Dichloroacetate Ameliorates Cardiac Dysfunction Caused by Ischemic Insults Through AMPK Signal Pathway—Not Only Shifts Metabolism

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ABSTRACT

Dichloroacetate (DCA), an inhibitor of pyruvate dehydrogenase kinase (PDK), regulates substrate metabolism in the heart. AMP-activated protein kinase (AMPK) is an age-related energy sensor that protects the heart from ischemic injury. This study aims to investigate whether DCA can protect the heart from ischemic injury through the AMPK signaling pathway. Young (3–4 months) and aged (20–24 months) male C57BL/6J mice were subjected to ligation of the left anterior descending coronary artery (LAD) for an *in vivo* ischemic model. The systolic function of the hearts was significantly decreased in both young and aged mice after 45 min of ischemia and 24 h of reperfusion. DCA treatment significantly improved cardiac function in both young and aged mice. The myocardial infarction analysis demonstrated that DCA treatment significantly reduced the infarction size caused by ischemia/reperfusion (I/R) in both young and aged mice. The isolated-cardiomyocyte experiments showed that DCA treatment ameliorated contractile dysfunction and improved the intracellular calcium signal of cardiomyocytes under hypoxia/reoxygenation (H/R) conditions. These cardioprotective functions of DCA can be attenuated by inhibiting AMPK activation. Furthermore, the metabolic measurements with an *ex vivo* working heart system demonstrated that the effects of DCA treatment on modulating the metabolic shift response to ischemia and reperfusion stress can be attenuated by inhibiting AMPK activity. The immunoblotting results showed that DCA treatment triggered cardiac AMPK signaling pathway by increasing the phosphorylation of AMPK's upstream kinase liver kinase B1 (LKB1) under both sham operations and I/R conditions. Thus, except from modulating metabolism in hearts, the cardioprotective function of DCA during I/R was mediated by the LKB1-AMPK pathway.

Key words: ischemia-reperfusion; dichloroacetate; AMP-activated protein kinase; metabolism.

Cardiovascular diseases (CVD) are a leading cause of death worldwide, especially in developed countries and regions. Even though the strategy of therapy is optimized, improving heart function, rescuing myocardium and decreasing infarct size is still a challenge for clinical practice. These are strongly associated with a patient's long-term results, especially in the elderly population (Shah *et al.*, 2007; Terkelsen *et al.*, 2009). Hence, more

researchers have focused on the efficiency of utilizing energy substrates in the heart, which could mediate injury or provide protection during I/R (Liu *et al.*, 2002; Lopaschuk *et al.*, 1990; Ussher *et al.*, 2012).

Ever since the early twentieth century, Dichloroacetate (DCA) has been known to have a variety of functions in pharmacology. However, none researches into its role as a clinical drug

were carried out until its discovery in lowering glucose was found to be beneficial in diabetic subjects in the 1970s, followed by identification of its metabolism attributes of glucose and lipids (Stacpoole, 1969). As an analog of pyruvate, it is thought that DCA inhibits PDK and promotes an upregulation of PDH, thereby facilitating glucose oxidation (Kankotia and Stacpoole, 2014). Today, DCA is used in the clinic for congenital lactic acidosis and cancer therapy (Allende-Vega et al., 2015; Michelaki et al., 2010; Stacpoole, 2011). Others have reported that administration of DCA had a protective function during I/R, although details of its mechanism are still not well understood (Sun et al., 2016; Ussher et al., 2012).

AMPK is an energy sensor, regulating the metabolism of glucose and fatty acids in cells, and has been shown to increase glucose uptake and glycolysis during stress (Hayashi et al., 2000; Marsin et al., 2000). Our lab and others have published evidence suggesting that activation of AMPK during I/R has a cardioprotective function (Chen et al., 2018; Kim et al., 2011; Ma et al., 2010; Miller et al., 2008; Morrison et al., 2011; Quan et al., 2017; Russell et al., 2004; Wang et al., 2011). Therefore, can DCA activate AMPK and, if so, does this activation regulate metabolism and provide protection against I/R injury? Based on these questions, we aimed to study the direct effect of DCA on cardiomyocytes and how it influences the signaling pathways in hearts during I/R.

MATERIALS AND METHODS

Animals and in vivo ischemia/reperfusion. Young (3–4 months) male C57BL/6J mice were obtained from Jackson Laboratory. Aged (20–24 months) male C57BL/6 mice were obtained from Charles River. All animal protocols in this study were approved by the Institutional Animal Care and Use Committee of the University of Mississippi Medical Center.

Cardiac-specific deletion of the *Pdha1* gene was generated by breeding pyruvate dehydrogenase (PDH) E1 $\alpha^{flox/flox}$ mice as previously described in Sidhu et al. (2008) and Sun et al. (2016). The inducible cardiac-specific PDH E1 α knockout (PDH-KO) mice (CreER^{T2}-PDH^{flox/flox}) were generated by administering Tamoxifen (TM) injection (0.08 mg/g, intraperitoneal for 5 days) to CreER^{T2}-PDH^{flox/flox} (8 weeks old) mice. PDH^{flox/flox} mice (8 weeks old) were also injected with TM and were used as controls. Two months post-TM injection were allowed for the PDH to degrade before mice were sacrificed for experiments.

