2-DG on intracellular ATP levels. As shown in Fig. 2A, 2-DG treatment resulted in a 17.9% reduction in ATP content compared with saline control (from 3.66 ± 0.15 to 3.01 ± 0.22, n = 6, p < 0.05), consistent with its role as a CR mimetic. DOX cardiotoxicity has been attributed at least partly to ATP depletion (53), and CR is able to mitigate DOX-induced cardiac damage and ATP depletion (31). These observations suggest that similar to CR, 2-DG may antagonize DOX cardiotoxicity through improved ATP homeostasis. To test this possibility, we treated cardiomyocytes with DOX and 2-DG either individually or in combination, and then measured the ATP contents. As shown in Fig. 2B, despite its ability to moderately reduce ATP concentration (by 17.9%), 2-DG completely prevented DOX-induced ATP depletion (DOX 36.7% versus 2-DG + DOX 17.1%, n = 6, p < 0.01). This result is in line with an earlier study in which 2-DG-induced reduction in ATP levels protected renal proximal tubule cells against a more severe ATP depletion during ischemia (54). In summary, one mechanism by which 2-DG reduces DOX cardiotoxicity may be attributable to its ability to prevent DOX-induced ATP depletion.
AMPK Activation Is Essential for the Protective Effects of 2-DG. To further explore the molecular mechanisms underlying the protective effects of 2-DG, we determined if 2-DG can mimic CR to affect several important signaling pathways in cultured NRC. Cardiomyocytes were treated with 2-DG for 40 h and then harvested for Western blot analysis. As shown in Fig. 2C, 2-DG reduced the phosphorylation of Akt (Ser-473 and Thr-308) and its downstream effectors FoxO1 (Thr-24) and GSK3β (Ser-9). Middle panel, mTOR signaling was inhibited by 2-DG as shown by reduced phosphorylation of mTOR downstream effectors P70S6K (Thr-389) and S6 (Ser-235). Bottom panel, 2-DG enhanced AMPK signaling as shown by increased phosphorylation of AMPKα (Thr-172), AMPKβ (Ser-108), and its downstream effector ACC (Ser-79).

**FIGURE 1.** 2-DG protected from DOX-induced cardiomyocyte death. NRC were cultured in DMEM with 2% bovine serum and pretreated with 500 μM 2-DG for 24 h, after which 1 μM DOX was added and cells were cultured for another 16 h. Cardiomyocyte death was determined by PI staining (A, phase contrast and fluorescent images; B, quantification of PI-positive cells), DNA laddering (C), and cleavage of caspase 3 (cCasp3) and PARP (D). Data in B and D are expressed as mean ± S.E. and analyzed by two-way ANOVA (n = 4) followed by the Bonferroni post test. *, p < 0.05; **, p < 0.01 compared with control (CON) group; ##, p < 0.01 compared with DOX group. The scale bars in A represent 200 μm.

