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Original Article

Palbociclib improves cardiac dysfunction in diabetic cardiomyopathy by regulating Rb phosphorylation

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Abstract: Diabetic cardiomyopathy (DCM) is a condition associated with significant structural changes including cardiac tissue necrosis, localized fibrosis, and hypertrophy of cardiomyocytes. This study sought to assess whether and how CDK4/6 inhibitor, Palbociclib, can attenuate DCM using a streptozotocin (STZ)-induced DCM model system. In this study, we found CDK4 and CDK6 expression are significantly increased the cardiac tissue of these mice. Palbociclib treatment after initial STZ administration attenuated oxidative stress and inflammation, thereby reducing cardiomyocyte death and preserving cardiac function in these animals. In addition, Rb phosphorylation induction was found in STZ-treated mice, which was inhibited by Palbociclib treatment. In summary, Palbociclib protects mice from damage associated with DCM pathway activation, making Palbociclib is a relevant therapeutic target in the context of DCM.

Keywords: CDK4/6 inhibitor, DCM, inflammation, oxidative stress, apoptosis

Introduction

Diabetes mellitus (DM) is a state of chronic hyperglycemic condition, caused by a deficiency of insulin secretion and may or may not be related to insulin resistance [1, 2]. Diabetic cardiomyopathy (DCM) is associated with myocardial dysfunction independent of high blood pressure and coronary artery disease [3, 4]. DCM involves the loss of cardiomyocytes and cardiac remodeling, which might lead to cardiac failure [5, 6]. The rates of cardiac failure in male and female diabetic patients are, respectively, two- and five-fold higher than those in people who do not have diabetes [7, 8]. The prevalence of diastolic dysfunction is 40%-60% in diabetic patients who do not suffer from coronary artery disease [5, 9]. Furthermore, the pathological complications of DCM cannot be successfully reversed even after rigorous blood glucose control [10, 11]. Consequently, it is crucial to develop drugs that selectively hinder the pathogenesis of DCM.

Diabetic cardiac dysfunction is specifically mediated by a series of pathogenic factors [12, 13]. Hyperglycaemia and inflammation lead to the excessive reactive oxygen species (ROS) production, which stimulates lipid peroxidation and reduce antioxidant efficiency, ultimately resulting in the loss of myocytes [14-16]. Hence, there is an urgent need for the development of an effectual strategy to inhibit the excessive production of ROS, inflammation and cardiomyocyte apoptosis for diabetic patients.

Palbociclib (PD-0332991) is an orally-available and extremely selective CDK4/6 kinase inhibitor which inhibits Rb phosphorylation and consequently inhibits progression of the cell cycle [17-19]. Palbociclib was approved by the US Food and Drug Administration (FDA) as a therapeutic for estrogen receptor (ER) positive metastatic breast cancer in combination with letrozole [20-22]. However, the role of Palbociclib in DCM is remain not well understood.

In the current study, we aimed to investigate whether Palbociclib could alleviate streptozotocin (STZ)-induced DCM and to explore the probable mechanism. We believe that this is the foremost account of the protective effect of Palbociclib in DCM.

Materials and methods

Animal models

Age-matched male C57BL/6 mice (10-week-old; 25-30 g) were maintained for 6 weeks at

22°C with humidity control and a 12-hrs light/dark cycle. They were separated into 3 diabetic groups (diabetic, diabetic + Palbociclib and diabetic + vehicle) and 1 control group of 12 mice each. The diabetic groups were injected intraperitoneally with freshly prepared STZ in citrate buffer (50 mg/kg; Sigma-Aldrich, St. Louis, MO, USA) for 5 successive days, whereas the control group was injected with buffer only. The mice with blood glucose ≥16.7 mmol/L were regarded as diabetic (typical value: 5-8 mmol/L). The diabetic + Palbociclib group was administered with Palbociclib (100 mg/kg; Selleckchem, Dallas, TX, USA) every day by oral gavage. The diabetic + vehicle group was administered an equal quantity of DMSO as the vehicle. All the investigations were conducted according to the Guide for the Care and Use of Laboratory Animals of the US National Institutes of Health and was approved by the Research Ethics Committee of The First Hospital of Jilin University.