Mice were anesthetized with 2%–3% Isoflurane and placed on a heating pad to maintain body temperature at 37°C. After intubation, a ventilator (Harvard Apparatus) was connected. We then performed left lateral thoracotomy to expose the heart. The left anterior descending artery (LAD) was occluded by 8–0 nylon suture for 45 min followed by 24-h reperfusion. A polyethylene tube was placed in the surgical knot to protect the LAD from direct injury during ligation. Success of surgery was confirmed by ischemic repolarization changes in ECG (ST-segment elevation) and blanching of the left ventricle. Twenty minutes before ischemia, 100 mg/kg DCA (Sigma) was injected to mice via intraperitoneal (i.p.). For ischemia/reperfusion model, a 50 mg/kg DCA injection was given through i.p. every hour for 8 h postischemia, and then maintained dosage through 110 mM DCA within the drinking water for the following 16 h (Ussher et al., 2012). For ischemia model without reperfusion, the hearts were harvested after 10 min ischemia. The ischemic region of the left ventricle was isolated before being flash frozen in liquid nitrogen for further signal pathway analysis by immunoblotting.

Mice randomly selected from young wild type (WT) were treated with compound C (ApexBio, B1372), a potent inhibitor of AMPK, and labeled as YC group. 0.1 μ g/g of Compound C was applied through i.p. injection 1 h before experiments to inhibit the AMPK signaling pathway (Liu et al., 2016). The same volume of 1X phosphate buffered saline (PBS) was used as vehicle.

Immunoblotting. Immunoblotting and immunoprecipitation were performed as previously described in our publications (Morrison et al., 2015; Quan et al., 2017; Yang et al., 2016). Briefly, the concentration of proteins was measured by using a Bradford dye-binding method (Bio-Rad). The target proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (Millipore). Primary rabbit antibodies p-LKB1 (3482s), LKB1 (3047s), p-AMPK α (2535s), AMPK α (2532s), p-acetyl-CoA carboxylase (p-ACC) (3661s), ACC (3676s), PDK (3820s), and PDH E1 α (3205s) were purchased from Cell Signaling and p-PDH E1 α (ABS2082) was purchased from EMD Millipore. Horseradish peroxidase (HRP)-coupled antirabbit secondary antibody was purchased from Cell Signaling.

Infarct size assessment. After 24 h of reperfusion, mice were anesthetized and ventilated as aforementioned. The hearts were then excised and stained with 2,3,5-triphenyltetrazolium (TTC) and Evan's blue dye. The hearts were left in 10% formaldehyde overnight, then fixed, sectioned into 1 mm slices, photographed with a Leica microscope (Leica Microsystems), and analyzed with Image J software (National Institutes of Health) (Morrison et al., 2011; Quan et al., 2017). The region stained blue from Evans Blue signified the nonischemic region whereas the area stained red from TTC indicated the ischemic area at risk (AAR). The infarcted area (INF) could not be stained and appeared white. The myocardial infarction size was calculated as a percentage of AAR.

Echocardiography. Randomly selected animals from each group were anesthetized (isoflurane) and transthoracic M-mode echocardiography (Vevo 3100, Visualsonics) were performed to measure systolic function. Simpson's measurements were performed to obtain an averaged ejection fraction (EF), fraction shortening (FS), and cardiac output (CO) of all LAD ligation animals and 5 representative samples of each sham group. Only a representative sample of the sham hearts was collected because wall motions were observed to be synchronous, whereas hearts having undergone ischemia and reperfusion injury had asynchronous wall motion. Because all LAD ligation mice were expected to have infarction and asynchronous wall motion, the averaged EF and FS were calculated by using Simpson's measurements.

Histological examination. Hearts were harvested from young, aged, and YC groups at the end of reperfusion, fixed overnight in 10% formalin, and embedded in paraffin. Serial 5-mm heart sections from each group were analyzed. Samples were stained with hematoxylin and eosin stain (H&E) for routine histologic examination (Sun et al., 2016; Zhang et al., 2017).

Measurement of glucose/oleate oxidation. In our system, we set up the working heart preload at 10 cm H₂O, and the afterload at 80 cm H₂O (Ma et al., 2015; Quan et al., 2017; Yang et al., 2016). The flow rate was kept at 15 ml/min. The buffer used was Krebs-Henseleit buffer containing glucose (7 mM), oleate (0.4 mM), bovine serum albumin (BSA) (1%), and a low fasting concentration

of insulin (10 IU/ml). [9,10]-³H-oleate (50 μ Ci/l) and ¹⁴C-glucose (20 μ Ci/l) labeled buffer was perfused into the heart through the right pulmonary vein and pumped out through the aorta. The perfusate pumped out from the aorta and outflowed from the coronary venous was recycled and collected every 10 min to measure the radioactivity. The fatty acids oxidation level was determined through the production of ³H₂O from [9,10]-³H-oleate. Metabolized ³H₂O was separated from [9,10]-³H-oleate by filtering through an anion exchange resin (Bio-Rad). Glucose oxidation was measured by metabolized ¹⁴CO₂ dissolved in buffer or gaseous ¹⁴CO₂ captured in sodium hydroxide. To separate ¹⁴CO₂ from ¹⁴C-glucose, sulfuric acid was added to perfusate samples and ¹⁴CO₂ was captured on hyamine hydroxide soaked filter paper. The ³H and ¹⁴C signals were detected to discriminate metabolic products from fatty acid and glucose, respectively.

