

Original Article

Effects of PPAR α /PGC-1 α on the energy metabolism remodeling and apoptosis in the doxorubicin induced mice cardiomyocytes *in vitro*

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Abstract: Dilated cardiomyopathy is the most frequent form of myocardial disease. Many factors contribute to dilated cardiomyopathy, for instance, long-term use of doxorubicin, one of the anthracyclines clinically used for cancer chemotherapy, result in dilated cardiomyopathy and congestive heart failure. However, the mechanism underlining doxorubicin-induced cardiomyocyte is still not fully understood. In this study, we evaluate the effects and their mechanisms of PPAR α and PGC-1 α pathways in doxorubicin induced mice cardiomyocytes. *In vitro*, cardiomyocytes isolated from hearts of adult FVB/NJ mice were treated with doxorubicin, GW 6471 (PPAR α inhibitors) and WY14643 (PPAR α agonists). The expression of PPAR α and PGC-1 α were detected via western blotting and Quantitative Real-Time PCR methods. Changes in energy and substrate metabolism were analyzed. MTT and flow cytometry were used for cell proliferation and apoptosis analysis. We detected expression of PPAR α and PGC-1 α was significantly higher in control group than doxorubicin group. Mitochondrial dysfunction was found in doxorubicin group including lower content of high-energy phosphates, significantly decreased mitochondrial ANT transport activity and markedly reduced mitochondrial membrane potential compared with control group. Metabolic remodeling existed in doxorubicin group because of higher concentration of free fatty acid and glucose consumption than of control group. More accumulations of reactive oxygen species were detected in doxorubicin group. The decreased cell viability and increased cell apoptosis observed in doxorubicin group. Severe apoptosis in doxorubicin group was verified by a set of markers including Bax, Bcl-2, cytosolic cytochrome c and caspase-3 up-regulation expression. These findings indicate that the PPAR α and PGC-1 α are closely involved in energy metabolism remodeling and apoptosis in cardiomyocytes.

Keywords: PPAR α , PGC-1 α , doxorubicin, energy metabolism, *in vitro*

Introduction

Dilated cardiomyopathy (DCM) is the most frequent form of myocardial disease, characterized with progressive ventricular dilatation and systolic dysfunction [1]. Many factors contribute to DCM, for instance, long-term use of doxorubicin (DOX), one of the anthracyclines clinically used for cancer chemotherapy [2], can cause high activation of sympathetic nervous system and left ventricular dysfunction [3]. The major side effect of DOX is cardiac toxicity, which could further result in dilated cardiomyopathy and congestive heart failure [4]. It has been reported that DOX can increase oxidative stress, decrease ATP yield, suppress gene expression, and cause mitochondrial abnormality as well as cardiomyocyte apoptotic death [5-7]. However, the mechanism underlining DOX-induced cardiomyocyte is still not fully understood.

Heart is an organ with a very high and dynamic demand for ATP. In the adult human, most of energy comes from fatty acid oxidation responsible for 70-80% of myocardial ATP production [8]. Emerging evidences demonstrated that peroxisome proliferator activated receptor α (PPAR α) in collaboration with PGC-1 α (a co-activator of PPAR α) modulates energy metabolism [9-11]. PPAR α , one of the three subtypes of PPAR besides β/δ and γ subunits [12], regulates cardiac metabolism [13, 14] and energy metabolism-related pathways [15] by affecting

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Table 1. Concentration of adenylate in different groups

Group	ATP (nmol/mg)	ADP (nmol/mg)	AMP (nmol/mg)
Control	1.85	3.36	3.82
DOX	1.13*	1.35*	2.21*
PPAR α -	1.05*	1.24*	1.92*
PPAR α +	1.33*	1.51*	2.39*

The adenine nucleotide in cardiomyocytes was determined using HPLC method. Data are the mean \pm SEM, *compared with control group, $P < 0.05$.

the activities of enzymes involved in free fatty acid oxidation [16]. PGC-1 α , serving as a co-activator of PPAR α , can enhance mitochondrial biogenesis and augment the oxidation of fatty acids through its synergistic effects with PPAR α [17, 18].

Our previous work showed that PPAR α and PGC-1 α are closely involved in energy metabolism in the DOX-induced mouse model of DCM [19]. Here, we reported the effect of PPAR α /PGC-1 α expression on the energy metabolism remodeling and apoptosis in DOX-induced cardiomyocytes *in vitro*.

Materials and methods

Cell culture and treatments

Cardiomyocytes were isolated by enzymatic digesting hearts of adult FVB/NJ mice. The isolated cardiomyocytes were cultured in DMEM medium containing 10% FBS and cultivated in 37°C, 50 ml/L CO₂ atmosphere. After 24 h of plating, cardiomyocytes were divided into four groups, control group, DOX group, PPAR α inhibition (PPAR α -) group and PPAR α activation (PPAR α +) group. DOX group used 5 μ M doxorubicin (Sigma-Aldrich), PPAR α - group used 10 μ M PPAR α inhibitors GW 6471 (Sigma-Aldrich) and PPAR α + group used 100 μ M PPAR α agonists WY14643 (Sigma-Aldrich).