Echocardiography

Mice were anesthetized using 2.0% isoflurane and echocardiography was performed using Vevo 770 (VisualSonics, Canada). Left ventricular ejection fraction (LVEF), fractional shortening (FS), early and late mitral inflow velocity ratio (E/A), LV internal diameter diastole (LVIDd), LV internal diameter systole (LVIDs), interventricular septal thickness (IVS), LV posterior wall thickness (LVPWd) and dp/dt max and dp/dt min were measured.

Histopathology

The 4% paraformaldehyde-fixed dissected mouse hearts were enclosed in paraffin and sliced into 5 μm sections. Subsequently, the echocardiographic measurements were validated histologically through staining with Masson's trichrome and Sirius red. The sections were treated with the corresponding primary antibodies overnight at 4°C, rinsed with PBS and then treated with the corresponding secondary antibodies for 120 min. The signal amplification was done using diaminobenzidine and counterstained with hematoxylin.

Real-time RT-PCR

The total RNA was extracted with TRIzol (Invitrogen, Carlsbad, CA, USA) and the samples were analyzed through spectroscopic methods. Briefly, total RNA was utilized to synthesize cDNA by means of SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). PCR was carried out using SsoFasr™ Probes Supermix (20 μL; Bio-Rad, Hercules, CA, USA) using gene-specific primer/probe sets and thermal cycling (35 cycles) on a Bio-Rad CFX96™ Real-time PCR System. The fold change was determined using the $2^{-\Delta\Delta Ct}$ method. The primers used in this study are list as followed: p67phox, F/R: 5'-CGTGTGTTGTTTGGCTTTGTG-3'/5'-CTGAGGCTGCGACTGAGG-3'; gp91phox, F/R: 5'-TAGCATCCATATCCGCATTG-3'/5'-CTAACATCACCACCTCATAGC-3'; β-actin, F/R: 5'-GGCACCACACCTTCTACAATG-3'/5'-GGGGTGTTGAAGGTCTCAAAC-3'.

Measurement of activities of oxidants, antioxidants

The freshly excised heart tissues (80-120 mg) were homogenized and subjected to centrifugation to obtain the supernatants. The activities of superoxide dismutase (SOD), NADPH oxidase and cAMP, along with the extent of lipid peroxidation, were measured by their respective commercial kits (Abcam, Cambridge, MA, USA).

Western blotting

The proteins were extracted from the cells in RIPA buffer. They (10 μg) were purified by 10% SDS-PAGE, blotted on nitrocellulose membranes and treated with primary antibodies against CDK4, CDK6 (Santa Cruz Biotechnology, Dallas, TX, USA), Bcl-2, Bax, p67phox (Abcam, Cambridge, MA, USA), cleaved caspase 3, p-RB, RB, p-AKT, AKT and β-actin (Cell Signaling Technology, Danvers, MA, USA). They were next incubated with peroxidase-labeled secondary antibodies. The Protein bands were visualized using luminol reagent and peroxide solution (1:1; Millipore, Cleveland, OH, USA), and the images were obtained.

ROS detection

For detection of ROS, the indicated cells were incubated with 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA; Sigma-Aldrich, St. Louis, MO, USA) for 30 min at 37°C. To quantify the ROS production, the fluorescence intensity was measured by flow cytometry.

Statistical analysis

Data were presented as the mean ± SD. All statistical comparisons were conducted in GraphPad Prism 6.0 through one-way ANOVA and the

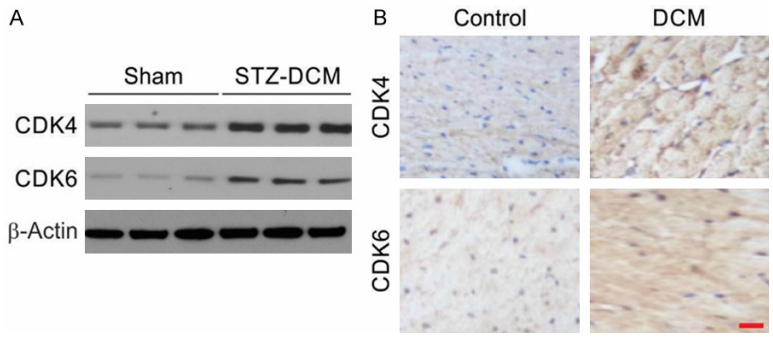

Figure 1. The expression of CDK4 and CDK6 was downregulated in the heart in STZ-induced diabetic mice. A. The expression of CDK4 and CDK6 in STZ-induced diabetic hearts were analyzed by western blotting. B. Immunohistochemistry of CDK4 and CDK6 in STZ-induced diabetic hearts. Scale bar, 50 μm.