Isolation of mice cardiomyocytes. Mice were given 1000 units/kg of heparin IV (Fresenius Kabi) for anticoagulation (Ma and Li, 2015) before being anesthetized with 2%–3% isoflurane. The heart was excised, cannulated and connected onto the cardiomyocyte perfusion apparatus (Radnoti) and perfusion was initiated in the Langendorff mode. The heart was perfused at 37°C with a Ca²⁺-free based buffer (pH 7.2) containing: 135 mM NaCl, 4 mM KCl, 1 mM MgCl₂, 10 mM HEPES, 0.33 mM NaH₂PO₄, 10 mM glucose, 10 mM 2, 3-butanedione monoxime (Sigma), and 5 mM taurine (Sigma) that was bubbled with 95% O₂/5% CO₂. After 3–5 min of recycling, the heart was then digested with 0.3 mg/g body weight collagenase D (Roche), 0.4 mg/g body weight collagenase B (Roche), and 0.05 mg/g body weight protease type XIV (Sigma) dissolved in 25 ml perfusion buffer. After completely digested, the heart was removed and minced. Extracellular Ca²⁺ was added back to the cells gradually, from 0.06 mM to a final concentration of 1.2 mM. The interval of each concentration of Ca²⁺ was 15 min.

Hypoxia and reoxygenation treatment. Isolated cardiomyocytes were treated with vehicle (PBS 1 $\times$) or 1.5 mM DCA before being placed in a nitrogen chamber containing 95%N₂/5%CO₂ for 20 min to induce hypoxia. The cells were then removed from the nitrogen chamber and allowed to reoxygenate for 20 min at 37°C under normal atmospheric oxygen levels. Normoxia cells were treated with vehicle or 1.5 mM DCA and placed into 37°C incubator under normal atmospheric oxygen levels.

Measurement of contractility in mice cardiomyocytes. The contractile properties of cardiomyocytes were assessed by a SoftEdge Myocam system (IonOptix Corporation). Cardiomyocytes were placed in a chamber and stimulated with a supra-threshold voltage at a frequency of 1 Hz (Gruntzig, 1978; Moshal et al., 2008, 2009). IonOptix SoftEdge software was used to record the changes in sarcomere length and duration of shortening and relengthening. The following parameters were used to evaluate cardiomyocytes: resting sarcomere length; the maximum velocity of shortening ($-dL/dt$); the maximum velocity of relengthening ($+dL/dt$); peak height, maximum change of sarcomere length during contraction; peak shortening, peak height normalized to the resting sarcomere length; time-to-90% relengthening, the duration of myocytes to reach 90% lengthening.

Intracellular Ca²⁺ transient measurement. Intracellular Ca²⁺ was measured using a dual-excitation, single emission

photomultiplier system (IonOptix) (Xue et al., 2017; Zhao et al., 2009). Cardiomyocytes were treated with Fura 2-AM (2 μ M) at 37°C for 20 min and then exposed to light emitted by a 75 W halogen lamp through either a 340- or 380-nm filter while being stimulated to contract at a frequency of 1 Hz. Fluorescence emissions were detected (Chen et al., 2015). The following parameters were recorded: baseline of calcium signal (F340/380); peak calcium signal, maximum changes of calcium signal during contraction ($\Delta F_{340/380}$); %peak calcium changes (% $\Delta F_{340/380}$), peak calcium signal normalized to baseline; time-to-90% peak calcium changes (TP90), the maximum velocity of calcium change during contraction ($+dF/dt$); the maximum rate of calcium change during relaxation ($-dF/dt$).

Mitochondrial superoxide measurements. MitoSOX Red (Invitrogen) was used to measure the production of mitochondrial superoxide, a type of reactive oxygen species (ROS). Isolated cardiomyocytes were loaded with MitoSOX Red (5 μ M) for 10 min at 37°C protected from light, followed by wash out. Images were obtained of cardiomyocytes by use of fluorescence microscopes (excitation at 514 nm and measuring the emitted light at 585 nm).

Statistical analysis. Data were expressed as means $\pm$ standard error of the means (SEM). Two-tailed Student's *t* test and one-way ANOVA with Tukey's test for post hoc comparisons were used by Prism 7.0 (GraphPad Software). *p* < .05 was considered as significant difference.

RESULTS

DCA Treatment Activates Cardiac AMPK Signaling Pathway

To determine whether DCA treatment could activate AMPK, we detected the signaling pathway during myocardium ischemia. We injected vehicle or DCA (100 mg/kg) via i.p. to young, aged, and YC mice, 20 min prior to ischemia. After carefully ligating LAD for 10 min, we harvested the heart samples for immunoblotting analysis. The results demonstrated that the phosphorylation of AMPK in aged hearts were significantly lower than that in young hearts. DCA treatment increased the activation of AMPK in sham and ischemia groups compared with vehicle groups (Figure 1A). The main downstream protein of AMPK, acetyl CoA carboxylase (ACC), was also significantly phosphorylated in DCA-treated groups. The results also showed that, after Compound C treatment, activation of AMPK and ACC were already inhibited.