Measurement of cell proliferation

Cell viability was determined by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, cardiomyocytes were plated on 96-well plates at a density of 1×10^4 cells/well. After doxorubicin, GW 6471, or WY14643 treatments, 20 μ L MTT (5 mg/mL) was added to each well and incubated for 4 h.

The medium was then removed, and the formazan crystals were dissolved with dimethyl sulphoxide (DMSO). Absorbance was read at 570 nm on microplate reader (Multiskan Spectrum, Thermo Fisher).

Flow cytometry

Apoptotic cells can be recognized and distinguished from normal cells using an Annexin V-FITC/PI apoptosis kit for flow cytometry according to the manufacturer's instructions (Invitrogen). After doxorubicin, GW 6471, or WY14643 treatments, cardiomyocytes were harvested and washed twice with cold PBS, and then incubated with 5 μ l FITC-Annexin V and 1 μ l PI working solution (100 μ g/ml) for 15 min in the dark at room temperature. Cellular fluorescence was measured by flow cytometry analysis (FACStation, BD Biosciences) [20].

Reactive oxygen species (ROS) determination

The ROS generation from cardiomyocytes were measured by CM-H₂DCFDA (chloromethyl-2',7'-dichlorodihydro fluorescein diacetate) staining. For CM-H₂DCFDA staining, cells at appropriate conditions were washed with ice cold PBS and treated with 10 μ M CM-H₂DCFDA (Invitrogen) in DMEM medium for 45 min. Then, cells were trypsinized and suspended in PBS, and the fluorescence was studied at 538 nm.

Western blotting

Total proteins were prepared from the cultured cardiomyocytes of each group. The protein concentration was determined using Bio-Rad protein assay system. Proteins were analyzed with SDS polyacrylamide gel electrophoresis. After electrophoresis, they were electro-transferred to the PVDF membrane. The membrane containing the proteins was used for immunoblotting with appropriate antibodies. The protein bands were scanned and quantified.

RNA isolation and quantitative real-time PCR analysis

Total RNA were extracted from the cultured cardiomyocytes of each group using TRIZOL Regent (Invitrogen). The cDNA Synthesis Kit (Takara) was used for the synthesis of cDNA according to the manufacturer's instructions. Quantitative PCR was accomplished using the

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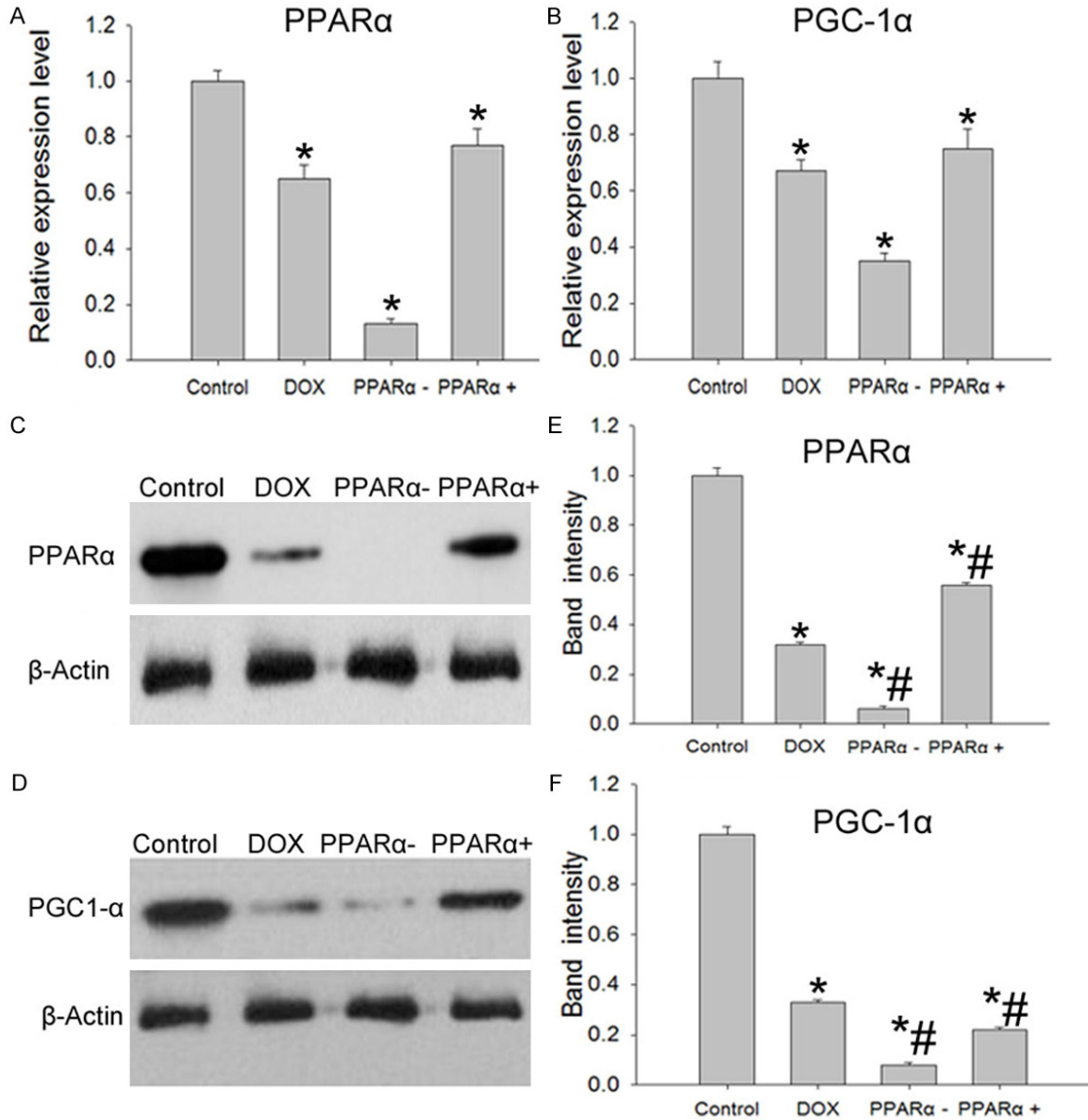


