

# Ischemic post-conditioning promotes mitochondrial translocation of DJ-1 and stabilizes binding to Bcl-xL to attenuate myocardial ischemic-reperfusion injury in STZ-induced diabetic rats

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## Research Article

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# Abstract

**Background** Ischemic post-conditioning (IPO) is a strategy in reducing myocardial ischemic-reperfusion (I/R) injury, but its specific molecular mechanism is incompletely understood. Deletions or mutations in the DJ-1 gene are directly linked to the cardiovascular system. DJ-1 plays a critical role in regulating mitochondrial homeostasis in response to stress through translocation of DJ-1 from the cytoplasm into the nucleus. Meanwhile, how the DJ-1 is removed in myocardial I/R injury and regulating apoptosis is needed to further verified. Given the discovery mentioned above, we hypothesize that DJ-1 translocate to mitochondria recover IPO induced cardioprotection in STZ-induced diabetic rats. To evaluate our hypothesis, we overexpressed the DJ-1 protein under high glucose condition, subjected IPO or not. And then measure the expression of DJ-1 in mitochondria and cytoplasm after myocardial I/R.

**Results** We found that DJ-1 translocated to mitochondria combined with Bcl-xL was reduced cardiomyocyte injury and apoptosis in diabetic myocardial I/R heart. Additionally, the binding of DJ-1 and Bcl-xL is dependent on the oxidative state of DJ-1(oxidation of DJ-1 at Cys-106 is important for its protective effect).

**Conclusions** If our hypothesis is correct, promote DJ-1 mitochondrial transfer may be critical with respect to restoring myocardial responsiveness to IPO in diabetes.

## 1 Introduction

Among the cardiovascular diseases, ischemic heart disease serves the most common cause of death in diabetic conditions. Due to its high morbidity and mortality it represents a major socioeconomic health problem[1]. The restoration of blood supply is the mainstay of treatment in ischemic heart disease. One or more brief cycles of IPO can protect the heart from acute myocardial infarction and myocardial reperfusion injury[2]. This effect was achieved by the potentiation of restore mitochondrial function, antioxidative, activates Akt signaling and anti-inflammatory to confer cardioprotection[3]. But in diabetic patients, the detrimental effect of myocardial reperfusion has been shown to be more pronounced (generation of reactive oxygen species, inflammatory response and accelerated apoptosis)[4-6]. Therefore, the responsiveness of diabetic hearts to IPO is impaired.

Mitochondrial dysfunction is one of the key pathological processes involved in the diabetic I/R[7]. B-cell lymphoma-extra large (Bcl-xL) is a pro-survival protein prominently localized to mitochondrial membrane[8]. Bcl-xL belongs to the Bcl-2 protein family, which serves critical role in the regulation of intrinsic apoptosis by controlling the mitochondrial outer membrane permeability and the release of cytochrome C. Bcl-2 family proteins are classified into anti-apoptotic (Bcl2, Bcl-xL, and Mcl-1) and pro-apoptotic (Bax, Bid, and Bad) members[9]. In diabetic myocardial I/R injury, Bcl-xL can regulate apoptosis and autophagy, regulate cytosolic cytochrome C release[10]. In the present study, we explored how Bcl-xL provides cardiac protection against myocardial I/R injury.

DJ-1 was originally identified as an oncogene product and aberrant expression of DJ-1 is associated with tumorigenesis in several cancer tissues[11]. It should be noted that DJ-1 protects the heart against I/R injury by regulating mitochondrial fission[12]. Two recent studies report that mice deficient in DJ-1 developed more severe heart failure in response to aortic banding and displayed exaggerated myocardial injury in response to ischemia because of mitochondrial dysfunction in the heart[12-14]. While these studies suggest DJ-1 plays a protective role by transfer to mitochondria in the heart, neither study investigated if stress induced its activation. Following exposure to ultraviolet B irradiation, DJ-1 translocates to the mitochondria and interacts with the mitochondrial protein Bcl-xL, DJ-1 stabilized Bcl-xL is resistant to the ultraviolet B irradiation induced degradation, which prevents cell death[15, 16]. Other reports suggest that dysfunction of DJ-1 contributes to onset and severity of various diseases including Parkinson's disease, diabetes, hypertension, obesity, and allergy as a result of oxidative stress[17-20]. However, to our knowledge, how the role of DJ-1 is transferred to the mitochondria to protect the myocardium remains unclear in diabetes.