Dunnet's post-hoc test. $P<0.05$ was defined as statistically significant.

Results

CDK4 and CDK6 was upregulated in the STZ-induced diabetic mice hearts

To investigate the role of Palbociclib in STZ-induced diabetic mice hearts, the expression of CDK4 and CDK6 were analyzed by western blotting. As shown in **Figure 1A**, CDK4 and CDK6 expressions were evaluated in the diabetic mice hearts which revealed their upregulated expressions. Immunostaining analyses demonstrated that CDK4 and CDK6 levels were noticeably induced in the hearts of STZ-induced diabetic mice (**Figure 1B**). These results indicated CDK4 and CDK6 may play a role in DCM.

Palbociclib hindered STZ-mediated cardiac injury and improved cardiac function

STZ-treated mice exhibited typical diabetic symptoms, including polydipsia, polyuria, and hyperglycemia (Data not shown). Increased blood glucose level and reduced bodyweight was also observed in the STZ-treated mice (**Figure 2A** and **2B**). However, Palbociclib treatment did not alter blood glucose and bodyweight (**Figure 2A** and **2B**). Mice administered with STZ for 16 weeks displayed an impaired cardiac function with noticeably diminished LVEF, FS and E/A ratio. Palbociclib treatment attenuated the decreased cardiac function (**Figure 2C-E**). In addition, LVIDd, LVIDs, IVS, and LVPWd level were increased in STZ-treated mice. However, Palbociclib treatment attenuated these increasement (**Figure 2F-I**). Moreover, the STZ-treated mice exhibited systolic (dP/dt(max)) and diastolic dysfunctions (dP/dt(min)) (**Figure 2J** and **2K**). Nevertheless, such diabetes-associated cardiac functional changes were hindered following Palbociclib treatment. Palbociclib treatment also protected diabetic mice against the reduced heart weight-to-body weight ratio and the heart weight-to-tibia ratio (**Figure 2L** and **2M**).

Palbociclib treatment reduced oxidative damage

Previously studies indicated that oxidative stress is a key feature of DCM [23, 24]. Western blotting results revealed that Palbociclib treatment significantly reduced the NADPH oxidase subunit p67phox level in diabetic mouse hearts (**Figure 3A**). Moreover, Palbociclib reduced the upregulated P67phox and Gp91phox mRNA expressions induced by STZ in diabetic mice (**Figure 3B** and **3C**), along with reducing the abnormal activity of NADPH oxidase (**Figure 3D**). Relative to mice in the control group, the reduced total SOD activity in diabetic mice was considerably increased by Palbociclib treatment (**Figure 3E**). Palbociclib treatment also diminished the myocardial lipid peroxidation in diabetic mice (**Figure 3F**). The above fata demonstrate that Palbociclib treatment reduced oxidative damage in the heart of diabetic mouse.

Palbociclib treatment reduced inflammation

Next, we investigated the effect of Palbociclib on inflammation. Our findings demonstrated that Palbociclib displays noteworthy effect on myocardial TNF-α, Il-1β and Il-6 in diabetic mice (**Figure 4A-C**). Our findings also showed that Palbociclib treatment suppressed STZ-induced cytokines upregulation (**Figure 4A-C**). CD68-labeled macrophage and CD45-labeled leukocyte infiltration were found increased in STZ-treated mice. However, Palbociclib treatment attenuated the inflammatory response in the heart of STZ-treated diabetic mice (**Figure 4D** and **4E**).