**FIGURE 2.** Effects of 2-DG on ATP content and Akt, mTOR, and AMPK signaling. A. Cardiomyocytes were treated with 2-DG (500 μM) for 40 h. The intracellular ATP levels were measured using an ATP colorimetric/fluorometric assay kit. Data are expressed as mean ± S.E. and analyzed by t test (n = 6). *, p < 0.05 compared with control (Con) group. B. Cardiomyocytes were pretreated with 2-DG (500 μM) for 24 h and then treated with 1 μM DOX for another 16 h. The ATP levels are expressed as mean ± S.E. and analyzed by one-way ANOVA (n = 6) followed by the Bonferroni post test. *, p < 0.05; **, p < 0.01 compared with control group; ##, p < 0.01 compared with DOX group. C. Cardiomyocytes were treated with 2-DG (500 μM) for 40 h and then harvested for Western blot analysis. Top panel, Akt signaling was inhibited by 2-DG as shown by reduced phosphorylation of Akt (Ser-473 and Thr-308), FOXO1 (Thr-24), and GSK3β (Ser-9). Middle panel, mTOR signaling was inhibited by 2-DG as shown by reduced phosphorylation of mTOR downstream effectors P70S6K (Thr-389) and S6 (Ser-235). Bottom panel, 2-DG enhanced AMPK signaling as shown by increased phosphorylation of AMPKα (Thr-172), AMPKβ (Ser-108), and its downstream effector ACC (Ser-79).
GSK3β (Ser-9), suggesting that 2-DG largely inhibited Akt signaling. Similarly, 2-DG suppressed mTOR signaling as indicated by reduced phosphorylation of mTOR downstream targets S6K (Thr-389) and S6 (Ser-235). Given the pro-survival nature of Akt and mTOR, the inhibition of these two pathways may not be directly responsible for 2-DG-induced cardioprotective effects. By contrast, 2-DG markedly enhanced the activity of AMPK as shown by the increased phosphorylation of AMPKα (Thr-172), AMPKβ (Ser-108), and its downstream target ACC (Ser-79). AMPK is a highly conserved heterotrimeric protein kinase composed of a catalytic α subunit and two regulatory subunits (β and γ). AMPK is an energy sensor that can be activated by reduced ATP or increased AMP content. Importantly, AMPK has been shown to protect the heart under various conditions such as ischemia (55–57). Thus, the fact that 2-DG activates AMPK suggests that AMPK may mediate 2-DG-induced cardioprotection.

To test if AMPK plays a role in 2-DG-induced survival, we treated NRC with 5 µM compound C (Cpd C), a commonly used AMPK inhibitor (58), which was followed by 2-DG and DOX treatment. The effectiveness of 5 µM Cpd C to inhibit AMPK activity is shown by dramatically diminished phosphorylation of AMPKα and β subunits (pAMPKα and pAMPKβ). Cardiomyocyte death was determined by PI staining (B, phase contrast and fluorescent images; C, quantification of PI-positive cells), DNA laddering (D), and cleavage of caspase 3 (cCasp3) and PARP (E). Data in C (percentage PI-positive cells) and E (densitometric analysis of Western blots of cCasp3 and PARP) are expressed as mean ± S.E. and analyzed by one-way ANOVA (n = 4 –5) followed by the Bonferroni post test. **, p < 0.01 compared with control group; ##, p < 0.01 compared with DOX group; $$$, p < 0.01 compared with 2-DG plus DOX group. The scale bars in B represent 200 µm.

FIGURE 3. Inhibition of AMPK attenuated the protective effects of 2-DG. Cardiomyocytes were pretreated with 5 µM Cpd C (AMPK inhibitor) for 6 h and followed by 2-DG and DOX treatment. The effectiveness of 5 µM Cpd C to inhibit AMPK activity is shown by dramatically diminished phosphorylation of AMPKα and β subunits (A, pAMPKα and -β). Cardiomyocyte death was determined by PI staining (B, phase contrast and fluorescent images; C, quantification of PI-positive cells), DNA laddering (D), and cleavage of caspase 3 (cCasp3) and PARP (E). Data in C (percentage PI-positive cells) and E (densitometric analysis of Western blots of cCasp3 and PARP) are expressed as mean ± S.E. and analyzed by one-way ANOVA (n = 4 –5) followed by the Bonferroni post test. **, p < 0.01 compared with control group; ##, p < 0.01 compared with DOX group; $$$, p < 0.01 compared with 2-DG plus DOX group. The scale bars in B represent 200 µm.
AMPK Is Sufficient to Protect against DOX Cardiotoxicity—If AMPK mediates the protective effects of 2-DG, AMPK itself should be able to attenuate DOX-induced cardiomyocyte death. Therefore, we tested the ability of AMPK to provide cardioprotection by using AICAR, a potent AMPK activator (58), and adenovirus-mediated expression of a constitutively active AMPKα/H9251 mutant (AMPK-CA). This mutant encodes a truncated AMPKα/H9251 subunit (amino acid 1–312), which lacks the H9252/H9253 binding domain and the autoinhibitory domain but still retains the phosphorylation site for upstream kinase (59). The ability of AICAR to activate AMPK in a dose-dependent manner is shown in Fig. 5A by increased phosphorylation of AMPKα and -β. As suspected, either AICAR (1 mM) or AMPK-CA (100 m.o.i.) alone is sufficient to reduce DOX-induced cardiomyocyte death as indicated by multiple parameters (Fig. 5, B–E). In addition, overexpression of AMPK-CA did not further enhance the protective effects of 2-DG. Collectively, our data suggest that AMPK is not only sufficient to confer cardioprotection on its own but also necessary for 2-DG to protect against DOX-induced cardiomyocyte death.