Figure 1. PPAR α and PGC-1 α expression in different groups. A. Q-PCR results of PPAR α expression in different group. B. Q-PCR results of PGC-1 α expression in different group. C and E. Western blotting results of PPAR α expression in different group. D and F. Western blotting results of PGC-1 α expression in different group. Data are the mean \pm SEM (n=3), Each bar represents the mean of three independent experiments carried out in triplicate. *Compared with control group, $P < 0.05$, #Compared with DOX group, $P < 0.05$.

iCycler iQ Detection System (Bio-Rad, Richmond, CA) and interaction dye SYBR Green. Primer sequences are listed in [Supplementary Table 1](#).

Analysis of adenylate in cardiomyocytes

The adenine nucleotide from cardiomyocytes of each group was determined according to the method of Yongyao Yang [19]. The wavelength of HPLC was 254 nm, and sensitivity was 0.01

AUFS. Chromatographic column was 4.6 nm \times 250 nm, YWG-ODS C18 10 μ m. The mobile phase was 2 mmol/L PBS (pH 5.5). The speed was 1 mL/min.

Determination of mitochondrial ANT transport activity in cardiomyocytes

We determined mitochondrial ANT transport activity use inhibitors termination method. 20 μ l of 3 H-ADP solution (0.3 μ mol/L) was added

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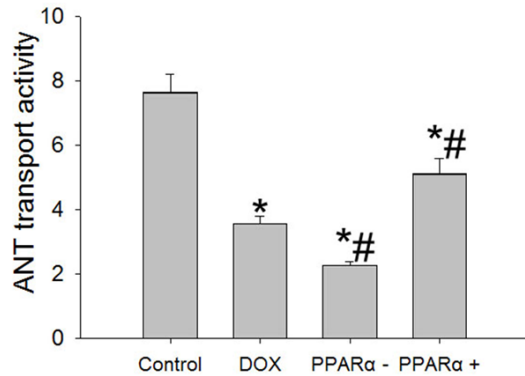


Figure 2. Changes of mitochondrial ANT transport activity in different groups. Data are the mean \pm SEM (n=3), each bar represents the mean of three independent experiments carried out in triplicate. *Compared with control group, $P < 0.05$; #Compared with DOX group, $P < 0.05$.

into 50 μ l of prepared mitochondrial suspension for 10 s, then 50 μ l of ANT inhibitor ATR (3.2 nmol/L) was added and mixed immediately to terminate ANT transporter function. The mixture was centrifuged for 20 min with 12000 r/min speed at 4°C and the supernatant was discarded. 400 μ l of H₂O₂ (8.8 mol/L) was added into the precipitation after being dissolved and cleaned with 1 ml separation medium, then it was digested for 40 min in 70°C water bath. The radioactivity was measured by liquid scintillation counting method.

Determination of free fatty acid in cardiomyocytes

Free fatty acid was separated from cardiomyocytes from each group and measured by HPLC method referring to Michael Puttmann [21].

Measurement of glucose consumption in cardiomyocytes

The glucose consumption of cardiomyocytes was measured in small glass spinner flasks refer to J.P. FREYER [22]. The concentration of glucose in complete medium was carried out with a standard enzyme reagent kit (Sigma) according to the manufacturer's instructions.