Our previous studies have confirmed that DJ-1 expression is decreased in diabetic myocardium, while overexpression of DJ-1 does not reduce myocardial I/R damage[2]. However, DJ-1 combined with IPO can reduce myocardial damage and restore cardiac function, the specific molecular mechanism needed be exploration. Mitochondrial DJ-1 expression was significantly reduced after diabetic myocardial I/R injury. We hypothesized that decreased mitochondrial transfer may be important causes of IPO in activation. We aimed to investigate whether IPO can reduce myocardial damage by promoting DJ-1 mitochondrial transfer and activating the endogenous protective protein Bcl-xL.

## 2 Materials And Methods

### 2.1 Experimental animals

Male adult Sprague-Dawley rats ( $250 \pm 10$  g, 6-8 weeks of age) were supplied by the Laboratory Animal Service Center of Wuhan University. All the rats were housed in an environment with a controlled temperature of  $24^{\circ}\text{C}$ , a relative humidity of  $50 \pm 10\%$ , and a fixed light/dark schedule (12 h light/12 h dark) and were allowed free access to food and water. All the experimental protocols were performed in accordance with the animal care principles of Wuhan University and were approved by the Committee for the Use of Live Animals in Teaching and Research.

### 2.2 Reagents

Streptozotocin (STZ), triphenyl tetrazolium chloride (TTC), and Evans blue were obtained from Sigma Chemical Co. (MO, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco Laboratories (Grand Island, NY, USA). Antibodies directed against DJ-1, Bcl-xL, Cleaved Caspase-3, Bax, Bcl-2, Mcl-1, COX IV and GAPDH were obtained from Cell Signaling Technology (CST, Beverly, CA, USA). All other chemicals used in the study were purchased from Sigma Chemical Co., unless otherwise stated.

## **2.3 Diabetes, myocardial I/R injury models and myocardial IPO models**

After 3 days of acclimatization, the rats were fasted 12 h for diabetes induction. The diabetes model was induced via a single intraperitoneal injection of 60 mg/kg STZ, as described previously[2]. Rats exhibiting hyperglycaemia (blood glucose level  $\geq 16.7$  mmol/l) were considered diabetic rats. The I/R injury model was achieved by occluding the left anterior descending (LAD) artery for 30 min, followed by 120 min of reperfusion. IPO was completed after 30 min of ischemia, it consisted of three cycles of 10 s of reperfusion and ischemia, and then followed by 120 min of reperfusion. Sham-operated rats underwent the same surgical procedures without LAD occlusion. Blood and tissue samples were collected for additional analyses at the end of reperfusion.

## **2.4 AAV infection**

AAV9 vectors, which carry a CMV promoter for high-level gene expression, were produced by Hanbio Biotechnology Co. via a modified 3-plasmid co-transfection method. AAV9-CMV or AAV9-CMV-DJ-1 was administered via tail vein injection at a dose of  $2 \times 10^{12}$  vg/kg 3 weeks before myocardial I/R induction (5 weeks after diabetes induction).

## **2.5 Cardiac function assessment**

To evaluate myocardial function, we performed invasive haemodynamic monitoring. Rats were subjected to electrophysiology at three different states i.e., before starting the IR, after IR and IPO. Following anesthesia with sevoflurane, a fluid-filled catheter was advanced through the right carotid artery into the left ventricle. Heart rate, LVSP (left ventricular systolic pressure),  $\pm dp/dt$  (maximal rates of left ventricular-developed pressure increases and decreases) were continuously monitored via electrophysiology (BioPAC, MH150), and the data were derived using AcqKnowledge 4.0 software.

## **2.6 Infarct size determination**

Infarct size was measured as described previously[2]. We used 3% Evans Blue dye and 1% 2,3,5-triphenyltetrazolium chloride (pH 7.4) for myocardial staining. The stained myocardial slices were scanned (Epson v30) and assessed using an image analysis system (Image J).

## **2.7 Myocardial creatine kinase-MB assay**

CK-MB (creatine kinase-MB) is a commonly used specific indicator of myocardial injury. Blood samples were collected at the end of reperfusion and centrifuged at 2000 rpm for 15 min to detect CK-MB expression via enzyme immunoassay using a commercial ELISA kit (Jiancheng Bio).