2-DG Induced Autophagy in Neonatal Rat Cardiomyocytes—Autophagy is the primary cellular pathway for lysosomal degradation and recycling of long lived proteins and organelles. CR has been shown to induce autophagy in previous studies (3, 60, 61). We show here that the CR mimetic 2-DG increased the activity of AMPK (Fig. 2C), a positive regulator of autophagy (62), and reduced the activity of Akt and mTOR (Fig. 2C), two pathways that negatively regulate autophagy. Thus, it is very likely that 2-DG could induce autophagy in cardiomyocytes as it did in other cell types (63, 64). To test if 2-DG can indeed trigger autophagy in cardiomyocytes, we infected NRC with AdGFP-LC3 and then treated cells with 500 μM 2-DG. As suspected, 2-DG induced the formation of numerous GFP-LC3 punctate structures or dots indicative of AVs in the cytoplasm (Fig. 6A). We counted cells with more than 25 dots, an arbitrary cutoff number that defines a cell as positive for increased AVs. 2-DG increased the percentage of AV-positive cells from 12.8 ± 3.9% at baseline to 32.5 ± 6.9% (Fig. 6B). To rule out the possibility that the increased number of AVs is due to an inhibited AV degradation rather than AV formation, we measured...
endogenous autophagic flux by the difference in the levels of LC3-II protein in the absence and presence of BFA (50 nM), an inhibitor of AV degradation (65). As shown in Fig. 6C, LC3-II was increased in 2-DG-treated cells compared with untreated cells. BFA further increased LC3-II levels in both 2-DG and control cells, but the increase in 2-DG-treated cells was much larger than that in control cells (Fig. 6D). The net increase in LC3-II by BFA was defined as autophagic flux and plotted in Fig. 6E (control 1.25 ± 0.24 versus 2-DG 2.78 ± 0.49, n = 4, p < 0.01). The results indicate that 2-DG indeed accelerated autophagic flux in cardiomyocytes.

**Autophagy Induction Is Not Necessary for 2-DG to Protect against DOX Cardiotoxicity**—CR may induce autophagy as a means to promote survival and longevity (3, 60, 61). Consistently, autophagy is required for the survival of starved neonatal mice (66) and for CR-mediated life span extension in *C. elegans* (67). Given the ability of 2-DG to induce autophagy (Fig. 6), it is possible that autophagy induction may contribute to 2-DG-mediated cardioprotection against DOX toxicity. Therefore, we determined the functional significance of autophagy in the protective effects of 2-DG by using 3-MA (2.5 mM), a class III PI3K inhibitor that blocks autophagy initiation, and short hairpin RNA (shRNA)-mediated gene silencing of *beclin 1* (shBCN1), a gene essential for autophagy. The shBCN1 was delivered into cardiomyocytes by adeno viral infection (100 m.o.i.) as described previously (44). A m.o.i. of 100 was chosen because it knocked down Beclin 1 protein by 70% without affecting cell viability (Fig. 7A).