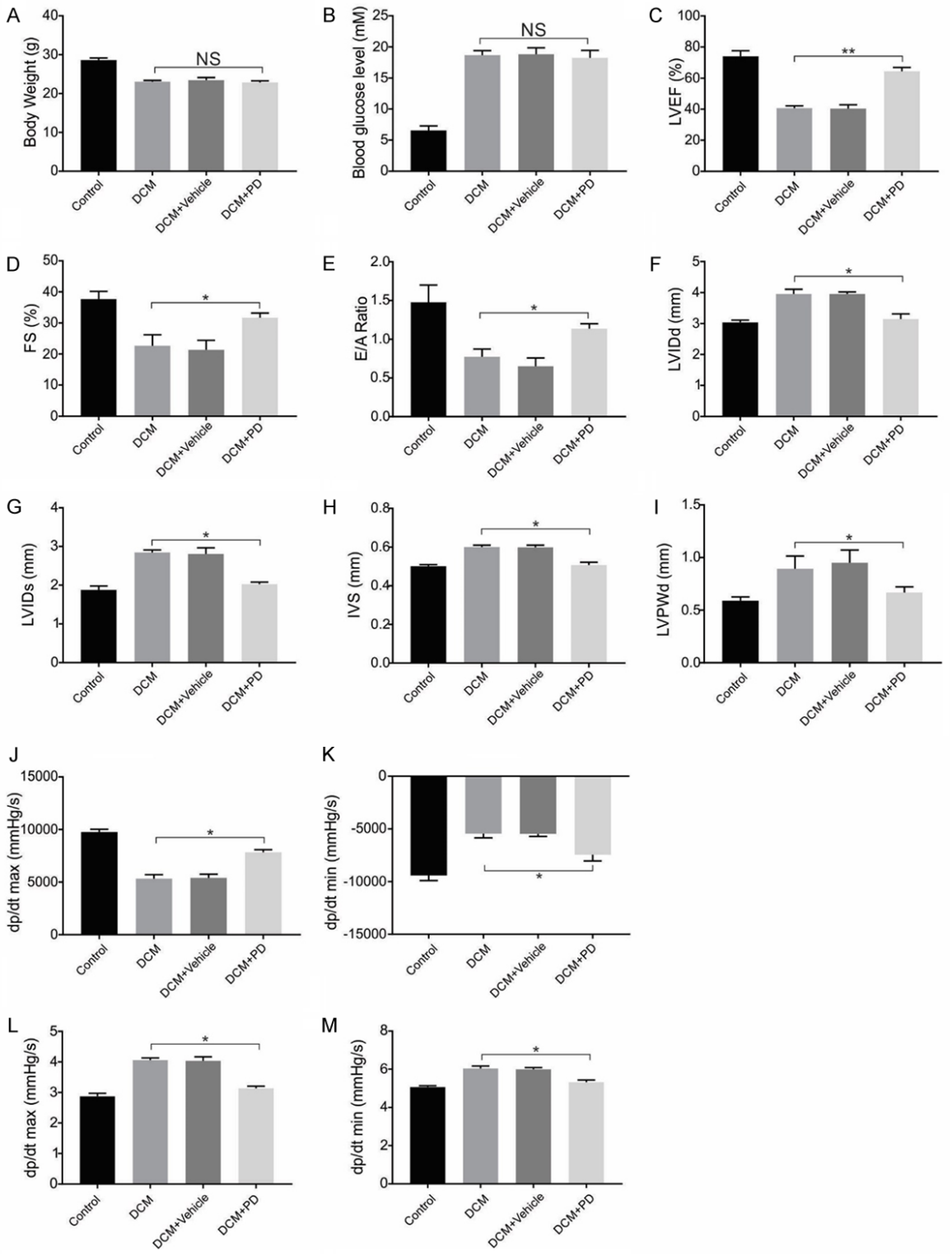

Figure 2. Palbociclib improved diabetes-induced cardiac dysfunction *in vivo*. Doppler echocardiogram was used to detect the effect of Palbociclib on cardiac function at the end of 16 weeks. Evaluations of body weight (A), Blood glucose level (B), left ventricular ejection fraction (LVEF) (C), fractional shortening (FS) (D), early to latemitral flow ratio (E/A ratio) (E), LV internal dimesion at diastole (LVIDd) (F), LV internal dimension at systole (LVIDs) (G), interventricular septal diastolic wall thickness (IVS) (H), and left ventricular posterior wall thickness (LVPWd) (I). (J and K) Effect of Palbociclib on hemodynamic measurements. (L and M) The ratio of heart weight (HW) to body weight (BW) and to tibia length (TL). Results in (A-M) were analyzed by one-way ANOVA with Dunnet's post-hoc test. Data represent the mean ± SD of three independent experiments. *, $P<0.05$.