DCA Improves Cardiac Function During Ischemia/Reperfusion

The echocardiographic measurements demonstrated that under I/R condition versus sham, the cardiac function, including EF, FS, and CO, was significantly decreased (Figs. 1B and 1C and Table 1). Aged and YC groups had even worse outcomes. DCA was shown to improve the cardiac function under I/R stress, with the EF, FS, and CO significantly increased compared with the vehicle groups. Interestingly, after treatment of DCA, the systolic function of aged hearts was similar to young hearts under I/R stress. According to the results, even in YC group, DCA can also slightly improve the systolic function. To demonstrate whether AMPK activation would affect the cardioprotective function of DCA, we compared the extent of improvement of systolic function between vehicle and DCA after I/R in young and YC groups. However, the cardioprotection of DCA was

Figure 1. DCA treatment augmented cardiac AMPK activation by ischemia in both young and aged mice. DCA was given as described in Materials and Methods section. **A**, The representative immunoblotting was shown in the left panels. The relative levels of p-AMPK and p-ACC were shown in the right panels. Values are means $\pm$ SEM, $n = 4-8$ per group. * $p < .05$ versus sham vehicle, respectively; $^{\dagger}p < .05$ versus young ischemic vehicle; $^{\ddagger}p < .05$ between two groups. **B**, The representative echocardiography of sham and I/R groups treated by vehicle or DCA in young, aged, and YC groups. **C**, The systolic functions, including the left ventricular ejection fraction (EF), fractional shortening (FS), and cardiac output (CO) during ischemia/reperfusion (I/R), were significantly reduced in all groups. DCA treatment restored the systolic functions after I/R. Values are means $\pm$ SEM, $n = 4-8$ per group. * $p < .05$ versus sham vehicle, respectively; $^{\dagger}p < .05$ versus young I/R vehicle; $^{\ddagger}p < .05$ between two groups. **D**, The percentage of improvement of systolic functions during I/R in young and YC groups were shown. Compared with young group, the cardiac protection of in YC group was attenuated. Values are means $\pm$ SEM, $n = 4-8$ per group. $^{\ddagger}p < .05$ between two groups.

attenuated in YC group (Figure 1D). It is indicated that AMPK was involved in cardioprotection of DCA. In addition, DCA did not change any systolic functions of young, aged, and YC group mice under physiological condition (Figs. 1B and 1C).

DCA Treatment Limits Myocardial Infarction by Ischemia/Reperfusion

The young, aged, or YC group mice were subjected to 45 min of ischemia by ligation of LAD and 24 h of reperfusion by release of

Table 1. Echocardiographic Assessment of Cardiac Function of Young, Aged, and Compound C Treated Mice Under Normal Physiological and Ischemia/Reperfusion Conditions

n = 4–8	Young Sham		Young I/R		Aged Sham		Aged I/R		YC Sham		YC I/R	
	Vehicle	DCA	Vehicle	DCA	Vehicle	DCA	Vehicle	DCA	Vehicle	DCA	Vehicle	DCA
EF (%)	63.4 ± 1.9	64.2 ± 5.3	45.1 ± 1.1*	56.0 ± 1.9**	58.4 ± 1.2	57.3 ± 0.9	33.1 ± 2.2***	51.6 ± 1.0***	58.3 ± 3.3	58.2 ± 1.9	34.6 ± 1.5*,**	40.2 ± 1.3***
FS (%)	33.9 ± 1.4	34.7 ± 4.1	22.1 ± 0.6*	28.8 ± 1.1**	30.4 ± 0.7	29.7 ± 0.6	15.4 ± 1.2***	26.0 ± 0.6***	30.5 ± 2.2	30.1 ± 1.2	16.4 ± 0.8*,**	19.8 ± 0.8***
IVSs (mm)	1.21 ± 0.04	1.06 ± 0.05	1.00 ± 0.03	1.12 ± 0.07	1.32 ± 0.06	1.19 ± 0.07	1.01 ± 0.13	1.05 ± 0.10	1.12 ± 0.03	1.02 ± 0.03	0.90 ± 0.01	0.83 ± 0.04
IVSd (mm)	0.80 ± 0.05	0.68 ± 0.01	0.68 ± 0.02	0.80 ± 0.05	0.91 ± 0.04	0.83 ± 0.04	0.71 ± 0.05	0.78 ± 0.09	0.74 ± 0.02	0.70 ± 0.04	0.72 ± 0.03	0.59 ± 0.02
LVPWVs (mm)	1.28 ± 0.05	1.14 ± 0.15	0.91 ± 0.03	1.16 ± 0.13	1.26 ± 0.06	1.33 ± 0.03	0.90 ± 0.08	1.16 ± 0.06	1.09 ± 0.14	1.16 ± 0.03	1.03 ± 0.12	0.99 ± 0.07
LVPWd (mm)	0.83 ± 0.04	0.74 ± 0.10	0.74 ± 0.02	0.87 ± 0.09	0.98 ± 0.03	0.98 ± 0.05	0.86 ± 0.08	0.89 ± 0.09	0.94 ± 0.07	0.88 ± 0.04	0.91 ± 0.09	0.86 ± 0.08
LVIDs (mm)	2.45 ± 0.13	2.32 ± 0.30	3.05 ± 0.07	2.73 ± 0.14	2.79 ± 0.21	2.83 ± 0.19	3.17 ± 0.17	3.07 ± 0.09	2.84 ± 0.21	2.58 ± 0.16	3.56 ± 0.09	2.99 ± 0.15
LVIDd (mm)	3.81 ± 0.09	3.53 ± 0.33	3.91 ± 0.08	3.76 ± 0.19	4.00 ± 0.29	4.02 ± 0.28	3.75 ± 0.21	4.11 ± 0.08	4.08 ± 0.25	3.68 ± 0.19	4.25 ± 0.10	3.52 ± 0.17
CO (ml/min)	20.5 ± 1.0	20.8 ± 0.3	12.5 ± 0.7*	17.9 ± 1.0**	19.9 ± 0.5	20.6 ± 0.5	8.5 ± 0.8***	16.2 ± 0.7***	20.1 ± 0.8	20.4 ± 0.3	8.6 ± 0.7*,**	10.4 ± 0.3***
HR (BPM)	492.3 ± 28.6	389.1 ± 31.0	461.1 ± 9.6	405.5 ± 25.4	443.2 ± 23.5	408.2 ± 13.8	426.5 ± 24.8	403.8 ± 31.3	431.6 ± 19.3	399.1 ± 4.4	448.3 ± 38.8	409.3 ± 23.5