The ability of shBCN and 3-MA to inhibit autophagic flux was determined by the difference in the levels of endogenous LC3-II protein or in the numbers of AV-positive cells in the absence and presence of BFA. As shown in Fig. 7, LC3-II was increased from 1.05 ± 0.02 to 2.47 ± 0.1 by BFA (Fig. 7C), resulting in an autophagic flux of 1.47 ± 0.12 (Fig. 7D) in control cells. This base-line autophagic flux was inhibited 49.9 ± 6.3% by 3-MA and 54.7 ± 5.3% by shBCN1 (n = 4, p < 0.01). Similarly, as shown in Fig. 7, the AV-positive cells (Fig. 7E) was increased from 12.3 ± 0.79 to 30.4 ± 1.48% by BFA (Fig. 7F), leading to an autophagic flux of 18.1 ± 1.15% (Fig. 7G), which was suppressed 36.3 ± 5.5% by 3-MA and 42.0 ± 6.8% by shBCN1 (n = 4, p < 0.005).
results indicate that either 3-MA or shBCN1 is sufficient to inhibit autophagic flux in cardiomyocytes. The ability of 2-DG to antagonize DOX cardiotoxicity was determined with or without autophagy inhibition by 3-MA or shBCN1. As shown in Fig. 8, 2-DG drastically attenuated DOX-induced cardiomyocyte death as indicated by reduced PI-positive cells (Fig. 8, A and B), diminished DNA laddering (Fig. 8C), and decreased cleavage of caspase 3 and PARP (Fig. 8D). However, the protective effects of 2-DG were not affected by 3-MA (2.5 mM) or shBCN1, indicating that 2-DG does not require autophagy to protect against DOX cardiotoxicity. In other words, autophagy induction is irrelevant to the cytoprotective effects of 2DG.

**2-DG-mediated Cytoprotection Was Associated with an Inhibition of DOX-induced Detrimental Autophagy**—If autophagy is indeed the major mechanism of DOX cardiotoxicity, how could 2-DG reduce DOX toxic effects when 2-DG itself is able to induce autophagy? To resolve this paradox, we determined...
and compared the autophagic activities in cardiomyocytes treated with 2-DG (500 \mu M) and DOX (1 \mu M) either separately or in combination. Surprisingly, despite its ability to activate autophagy on its own, 2-DG antagonized DOX-induced detrimental autophagy instead of further enhancing it. Specifically, cardiomyocytes were infected with AdGFP-LC3 and then treated with drugs. Either DOX or 2-DG alone increased the number of AVs in cardiomyocytes as indicated by the GFP-LC3 dots. However, when cells were treated with both 2-DG and DOX, the number of AVs was drastically reduced (Fig. 10A), suggesting an antagonistic rather than an additive effect of 2-DG and DOX on autophagic activity. We further measured the autophagic flux under these treatments by the difference in the percentages of AV-positive cells (defined as having more than 25 GFP-LC3 dots) in the absence and presence of BFA. As shown in Fig. 10B, at base line, the AV-positive cells were increased to 25.4 ± 4.1% by BFA, resulting in an autophagic flux of 13.8%. DOX and 2-DG each increased AV-positive cells from base-line level to 38.5 ± 5.0 and 29.0 ± 3.7%, respectively, which was further elevated to 65.6 ± 7.5 and 55.1 ± 5.9% by BFA, giving rise to a flux of 27.2 and 26.2%, almost doubled compared with base-line flux. Strikingly, when treated with both 2-DG and DOX, AV-positive cells were just slightly increased to 20.2 ± 2.4%, much less than that
induced by 2-DG or DOX alone. Even after BFA treatment, this number was elevated only to 36.2 ± 6.2%, giving rise to a flux of 16.0% (Fig. 10B), similar to base-line flux (13.8%). To better appreciate the differential effects of the drugs, we plotted in Fig. 10C the calculated autophagic flux, i.e., the difference in the percentages of AV-positive cells with and without BFA (BFA-CON). Additionally, Western blot analysis indicated that either DOX or 2-DG alone increased endogenous LC3-II protein levels, but 2-DG plus DOX together brought LC3-II back to the control levels (Fig. 10D). Collectively, these results provide convincing evidence indicating that 2-DG antagonized DOX-induced detrimental autophagy.