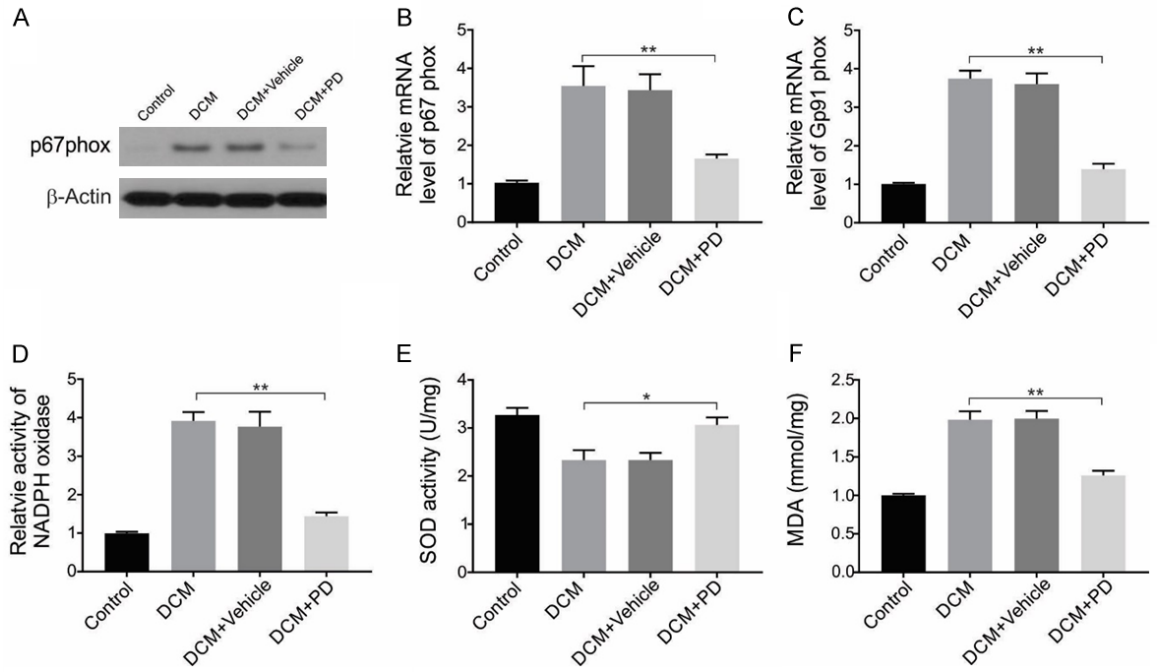

Figure 3. Palbociclib attenuated diabetes-induced oxidative injury and inflammation *in vivo*. (A) The expression of p67phox in diabetic mice hearts was analyzed by western blotting. (B) The mRNA level of p67phox in diabetic hearts was analyzed by real-time PCR. (C) The mRNA level of gp91phox in diabetic hearts was analyzed by real-time PCR. (D) NADPH oxidase activity in diabetic hearts by Palbociclib treatment. (E) Total SOD activity in diabetic hearts upon Palbociclib treatment. (F) Lipid peroxidation in diabetic hearts. Results in (B-F) were analyzed by one-way ANOVA with Dunnet's post-hoc test. Data represent the mean ± SD of three independent experiments. *, $P<0.05$; **, $P<0.01$.

Palbociclib treatment inhibited diabetes-triggered cell apoptosis in the hearts

A higher proportion of apoptotic cells were detected in diabetic heart, which was decreased considerably following Palbociclib treatment (**Figure 5A** and **5B**). The inhibitory effect of Palbociclib treatment on apoptosis was further confirmed by western blotting, which revealed the upregulated expression of Bcl-2 and diminished expression of Bax (**Figure 5C** and **5D**). The above data indicate that Palbociclib attenuate STZ-induced apoptosis in the diabetic heart.