Caspase 3 assay in cardiomyocytes

Caspase 3 activity was determined using the luminescent Caspase-Glo 3 assay (Promega). The assay was conducted according to the manufacturer's protocol.

Statistical analysis

All the original experimental data were analyzed by SPSS19.0. Comparison among the groups was carried out by One-way ANOVA. The comparison between two groups was carried out by LSD. $P < 0.05$ was considered as statistically significant differences.

Results

Expression of PPAR α and PGC1- α in different groups

The results of q-PCR showed that the expression of PPAR α and PGC-1 α in DOX group were lower than that of control group ($P < 0.05$). While the expression of PPAR α and PGC-1 α in PPAR α + group were higher compared with DOX and PPAR α - group ($P < 0.05$) (Figure 1A, 1B). The Western blotting results showed in Figure 1C-F.

Mitochondrial dysfunction in different groups

The essential function of cardiomyocytes is energy production. Firstly, we measured concentration of adenylate in different groups using HPLC method. Results established that compared with control group, ATP, ADP and AMP decreased significantly in DOX, PPAR α + and PPAR α - groups ($P < 0.05$) (Table 1). The decline in the high-energy phosphate pool might be indicative for impaired mitochondrial function.

The ATP produced in the mitochondrion is transported to the cytoplasm for important cellular work. Then, we analyzed mitochondrial ANT transport activity in different groups. Data showed that compared with control group, the mitochondrial ANT transport activity decreased significantly in DOX, PPAR α + and PPAR α - groups, the results were showed in Figure 2.

In addition, we also found that mitochondrial membrane potential significantly reduced in DOX group, PPAR α - group and PPAR α + group (Supplementary Figure 1).

Change in cardiac substrate utilization

The impaired mitochondrial function might be indicative for decline in oxidation of fatty acid and glucose. Free fatty acid content was detected and results revealed that palmitic acid, stearic acid, oleic acid, linoleic acid, linolenic acid,

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Table 2. Concentration of free fatty acid in different groups

Group	C (FFAs) $\mu\text{mol/L}$					
	Palmitic acid (c16:0)	Stearic acid (c18:0)	Oleic acid (c18:1)	Linoleic acid (c18:2)	Linolenic acid (c18:3)	Arachidonic acid (c20:4)
Control	298.73 \pm 10.01	179.63 \pm 6.65	322.5 \pm 19.84	514.23 \pm 14.52	29.12 \pm 2.56	91.23 \pm 6.54
DOX	364.98 \pm 12.45*	239.01 \pm 9.14*	385.2 \pm 17.89*	555.62 \pm 19.54*	59.6 \pm 3.89*	118.94 \pm 8.79*
PPAR α -	412.01 \pm 15.96*	264.53 \pm 8.78*	411.2 \pm 19.24*	599.87 \pm 18.74*	77.08 \pm 4.52*	132.25 \pm 5.96*
PPAR α +	340.68 \pm 11.15*	211.01 \pm 6.56*	355.78 \pm 18.74*	524.12 \pm 16.58*	45.21 \pm 4.89*	101.03 \pm 7.89*

Free fatty acid was separated from cardiomyocytes and measured by HPLC method. Data are the mean \pm SEM, *compared with control group, $P < 0.05$.

Table 3. Glucose consumption in different groups

Group	12 h	24 h	36 h
Control	6.32 \pm 0.12	5.34 \pm 0.39	4.23 \pm 0.09
DOX	6.91 \pm 0.24*	5.98 \pm 0.26*	5.08 \pm 0.11*
PPAR α -	7.22 \pm 0.32*	6.31 \pm 0.21*	5.44 \pm 0.19*
PPAR α +	6.69 \pm 0.27*	5.71 \pm 0.29*	4.78 \pm 0.27*

The concentration of glucose in complete medium was carried out d a standard enzyme reagent kit. Data are the mean \pm SEM, *compared with control group, $P < 0.05$.

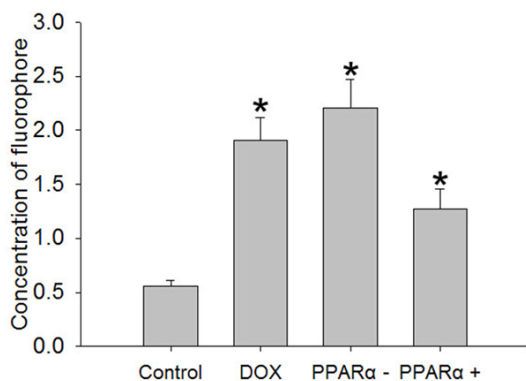


Figure 3. Reactive oxygens accumulate in different groups. The ROS generation from cardiomyocytes was measured by CM-H₂DCFDA. Data are the mean \pm SEM (n=3), Each bar represents the mean of three independent experiments carried out in triplicate. *Compared with control group, $P < 0.05$.

and arachidonic acid increased significantly in DOX group, PPAR α - group and PPAR α + than control group ($P < 0.05$) (Table 2).