Mechanisms That Mediate the Ability of 2-DG to Antagonize DOX-induced Autophagy—Our results not only established the harmful nature of DOX-induced autophagy, but they also suggested that 2-DG acts through distinct signaling pathways to induce a type of autophagy that can diminish DOX-induced detrimental autophagy. To explore the underlying molecular mechanisms, we examined several pathways known to either positively regulate autophagy such as AMPK or negatively regulate autophagy such as Akt and mTOR. Cardiomyocytes were treated with 2-DG and DOX either individually or in combination, and the functional states of these pathways were examined by Western blot analysis. DOX had no obvious effect on AMPK activity and markedly enhanced the activity of Akt and mTOR as shown by the increased phosphorylation of Akt, FoxO1, GSK3β, and P70S6K (Fig. 10D), suggesting that AMPK, Akt, and mTOR signaling pathways are unlikely directly responsible for DOX-induced autophagy. By contrast, 2-DG increased the activity of AMPK and reduced the activity of Akt and mTOR, indicating that these pathways may be involved in 2-DG-induced autophagy. However, when cells were treated with both DOX and 2-DG, the effects of 2-DG on these pathways still persisted albeit to a lesser degree due to the presence of DOX. Apparently, the changes in these pathways cannot explain the mechanism by which 2-DG antagonizes DOX-induced autophagy. Therefore, we further determined the expression levels of several autophagy-related genes, including Atg5, Atg7, and Atg12. All of them are essential for autophagy induction. Notably, the protein levels of Atg5, Atg12, and Atg5-Atg12 complex were increased by either DOX or 2-DG, which correlated with increased autophagic activity as shown by LC3-II levels (top row of Fig. 10D). Importantly, the effect of either DOX or 2-DG alone on the expression of Atg5, Atg12, or Atg5-
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Atg12 complex was abolished when cells were treated with both drugs at the same time, suggesting that the ability of 2-DG to attenuate DOX-induced autophagy is at least partly mediated by the reduced protein levels of Atg5 and Atg12. Additionally, we measured the protein levels of transcription factors GATA4, Bcl2, and BH3-only protein Bad under these conditions. GATA4 and Bcl2 are negative regulators of autophagy (44), and Bad is a pro-apoptotic protein that can also induce autophagy (72, 73). We found that DOX reduced GATA4 and Bcl2 but increased Bad protein levels, consistent with an induction of autophagy (data not shown). Although 2-DG on its own did not have any effect on GATA4, Bcl2, or Bad, it undermined the ability of DOX to deplete GATA4 and Bcl2 or to induce Bad.

Finally, 2-DG has been shown to induce autophagy in tumor cells through eEF2k (63, 74). Therefore, we tested this possibility in cardiomyocytes. In contrast to the results in tumor cells, 2-DG itself did not affect the protein levels of eEF2k or its phosphorylation at Ser-366. Instead, 2-DG prevented DOX-induced reduction in eEF2k phosphorylation (data not shown). Because phosphorylation inactivates eEF2k, concurrently promoting protein translation and inhibiting autophagy, this result provides additional insight into the mechanism by which 2-DG antagonizes DOX-induced autophagy.