Palbociclib treatment suppressed Rb phosphorylation and activated AKT signaling pathway in the diabetic hearts

We next investigated how Palbociclib treatment affects the downstream of CDK4/6. Compared to mice in the control group, diabetic mice exhibited increased phosphorylation of Rb in the hearts, and Palbociclib treatment drastically decreased the phosphorylation of Rb (**Figure 6A**). The PI3K/AKT signaling cascade is involved in cellular proliferation and survival. A previous study showed that suppression of Rb phosphorylation results in mTORC2-mediated activation of AKT [25]. Next, we investigated if Palbociclib treatment affects the downstream signaling of CDK4/6. Compared with the control group, diabetic mice exhibited decreased cardiac AKT phosphorylation and Palbociclib treatment significantly enhanced the phosphorylation (**Figure 6B**). Our results indicate that Rb phosphosrylation and AKT signaling pathway may be involved in the protection of Palbociclib in DCM.

Discussion

DCM involves mechanical, biochemical, and structural cardiac alterations, which might result in cardiac dysfunction [26, 27]. Nevertheless, the exact mechanism of DCM is not completely elucidated. We observed that Palbociclib treatment restored cardiac function, reduced hyperglycemia-induced inflammation and inhibited cardiomyocyte apoptosis in mice. These outcomes indicated that Palbociclib might be an efficacious drug for DCM.