To characterize whether free fatty acid metabolism was changed in different groups, we detected genes expression involved in free fatty acid oxidation pathway using real-time PCR method. The results showed that *M-CPT1*, *ACOX1*, *ACOX3*, and *LBP* expression markedly

down-regulation expression in DOX group, PPAR α - group and PPAR α + group respectively (Supplementary Figure 2).

Cardiomyocytes prior fatty acid to glucose for energy supporting, but in some heart disease marked shift in substrate preference away from fatty acids towards glucose. We conduct glucose consumption in different groups.

The data demonstrated that all the treatment groups possess higher glucose consumption regardless of 12 hr, 24 hr and 36 hr after treatment respectively than control group (Table 3).

Collectively, these observations indicate that metabolic remodeling in DOX, PPAR α - and PPAR α + groups.

Reactive oxygen species (ROS) accumulation in different groups

It is well known that higher concentration of glucose and free fatty acid have toxic effect on cells, and this toxicity is likely prompt more ROS production. ROS level was measured by CM-H₂DCFDA staining in different groups. As shown in Figure 3, we observed more ROS accumulation in DOX group, PPAR α - group and PPAR α + group than control group ($P < 0.05$).

Cell viability and apoptosis in different groups

To determine the effect of ROS on cardiomyocytes, MTT method was used to detect cell viability in different groups. Experiments exhibited that DOX group, PPAR α - group and PPAR α + group, cell viability decrease significantly compared with control group (Figure 4A).

Lower cell viability in treated groups made us to characterize whether cell apoptosis was changed in different groups by means of flow

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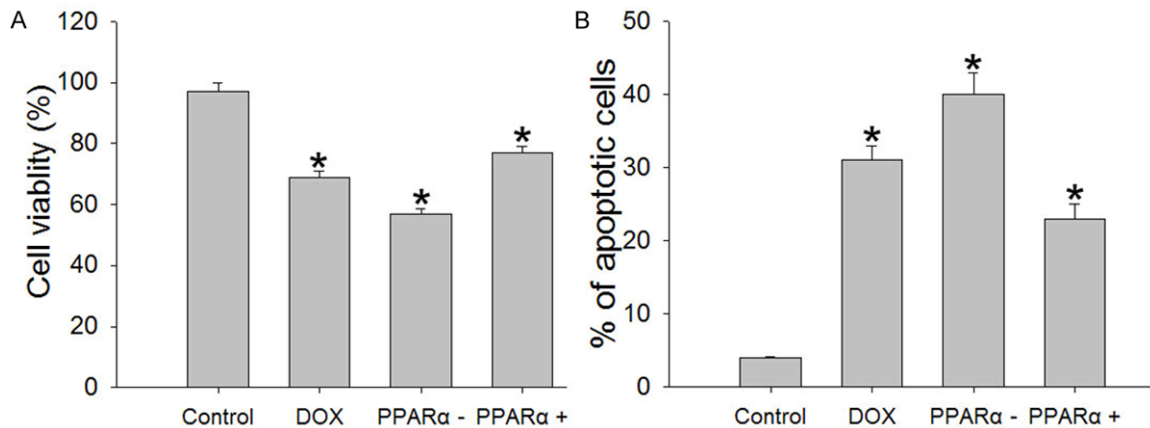


Figure 4. Cell viability and apoptosis in different groups. A. Cell viability was measured by the MTT colorimetric assay. B. Cell apoptosis in different groups. Apoptotic cells can be recognized and distinguished using an Annexin V-FITC/PI apoptosis kit for flow cytometry. Data are the mean \pm SEM (n=3), each bar represents the mean of three independent experiments carried out in triplicate. *Compared with control group, $P < 0.05$.

cytometry. As shown in **Figure 4B**, DOX significantly increased the number of apoptotic cells compared with control group ($P < 0.05$).

Markers for apoptosis in different groups

To confirm the reliability of apoptosis result, we performed western blotting analysis of a set of markers for apoptosis, including Bax, Bcl-2, cytosolic cytochrome c and caspase-3. We found that a strong increase in the expression of Bax and a reduced expression of Bcl-2 in DOX group, PPAR α - group and PPAR α + group compared with control group. Furthermore, Cytosolic cytochrome c and caspase-3 expression level also showed markedly increased in DOX group, PPAR α - group and PPAR α + group (**Figure 5**). In addition, we found that caspase-3 activity significantly higher in DOX group, PPAR α - group and PPAR α + group (**Figure 6**).