### DISCUSSION

Excessive calorie intake posts an increased risk for age-related pathologies, including cancer, diabetes, and cardiovascular disease. Conversely, CR can enhance human health by delaying the onset of these diseases. As a result, intense research has focused on identifying pharmacological agents that can mimic the beneficial effects of CR. 2-DG is one of the first compounds that have demonstrated this ability. Indeed, 2-DG can extend life span in C. elegans (19) and mimic many beneficial effects of CR in rodent models (15–17, 75). In this study, we demonstrated that 2-DG can dramatically attenuate cardiomyocyte death induced by DOX, a widely used anticancer agent whose clinical use is limited by a dose-dependent cardiomyopathy that often leads to congestive heart failure and death (20–22). Our results suggest a novel antitumor chemotherapeutic strategy in which 2-DG can be used to reduce the toxicity of DOX to non-tumor tissues, including the heart, despite evidence that 2-DG may not be a viable approach to prolong life in animals (18).

Additionally, 2-DG has been shown to enhance the antitumor activity of DOX in cell culture (42) and in tumor-bearing mice (43), further supporting that the combined use of 2-DG and DOX is likely an effective approach that can selectively destroy tumors while concurrently limiting cardiac damage. Of note, dexrazoxane is the only drug approved by the Food and Drug Administration to reduce DOX cardiotoxicity (76), yet its combined use with DOX has been limited, due to fears of its negative effect on antitumor efficacy of DOX (77). Without this concern, 2-DG may turn out to be a superior drug that will find increased use in DOX antitumor therapy in the near future.

The mechanisms by which 2-DG protects against DOX-induced cardiomyocyte death appear to be complicated, likely involving multiple cellular and molecular pathways. 2-DG itself can moderately reduce ATP production, but it completely prevented the DOX-induced more severe ATP depletion (Fig. 2, A and B), consistent with its protective role in ischemic cellular injury (54). Like CR, 2-DG-induced mild ATP reduction may constitute an important part of the beneficial adaptive response termed hormesis. Briefly, in response to mild stress such as CR or CR mimetics, cellular defense and repair systems are enhanced in a coordinated fashion to increase stress resistance and to retard aging (3, 27, 78). Thus, CR induces a general attenuation of oxidative damage (28), inflammation (29), and apoptosis (30). These processes are associated with drastic signaling changes in multiple pathways, including activation of AMPK, and inhibition of insulin, insulin-like growth factor 1 (IGF-1), Akt, and mTOR (3, 27, 79). Consistently, this study demonstrated that 2-DG increased AMPK activity although it...
inhibited Akt and mTOR signaling in cardiomyocytes (Fig. 2C). Together, these signaling changes may act separately or synergistically to protect against DOX-induced cardiomyocyte death. However, the relative importance of individual pathways in 2-DG-induced cytoprotection remains unclear.

AMPK is a highly conserved heterotrimeric protein kinase composed of a catalytic α subunit and two regulatory subunits (β and γ). 2-DG-induced relative energy deficits result in elevated intracellular AMP, which binds to AMPK, leading to phosphorylation and activation of AMPK. Activated AMPK impacts multiple metabolic pathways to maintain an energy homeostasis conducive to cell survival (80). Not surprisingly, AMPK has been implicated in CR-induced longevity (3, 27, 81) and has been shown to protect the ischemic heart (55, 56). Thus, it is highly likely that enhanced AMPK signaling may contribute to 2-DG-induced cardioprotection. Indeed, using both a chemical inhibitor and siRNA-mediated knockdown of AMPK, we demonstrated that AMPK is required for 2-DG to provide protection against DOX cardiotoxicity, suggesting that therapeutic strategies aiming to increase AMPK activity would be expected to reduce DOX cardiotoxicity. In this respect, metformin, a commonly prescribed drug for type 2 diabetes, is able to protect the heart under various pathological conditions (82–87). It can also enhance the ability of DOX to reduce tumor mass (88). All these effects have been associated with the ability of metformin to activate AMPK. Thus, metformin may turn out to be an AMPK activator that may increase the antitumor activity of DOX and simultaneously reduce its cardiotoxicity. In fact, metformin has been considered another CR mimetic that may prolong life by inhibiting tumor and enhancing cardiovascular health (68, 81). It is of interest to determine whether other AMPK activators such as AICAR could have similar actions as 2-DG and metformin.