Values are means ± SEM; *p < .05 versus corresponding sham vehicle; **p < .05 versus young I/R; ***p < .05 versus Aged I/R; ****p < .05 versus YC I/R.

LAD ligation. The dual stains with TTC and Evans blue were used to reveal the myocardial infarction size. The representative cardiac sections are shown in Figure 2A. There was no significant difference of AAR among young, aged, and YC groups, indicating that similar ischemic stresses were induced in these groups. The aged and YC vehicle groups had increased infarction sizes compared with young vehicle group. DCA treatment significantly decreased the infarct size compared with corresponding vehicle groups. We compared the reduction of infarct size by DCA treatment in young and YC groups. Intriguingly, compared with young group, the extent of reduction in infarct size ($\Delta\text{INF}/\text{AAR}$) was significantly lower in YC group, indicating that although DCA treatment can reduce infarct size in YC group after I/R, the protection was attenuated by inhibiting AMPK activity. Representative pictures of HE staining of different groups are shown in Figure 2B. The arrangement of cardiomyocytes was disorganized after I/R insults, especially in aged and YC groups. DCA treatment decreased the myocardial injury.

DCA Ameliorates the Contractile Dysfunction of Cardiomyocytes by Hypoxia and Reoxygenation

To determine whether DCA could directly affect contractile function, cardiomyocytes were isolated from mouse hearts and treated with vehicle or DCA followed by single cell measurements of contractile function through the IonOptix system. The representative traces of contractility of each group of cells are shown in Figures 3A and 3B. The resting sarcomere length in each group had no statistical difference (Figure 3C). The peak contraction of cardiomyocytes of young and aged groups was similar under normoxia conditions. However, under hypoxia/reoxygenation (H/R) conditions, the contractile properties were significantly decreased compared with normoxia conditions in both young and aged cardiomyocytes (Figure 3D). Moreover, age is a risk factor during H/R stress, where the contractility of aged cardiomyocytes under H/R was significantly impaired as compared with young H/R group (Figs. 3D–H). The peak shortening and maximum velocity of shortening and relengthening were significantly improved with DCA treatment in both young and aged cardiomyocytes under H/R conditions (Figs. 3D–G). The H/R induced prolonged time-to-90% relengthening (TR_{90}) was significantly improved by DCA treatment in both young and aged cardiomyocytes (Figure 3H). Interestingly, the effects of DCA treatment on cardiomyocytes' contractility were not observed under normoxia conditions in both young and aged cardiomyocytes (Figs. 3D–H). These results suggested that the aged cardiomyocytes versus young were more vulnerable to H/R stress, and DCA treatment had direct beneficial influence on the contractility of cardiomyocytes under H/R stress conditions in both young and aged cardiomyocytes.

DCA Treatment Influences Intracellular Ca^{2+} Signal of Cardiomyocytes

To investigate the potential mechanisms involved in DCA treatment against compromised contractility during H/R on cardiomyocytes, we evaluated the intracellular Ca^{2+} homeostasis using the fluorescent dye fura-2/AM. (Xue et al., 2017) The representative traces of intracellular calcium of each group are shown in Figures 4A and 4B. There was no significant difference in basal intracellular calcium signal under normoxia and H/R conditions (Figure 4C). However, there is a significant alteration in the intracellular calcium homeostasis in both young and aged cardiomyocytes under H/R stress conditions versus normoxia conditions. Declined peak calcium signal (Figs. 4D and 4E), reduced maximum velocity of increasing and decreasing

Figure 2. DCA treatment reduced myocardial infarction caused by ischemia and reperfusion in both young and aged mice. **A**, The extent of myocardial infarction in young, aged, and YC mice treated with vehicle or DCA following 45 min of ischemia and 24 h of reperfusion. Upper: representative sections of myocardial infarction staining with TTC and Evens Blue in young, aged, and YC group hearts. Lower: the ratio of the area at the risk (AAR) to the myocardial area; the ratio of the infarction area (INF) to AAR; and the extent of reduction of INF/AAR by DCA treatment in young and YC groups. Values are means $\pm$ SEM, $n = 3-5$ per group. * $p < .05$ versus young vehicle; † $p < .05$ between two groups. **B**, Representative HE staining images of left-ventricle sections after 45 min ischemia and 24 h reperfusion.

calcium signal (Figs. 4G and 4H), and prolonged duration to 90% peak signal (TPS₉₀) (Figure 4F) were also significantly altered under H/R conditions. Moreover, the impairment in calcium homeostasis was observed in the aged versus young cardiomyocytes under H/R stress conditions (Figs. 4D–H). Interestingly, DCA treatment significantly increased the peak signal (Figs. 4D and E), maximum velocity (Figs. 4G and H), and reduced the time-to-90% peak signal (TPS₉₀) (Figure 4F) in both young and aged cardiomyocytes under H/R stress conditions. This shows that DCA treatment contributes to maintaining calcium homeostasis in response to H/R stress conditions (Figs. 4B and D–H).