Discussion

Doxorubicin (DOX) was widely used as effective anticancer drug. However, DOX is a double-edged sword due to its use could cause a cardiomyopathy which lead to a refractory form of heart failure. DOX was used to induce the model of congestive cardiomyopathy these years [23, 24]. Dilated cardiomyopathy (DCM) is the most frequent form of myocardial disease. However, the mechanism of DCM induced by DOX still not fully understood until now. Generally, mitochondria are the main target organs in the pathogenesis of DOX and lead to

mitochondrial dysfunction occurred and energy imbalance, such as inhibiting the oxidative phosphorylation, blocking ATP generation [19].

Emerging evidence demonstrates the importance of PPAR α and PGC-1 α molecules in the transcriptional activation of genes expression involved in fatty acid oxidation [25, 26]. At present, the changes and regulations of myocardial energy metabolism in DCM are poorly understood, especially the transcriptional regulation of PPAR α and PGC-1 α on energy metabolism of drug-induced cardiomyopathy is rarely reported. Our previous work showed that PPAR α and PGC-1 α were closely involved in energy metabolism in the DOX-induced mouse model of DCM [19]. Here, we investigate the effect of PPAR α /PGC-1 α expression on the energy metabolism in DOX-induced cardiomyocytes.

Doxorubicin treatment lead to the expression of PPAR α and PGC-1 α decreased significantly in DOX group than that of control group. Lower content of high-energy phosphates and significantly decreased mitochondrial ANT transport activity implied that abnormal energy metabolism in DOX group compared with control group. These imbalances maybe further prompt higher concentration of free fatty acid and lower glucose consumption detected in DOX group. More ROS molecules, might from the disorder energy metabolism, were harmful to cells. The ROS will inflict mitochondrial damage and make the dysfunction of the respiratory chains. And this possible were the mainly reasons for lower