Similar to CR, 2-DG limits cellular energy availability and affects several signaling pathways in a coordinated fashion that favors autophagy induction. Indeed, we confirmed the ability of 2-DG to induce autophagy in cardiomyocytes (Fig. 6), consistent with findings in other cell types (63, 64). Autophagy is the primary cellular pathway for lysosomal degradation and recycling of long lived proteins and organelles, which plays an extremely important role in cytoplasmic quality control and maintaining cellular homeostasis under both normal and patho-
ological conditions. Nevertheless, activation of autophagy could be either beneficial or detrimental depending on the particular cell type, the subcellular environment, the nature and intensity of the stimuli, and the levels of autophagy induced (55, 69, 70). Thus, the functional significance of autophagy induction in different contexts has to be individually determined. In tumor cells, 2-DG has an overall cytotoxic effect, but it simultaneously stimulates autophagy, which turns out to be pro-survival because inhibiting autophagy accentuates 2-DG cytotoxicity (63). This led us to surmise that 2-DG-induced autophagy may be responsible, at least in part, for its ability to antagonize DOX-induced cardiomyocyte death. Surprisingly, however, inhibition of autophagy with siRNA-mediated depletion of beclin 1 or the chemical inhibitor 3-MA did not affect the protective effects of 2-DG (Fig. 8), suggesting that activation of autophagy is not necessary for 2-DG to reduce DOX cardiotoxicity. In other words, autophagy induction is irrelevant to the cytoprotective effects of 2DG. More intriguingly, despite its ability to activate autophagy on its own, 2-DG diminished DOX-induced detrimental autophagy (Fig. 10).

It is very clear that 2-DG and DOX act through distinct signaling pathways to induce autophagy of different natures. 2-DG-induced autophagy is associated with activation of AMPK, inhibition of Akt and mTOR, and increased expression of Atg5 and Atg12. In contrast, DOX-triggered autophagy is detrimental and attributable to increased expression of Atg5, Atg12, and Bad, diminished protein levels of GATA4 and Bcl2, and reduced phosphorylation of eEF2k (Fig. 10D) (data not shown). When cardiomyocytes were treated with both 2-DG and DOX, autophagy induction by either drug alone was largely prevented (Fig. 10, A–D), which correlated with nearly normalized protein levels of Atg5, Atg12, GATA4, Bcl2, and Bad as well as restored phosphorylation of eEF2k (Fig. 10D) (data not shown). Given their importance in the regulation of autophagy (44, 72–74), the specific changes in Atg5, Atg12, GATA4, Bcl2, and eEF2k may collectively underlie the mechanism by which 2-DG attenuates DOX-induced autophagy. Of note, GATA4 may play a central role in the prevention of DOX-induced autophagy by 2-DG because GATA4 positively regulates Bcl2 (46) and represses DOX-induced expression of Atg5 and Atg12 genes (44). However, it remains largely unknown how 2-DG is able to restore the expression of GATA4 and Bad as well as the phosphorylation of eEF2k. Further studies are clearly warranted to elucidate the molecular mechanisms that mediate the effects of 2-DG on these autophagy regulatory pathways.

In summary, the CR mimetic 2-DG is sufficient to protect against DOX-induced cardiomyocyte death, which is mediated through multiple mechanisms, including the preservation of ATP content, the activation of AMPK, and the inhibition of autophagy. It is possible that additional mechanisms yet to be identified may also contribute to the protective effect of 2-DG considering its pleiotropic effects on several neurohormonal systems and multiple signaling pathways. Given the well-established anti-cancer properties of 2-DG, the results from this study suggest that the combined use of 2-DG and DOX may turn out to be a viable regimen that can selectively destroy tumors while concurrently limiting cardiac damage. This possibility is currently being tested in our ongoing experiments using tumor-bearing animal models.

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