DCA Treatment Reduces the Production of Mitochondrial Superoxide Under H/R Stress Conditions

The production of mitochondrial superoxide was similar in young and YC cardiomyocytes under normoxia conditions. But,

mitochondrial superoxide level from YC cardiomyocytes under H/R condition was higher than that in young cardiomyocytes. DCA reduced mitochondrial superoxide under H/R condition in young but not YC group (Figure 5A).

DCA Can Still Increase AMPK Activation in PDH KO Hearts

To further investigate how DCA-activated AMPK and the relationship between PDH activity and AMPK activation, experiments were conducted in heart-specific PDH-KO mice. Although PDH was deleted in hearts, AMPK showed activation by DCA in both sham and ischemic groups (Figure 5B). However, in PDH KO hearts, the phosphorylation of AMPK during I/R was attenuated compared with corresponding PDH^{fllox/fllox} groups. When WT mice were treated with DCA, the phosphorylation of LKB1 was increased in both sham and ischemia groups (Figure 5C).

Figure 3. The contractile functions of isolated cardiomyocytes from young or aged mice treated with vehicle or DCA under normoxia or hypoxia/reoxygenation (H/R) conditions. The representative traces of cell contraction by time with a stimulation at a frequency of 1 Hz were shown under normoxia (A) or hypoxia/reoxygenation (B). C, The resting sarcomere length. D, Peak height, maximum change of sarcomere length. E, The peak shortening peak height normalized to the resting sarcomere length. F, The maximum velocity of shortening ($-dL/dt$). G, The maximum velocity of relengthening ($+dL/dt$). H, The time-to-90% relengthening (TR_{90}). Values are means $\pm$ SEM, $n = 30$ –80 cells per group derived from 3 to 4 mice. * $p < .05$ versus normoxia vehicle, respectively; † $p < .05$ versus young H/R vehicle; ‡ $p < .05$ between two groups.

DCA Treatment Modulating Substrate Metabolism Mediated by AMPK

The *ex vivo* working heart perfusion system were performed to measure the rate of glucose and fatty acid oxidation in the heart under basal perfusion or ischemia/reperfusion conditions with or without DCA treatment. The results demonstrated that the

aged and YC group hearts had lower glucose oxidation rates as compared with the young group hearts under basal perfusion conditions (Figure 6A). During I/R conditions, the glucose oxidation rate was significantly decreased in each group, especially in aged and YC group hearts (Figure 6A). However, DCA treatment increased glucose oxidation rate in

Figure 4. DCA treatment improved intercellular calcium signal of isolated cardiomyocytes from young and aged mouse hearts during H/R. The representative fluorescent traces of F340/380 ratios of cardiomyocytes loaded with Fura 2-AM calcium signal staining stimulated at 1 Hz frequency under normoxia (A) or hypoxia and reoxygenation (H/R) (B). C, The baseline of calcium signal (F340/380). D, Peak calcium signal, maximum changes of calcium signal during contraction ($\Delta F_{340/380}$). E, The percent peak calcium changes ($\% \Delta F_{340/380}$), peak calcium signal normalized to baseline. F, The time-to-90% peak calcium changes (TP_{90}). G, The maximum velocity of calcium change during contraction ($+dF/dt$). H, The maximum rate of calcium change during relaxation ($-dF/dt$). Values are means $\pm$ SEM, $n = 30$ –80 cells per group derived from 3 to 4 mice. * $p < .05$ versus normoxia vehicle, respectively; † $p < .05$ versus young H/R vehicle; ‡ $p < .05$ between two groups.

all groups (Figure 6A). The oleate oxidation rate was significantly increased in all groups during I/R conditions whereas DCA treatment significantly reduced it during I/R stress. We compared the changes of substrate oxidation by DCA treatment after I/R in young hearts with or without Compound C treatment. Intriguingly, the extent of increment of glucose oxidation and reduction of oleate oxidation after I/R by DCA treatment was less in YC group compared with young group (Figure 6B).

DCA Treatment Activating AMPK Is Time Dependent

According to the result, the modulation of AMPK signaling pathway by DCA treatment is time-dependent (Figure 6C). After 6 h of DCA treatment, the phosphorylation of AMPK would return to basal level. However, the immunoblotting results showed that, after ischemia of 45 min and reperfusion of 24 h in the hearts, the phosphorylation of PDH was significantly increased in response to I/R stress in both young and aged hearts. But DCA treatment abolished the phosphorylation of PDH in both young