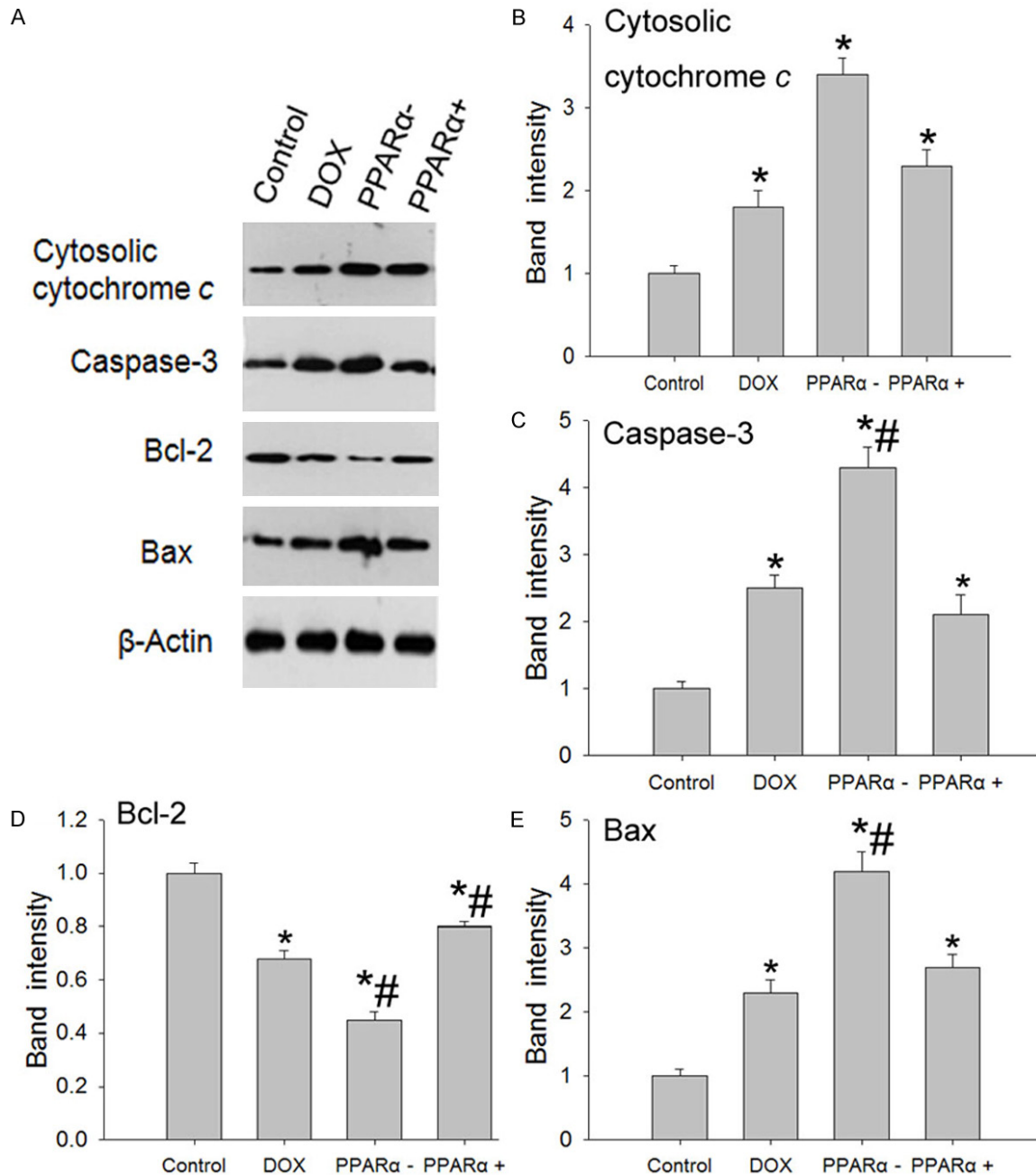


Figure 5. Expression level of markers for apoptosis in different groups. A. Western blotting results of markers for apoptosis expression in different groups. B-E. The graph represents a quantitative measure of band intensities of cytosolic cytochrome c, caspase-3, Bcl-2 and Bax, normalizing to β -actin in each lane. Data are the mean \pm SEM (n=3), each bar represents the mean of three independent experiments carried out in triplicate. *Compared with control group, $P < 0.05$, #compared with DOX group, $P < 0.05$.

cell viability and higher apoptosis in DOX, PPAR α - and PPAR α + groups.

The prognosis is significant for heart failure patients diagnosed. Few special new drugs have been designed to aim myocardial metabolic pathways in spite of more and more evi-

dences from fundamental researches reveal that heart failure because of an imbalance between myocardial energy demand and supply [27]. For heart failure, energy metabolism defect was closely correlated with heart dysfunction which is incapable of generating sufficient amounts of ATP. Down-regulation expres-

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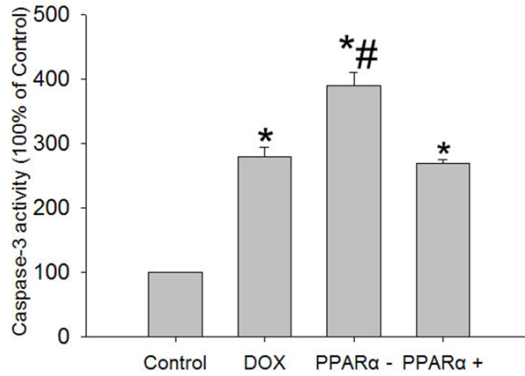


Figure 6. Enzymatic assay of caspase-3 activity. Activity of caspase-3 was measured by using a caspase-3 colorimetric protease assay kit. Data are the mean \pm SEM (n=3), each bar represents the mean of three independent experiments carried out in triplicate, *compared with control group, $P < 0.05$.

sion of fatty acid oxidation and glucose consumption likely urge the severity of this phenotype [28]. From aspect of energy metabolism, our results exhibit that lower capacity in producing energy maybe break normal cell life cycle, and cause cell apoptosis. An attempt to improve substrate oxidation and adenylate generation, maybe a new pharmaceutical therapies for heart failure patients induced by doxorubicin.

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Disclosure of conflict of interest

None.

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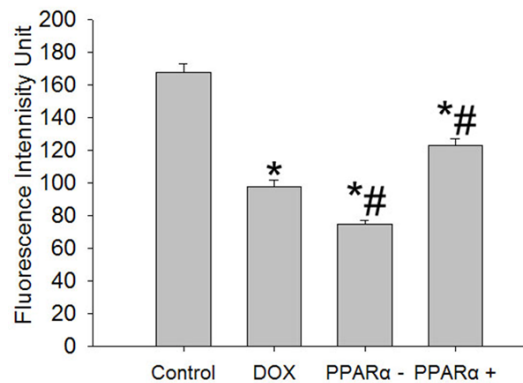
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Supplementary methods