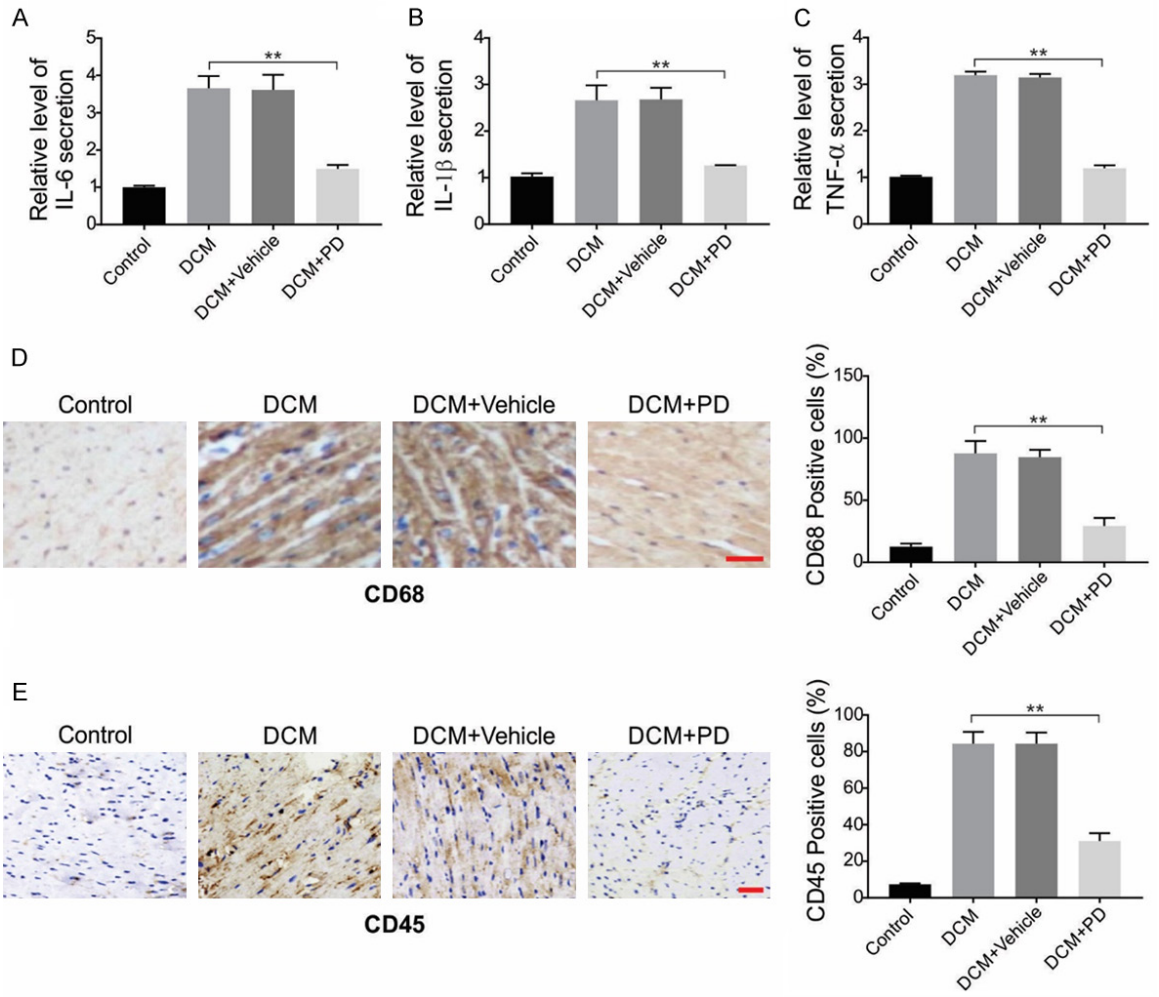

Figure 4. Palbociclib attenuated diabetes-induced inflammation *in vivo*. (A) mRNA level of myocardial IL-6 in mice with diabetes with or without Palbociclib treatment. (B) mRNA level of myocardial IL-1β in mice with diabetes with or without Palbociclib treatment. (C) mRNA level of myocardial TNF-α in mice with diabetes with or without Palbociclib treatment. (D) CD68 expression in the mice heart with diabetes with or without Palbociclib treatment was analyzed by immunohistochemistry. Scale bar, 50 μm. (E) CD45 expression in the mice heart with diabetes with or without Palbociclib treatment was analyzed by immunohistochemistry. Scale bar, 50 μm. Results in (A-E) were analyzed by one-way ANOVA with Dunnet's post-hoc test. Data represent the mean ± SD of three independent experiments. **, $P<0.01$.

This study demonstrated impaired cardiac function in STZ-induced DCM, while Palbociclib improved it compared to STZ alone. Moreover, Palbociclib treatment inhibited remodeling processes of DCM, including inflammation, oxidative stress and apoptosis, indicating that a cell cycle-mediated physiological process might be involved in the protection against STZ-induced DCM. These observations prompted us to scrutinize whether CDK4 and CDK6 levels in the hearts correlated with the attenuation of diabetes-related cardiac injury. Interestingly, Palbociclib treatment alleviated diabetes-induced cardiac dysfunction, signifying CDK4/6 as a potential target for the DCM treatment.

It was established that an extreme increase in oxidative stress accelerates the progression of DCM [28-30]. Elevated ROS levels, observed in HG-treated myocytes and in cardiomyocytes isolated from diabetic mice, induce lipid peroxidation and DNA damage, ultimately resulting in functional abnormalities of the heart [31-33]. Thus, it is essential to discover inhibitors of oxidative stress in diabetes. In the current study, we established with robust evidence that Palbociclib displayed a protective role against diabetes-mediated oxidative damage in vivo.

Inflammation is an additional characteristic of DCM [7, 34, 35]. Diabetic hearts exhibit ampli-