Figure 5. DCA treatment reduced the generation of mitochondrial superoxide during hypoxia and reoxygenation (H/R). **A**, Upper: representative images of mitochondrial superoxide in cardiomyocytes from young and YC groups under normoxia or hypoxia/reoxygenation conditions; Lower: quantitative analysis of the relative levels of mitochondrial superoxide in young and YC cardiomyocytes under normoxia or hypoxia/reoxygenation conditions. Values are means $\pm$ SEM, $n = 34$ –56 cells per group derived from 3 to 4 mice, $^*p < .05$ versus normoxia vehicle, respectively; $^\dagger p < .05$ versus young H/R vehicle; $^\ddagger p < .05$ between two groups. **B**, Left: the representative immunoblotting showed that DCA treatment increased the phosphorylation of AMPK in both PDH^{flox/flox} and PDH KO hearts under sham or 10-min ischemia conditions, Right: quantitative analysis of the relative levels of phosphorylation of AMPK and PDH under sham or ischemia conditions. Values are means $\pm$ SEM, $n = 4$ –5 per group. $^*p < .05$ versus sham vehicle, respectively; $^\dagger p < .05$ versus PDH^{flox/flox} ischemia vehicle; $^\ddagger p < .05$ versus PDH^{flox/flox} ischemia DCA; $^\S p < .05$ between two groups. **C**, Left: the representative immunoblotting showed the phosphorylation levels of AMPK upstream kinase LKB1 in WT hearts under sham or ischemia conditions. Right: quantitative analysis of the relative levels of phosphorylation of LKB1 under sham or ischemia conditions. Values are means $\pm$ SEM, $n = 6$ per group. $^*p < .05$ versus sham vehicle, respectively; $^\dagger p < .05$ versus ischemia vehicle.

and aged hearts under sham operations or I/R stress conditions (Figure 6D). It also demonstrated that there was no significant difference in PDK expression levels between young and aged hearts (Figure 6D), and DCA treatment did not affect

PDK protein levels. Interestingly, higher levels of phosphorylation of PDH were observed in the aged hearts versus young hearts under both sham operations and I/R conditions (Figure 6C).

Figure 6. AMPK plays a role in regulation of metabolism by DCA. **A**, DCA treatment modulates substrate metabolism in the *ex vivo* working heart perfusion during I/R stress conditions. DCA (1.5 mM) or vehicle was given at the beginning of the perfusion. The hearts were subjected to 10 min ischemia after 40 min balance, then followed by 40 min of reperfusion. The rate of glucose and oleate oxidation of the perfused hearts was measured. Values are means $\pm$ SEM, $n = 3$ –5 per group. * $p < .05$ versus basal vehicle, respectively; $^{\dagger}p < .05$ versus young I/R vehicle; $^{\ddagger}p < .05$ between two groups. **B**, The extent of changing in glucose and oleate oxidation by DCA treatment after I/R in young and YC groups was shown. Values are means $\pm$ SEM, $n = 3$ –5 per group. * $p < .05$ between two groups. **C**, WT mice were treated with DCA for different durations. Left: the representative immunoblotting showed that the activation of AMPK by DCA treatment is time dependent. Right: quantitative analysis of the relative levels of phosphorylation of AMPK. Values are means $\pm$ SEM, $n = 4$ –6 per group. * $p < .05$ versus basal. **D**, Left: the representative immunoblotting showed that DCA treatment abolished the phosphorylation of PDH in both young and aged hearts under sham or I/R stress conditions but did not influence the expression of PDK. Right: quantitative analysis of the relative levels of phosphorylation of PDH, and the protein levels of PDK in young and aged hearts under sham or I/R stress conditions. Values are means $\pm$ SEM, $n = 4$ –6 per group. * $p < .05$ versus sham vehicle, respectively; $^{\dagger}p < .05$ versus young I/R vehicle; $^{\ddagger}p < .05$ between two groups.

DISCUSSION

Myocardial ischemia is one of the most common causes of death. Apart from revascularization and standard medical management, therapy aimed at metabolism treatment could be a combined therapy for ischemic heart disease. Compared with fatty acid oxidation, glucose oxidation is more efficient by considering the consumption of oxygen. Glucose oxidation is able to yield more than 5 ATPs by consuming 1 molecule of O₂, whereas fatty acid oxidation, such as palmitate oxidation, only yields approximately 4.6 ATP with 1 molecule of O₂ (Fillmore and Lopaschuk, 2013). The excessive fatty acid oxidation produces more reactive oxygen species (ROS) which is detrimental for the fragile myocardium (Ma and Li, 2015). The uncoupling of glycolysis from glucose oxidation induces the accumulation of lactate and protons, forcing the myocardium to utilize a portion of ATP to rid of the byproducts. All of this further deteriorates the heart dysfunction (Liu et al., 1996a,b).

In our study, the results of the working heart perfusion showed that, under I/R conditions, glucose oxidation was increased whereas fatty acid oxidation was decreased significantly by DCA treatment. Thus, DCA could be an attractive therapeutic target for I/R injury. Our results were consistent with others, demonstrating the cardioprotective function of DCA during ischemia and reperfusion. The echocardiographic results of EF, FS, and CO were all significantly improved as compared with vehicle groups in response to I/R stress conditions. Furthermore, the TTC staining results demonstrated that DCA treatment reduces the infarcted areas during I/R. In addition, we also demonstrated that DCA treatment had a positive inotropic effect on isolated cardiomyocytes. DCA-treated groups had improved peak shortening and increased maximum velocity of shortening and relengthening under H/R condition. The analysis of calcium signals showed that DCA regulated the intracellular calcium signal to fortify the contractility of cardiomyocytes. This accumulation of evidence demonstrates that DCA had a positive effect to ameliorate hearts from I/R injury.

As we hypothesized, DCA is a modulator of metabolism and may influence the activation of AMPK. Our study demonstrated that DCA not only regulates phosphorylated level of PDH, but also modulates AMPK signaling pathway in mice hearts. The immunoblotting results showed that DCA was able to increase AMPK activation in young and aged mice under basal or ischemia conditions. Moreover, the main downstream kinase of AMPK, ACC was also significantly phosphorylated compared with vehicle groups.