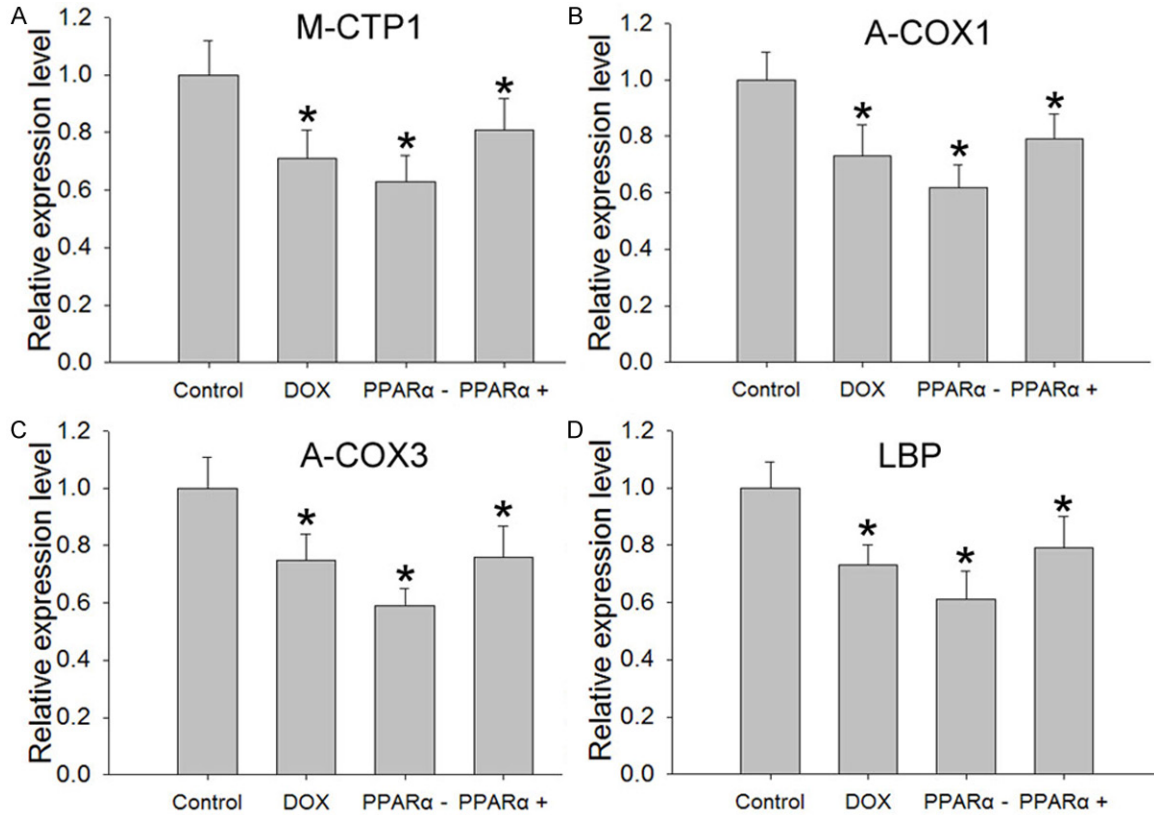
Measurement of mitochondrial membrane potential

Mitochondrial membrane potential ($\Delta\psi_m$) was analyzed used tetramethylrhodamine ethyl ester (TMRE), which distributes across the inner mitochondrial membrane in accordance with the Nernst equation. After doxorubicin, GW 6471, or WY14643 treatments, cardiomyocytes were incubated with 100 nmol/L TMRE for 30 min at 37°C and 5% CO₂. A Live/Dead® Near-IR Dead Cell stain kit (Invitrogen) was used to exclude dead cells. Viable cells were analyzed with a DAKO Cyan cytometer. The mitochondrial uncoupler carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP) was used as a control for depletion of the mitochondrial membrane potential.



Supplementary Figure 1. Changes of mitochondrial membrane potential in different groups of cardiomyocytes. Data are the mean \pm SEM (n=3), each bar represents the mean of three independent experiments carried out in triplicate. *Compared with control group, $P<0.05$; #Compared with DOX group, $P<0.05$.

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Supplementary Figure 2. Analysis of fatty acid oxidation related genes expression in different groups. A. *M-CPT1* (NM_009948.2). B. *A-COX1* (NM_015729.3). C. *A-COX3* (NM_030721.2). D. *LBP* (NM_008489.2). Data are the mean \pm SEM (n=3), each bar represents the mean of three independent experiments carried out in triplicate. *Compared with control group, $P < 0.05$.

Supplementary Table 1. Primers used for quantitative real-time PCR analysis

Gene	Name	Primers (5' to 3')
PPAR α	PPAR α -F	GGGTGGTTGAATCGTGAGG
	PPAR α -R	TGCCTTTTGCCAACAGTAGTAC
PGC-1 α	PGC-1 α -F	ATGGCACGCAGCCCTATTC
	PGC-1 α -R	GCATC-CTTTGGGGTCTTTG
A-COX1	A-COX1-F	CTCACTCGAAGCCAGCGTTA
	A-COX1-R	CGGTGCACAGAGTTTTTAAACCA
A-COX3	A-COX3-F	GGATCCGGGAGGAACATCAC
	A-COX3-R	ACGATAACTCTGCACACGGG
LBP	LBP-F	TTCAGAACTGTGAGCTGCGT
	LBP-R	CGGACACCGATGGAAGAGTC
M-CPT1	M-CPT1-F	CCGGAAAGGTATGGCCACTT
	M-CPT1-R	GAAGAAAATGCCTGTCGCC
Actin	Actin-F	GTCCACACCCGCCACAGTTC
	Actin-R	TCCCACCATCACACCCTGGTG

Primers were designed using Primer Express version 2.0 software. Primer specificity was confirmed using Primer-BLAST web software (National Centre for Biotechnology Information).