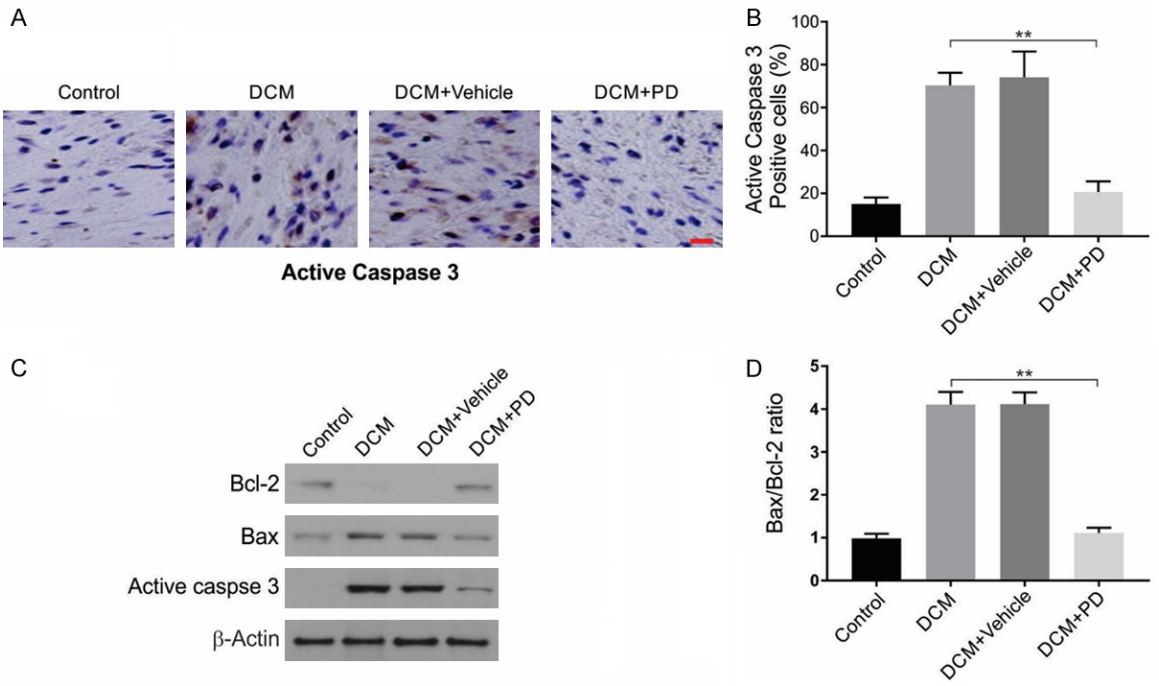

Figure 5. Palbociclib attenuated diabetes-induced apoptosis *in vivo*. (A and B) Active caspase 3 staining in diabetic hearts with or without Palbociclib treatment was analyzed by immunohistochemistry. Scale bar, 50 μm. (C) The expression of Bcl-2 and Bax in diabetic hearts with or without Palbociclib treatment was analyzed by western blotting. (D) The ratio of Bax to Bcl-2 was analyzed. Results in (B) and (D) were analyzed by one-way ANOVA with Dunnet's post-hoc test. Data represent the mean ± SD of three independent experiments. **, $P<0.01$.

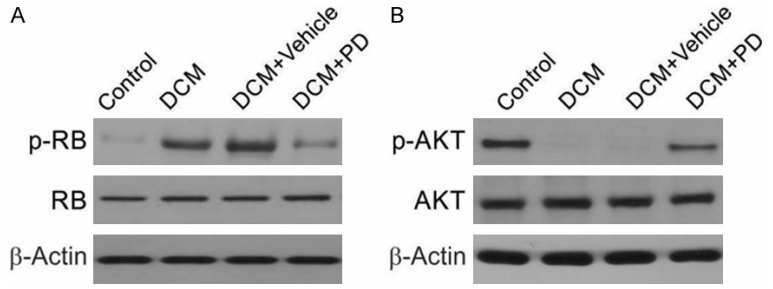

Figure 6. Palbociclib attenuated diabetes-induced RB phosphorylation and AKT dephosphorylation *in vivo*. A. The expression of phosphor- and totle-RB in the diabetic hearts with or without Palbociclib treatment was analyzed by western blotting. B. The expression of phosphor- and totle-AKT in the diabetic hearts with or without Palbociclib treatment was analyzed by western blotting.

fied levels of TNF-α, IL-6 and IL-1β, which may bring about cardiac dysfunction [36]. In the current study, attenuation of inflammation following CDK4/6 inhibitor treatment was observed in the diabetic mouse hearts. Altogether, these observations demonstrated the protective function of Palbociclib against apoptosis in diabetic hearts.

Rb status is the principal determinant of response to CDK4/6 inhibition. In breast carcinoma and GBM, Rb1 deficiency or inactivation induces palbociclib resistance [37, 38]. The PI3K/AKT signaling cascade is involved in cellular proliferation and survival [39]. An earlier study showed that suppression of Rb phosphorylation results in mTORC2-mediated activation of AKT [40, 41]. Our results demonstrated that Palbociclib treatment affects AKT activation.

In conclusion, this study offers a deeper comprehension of the regulatory role of Palbociclib in diabetic cardiac inflammation, oxidative stress and apoptosis, signifying that CDK4/6 could be a potential therapeutic target for DCM.

Disclosure of conflict of interest

None.

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