To determine the role of AMPK in cardioprotection of DCA during I/R, we introduced Compound C, a potent AMPK inhibitor to young mice. The results of echocardiography as well as the TTC staining showed that the protective function of DCA was attenuated after AMPK activation was inhibited by Compound C. This illustrates that DCA exerts its cardioprotective function through the AMPK pathway. In addition, in YC group, although DCA can increase glucose oxidation and decrease fatty acid oxidation under I/R condition, the effect is attenuated, indicating that DCA-shifted metabolism is mediated by the AMPK pathway. We also found that DCA reduced the excessive mitochondrial superoxide during H/R in cardiomyocytes through AMPK pathway.

The activation of AMPK in PDH-KO mice was attenuated compared with young WT, suggesting that PDH activity can also influence the activation of AMPK. Interestingly, DCA can still activate AMPK after the deletion of PDH in hearts, indicating that DCA may directly influence the activation of AMPK apart from

increasing PDH activity. It is reported that the phosphorylation of LKB1 at Ser428 can increase the ability of LKB1 to bind and activate AMPK (Xie et al., 2006). Our data showed that DCA can increase the phosphorylation of LKB1 by increasing its phosphorylation at Ser428. It is reported that DCA could increase the activity of Sirtuin 1 (SIRT1) (Subramani et al., 2017). And SIRT1 can increase the deacetylation of LKB1 and enhance its phosphorylation (Wang et al., 2018). These may account for how DCA increases the phosphorylation of LKB1, and then activates AMPK. In addition, our data showed that AMPK and PDH may have interaction. They can influence the activity of each other. This result was similar to the study of Dugan et al. (2013). They found in diabetic kidney, the impaired AMPK activity was associated with suppressed PDH. And the activity of PDH was enhanced after activation of AMPK.

It is interesting that DCA activating AMPK is time dependent. There was no significant effect on the phosphorylation of AMPK after continuous treatment of DCA for 24 h, even though the phosphorylation of PDH was still abolished under both physiological and pathological conditions. Based on these results, it is possible that DCA may protect hearts through the AMPK pathway in the period of acute ischemia and early reperfusion, but it can continue to protect hearts from I/R injury by increasing glucose oxidation.

Compared with the young mice, aged mice are more vulnerable to I/R stress, resulting in bigger infarction sizes, impaired contractility of cardiomyocytes *in vitro*, and worse cardiac function *in vivo*. These results are consistent with our previous and others' studies (Lakatta and Sollott, 2002; Mariani et al., 2000; Quan et al., 2017; Starnes et al., 1997). AMPK has a protective function for hearts during I/R, thus impaired activation of AMPK causes aged hearts to be more vulnerable to I/R injury. In addition, the intracellular calcium homeostasis is impaired even worse during H/R in aged versus young cardiomyocytes. The impaired intracellular calcium homeostasis could reduce the contractility of cardiomyocytes and affect the activities of several dehydrogenases in mitochondria, especially PDH (O'Donnell et al., 1998; Sun et al., 2015). The immunoblotting results showed that the phosphorylation levels of PDH in the aged hearts were significantly higher than that in young hearts during I/R. The working heart data also showed the significant impairment of glucose oxidation in aged hearts versus young hearts during I/R stress conditions. After DCA treatment, the metabolism of aged hearts was shifted and activation of AMPK was enhanced, resulting in improved outcomes for aged mice suffering from I/R.

According to our study, DCA seemed to have no effects on metabolism and function of hearts under normal condition. One reason is that under normal condition, the oxygen and energy supply are sufficient, the effects of DCA to the heart may be nonsignificant. Another reason could be that the modification at protein level may not induce physiological changes immediately sometimes. Furthermore, although DCA treatment can activate AMPK pathway at basal condition, the duration of basal condition in our working heart experiment might be not long enough to show the changes of metabolism. In the future study, we should increase the duration of DCA treatment and animal numbers to determine whether DCA have effects on the heart under normal condition.

Currently, DCA is used in the clinic for congenital lactate acidosis and as a supplementary medicine for cancer therapy (Kankotia and Stacpoole, 2014; Michelaki et al., 2010). This study provided evidence that DCA may apply for treatment of ischemic heart disease by reducing the ischemic insult. Because

Figure 7. The effects of DCA on cardiomyocytes. Under stress conditions, DCA can protect the heart by increasing glucose oxidation via suppressing PDK to increase the efficiency of metabolism. Furthermore, DCA can increase the activation of AMPK via phosphorylation of its upstream kinase LKB1 to exert cardioprotective function. Abbreviations: DCA, dichloroacetate; G-6-P, glucose 6-phosphorylase; LKB1, liver kinase B1; AMPK, AMP-activated protein kinase; PDH, pyruvate dehydrogenase; PDK, PDH kinase; TAC, tricarboxylic acid cycle.

elderly people are more vulnerable to I/R injury and worse prognosis, DCA represents a beneficial therapeutic solution for the aged population.

In conclusion, DCA has a cardioprotective function during ischemia and reperfusion. Our research suggests that in addition to modulating cardiac substrate metabolism towards increasing glucose oxidation, DCA can also reduce ischemic insults through the AMPK signaling pathway (Figure 7).

FUNDING

These studies were supported by American Diabetes Association, 1-17-IBS-296; NIH, R01AG049835, R01GM124108, P01HL051971, and P20GM104357.

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