## **2.8 Cell culture**

Embryonic rat cardiomyocyte-derived cell line H9c2 was obtained from the A.T.C.C. (Manassas, VA, U.S.A.), were cultured in the DMEM supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, and

100 mg/ml streptomycin at 37 °C and under 5% CO<sub>2</sub> in a humidified CO<sub>2</sub> incubator (Sanyo, Japan). The medium was replaced every other day and the H9c2 cells were allowed to grow to 60-80% confluence within 24 h prior to experiment.

## **2.9 Cell viability and lactate dehydrogenase release assay**

Cell viability was determined in 96-well plates using a CCK-8 assay kit (Dojindo), and lactate dehydrogenase (LDH) activity was measured using a cytotoxicity assay kit (Jiancheng Bio), according to the manufacturers' instructions.

## **2.10 Isolation of mitochondria**

Isolation of mitochondria and cytoplasm from H9c2 cells and heart tissue extracts were performed using Mitochondria Isolation Kit for Profiling Cultured Cells (Sigma, USA) according to the manufacturer's instructions. Protein levels were measured by BCA Assay (Servicebio, China) with bovine serum albumin as standard. The final pellet represents a crude mitochondrial fraction that be used for further experiments. All the isolation procedures should be performed at 2-8 °C with ice-cold solutions.

## **2.11 Co-immunoprecipitation assays**

1 mg protein sample was pre-cleared with 30 µl of protein A/G-agarose (Santa Cruz, USA) for at 4 °C for 2 h and then centrifuged at 14,000g, 4 °C for 30 min. Aliquots (50 ml) of the pre-clared supernatant were saved as input. The pre-cleared supernatant (250 µl) was incubated with antibody or normal IgG (used as negative control) overnight at 4 °C followed by incubation with protein A/G-agarose for 4 h. The agarose beads were collected by centrifugation, washed three times with cold PBS, and eluted by boiling for 5 min in 4×Laemmli sample buffer. The resulting immunoprecipitates and input were separated by SDS-PAGE and probed with antibodies in immunoblotting as described above.

## **2.12 Western blotting**

The expression levels of the proteins extracted from myocardial tissue samples or H9c2 cells were measured as described previously[2]. Briefly, tissues or cells were homogenized with a mixture of RIPA lysis buffer, and total protein concentrations were determined using a BCA kit (Servicebio, China). Equivalent amounts of protein (20-50 µg) were separated via SDS/polyacrylamide gel electrophoresis (5-15% gel) and electrotransferred onto PVDF membranes. After being blocked with 5% bovine serum albumin for 1 h at room temperature, the PVDF membranes were incubated with primary antibodies to DJ-1 (1:1000 dilution), Bcl-xL (1:1000 dilution), Cleaved Caspase-3 (1:1000 dilution) overnight at 4 °C, followed by incubation with the appropriate secondary antibody (1:12000 dilution) for 1 h at room temperature. GAPDH or COX IV were used as a control to ensure equal loading. Signals were detected and quantified using a fluorescence imaging scanner (Odyssey).

## **2.13 Statistical analysis**

All data were expressed as means  $\pm$  SEM. Statistical analyses were performed using one-way or two-way ANOVA, followed by the Tukey HSD test. Statistical analysis was performed using Graphpad Prism 8.0 for Windows (GraphPad Software, USA). Statistical significance was accepted at  $p < 0.05$ .

## 3 Results And Discussion

### 3 Results

#### 3.1 IPO is not effective in diabetic myocardial I/R injury rats

To investigate whether IPO has cardioprotective effects against myocardial I/R injury in diabetic rats, myocardial infarction size and biochemical markers of myocardial injury after I/R injury were examined. As shown in Figure 1(A), I/R infarction sizes in diabetic rats were larger than those in non-diabetic control rats. IPO significantly decreased infarct sizes in non-diabetic rats, but not in diabetic rats. As shown in Figure 1(B) and (C), CK-MB and LDH levels were significantly increased in diabetic rats compared with non-diabetic rats. IPO noticeably reduced CK-MB and LDH release in non-diabetic rats, but not in diabetic rats. These results showed that IPO improved cardiac functional recovery in non-diabetic rats subjected to I/R, but it had no effect on diabetic rats.

We next measured the changes in DJ-1, Bcl-xL and apoptosis related protein Cleaved Caspase-3 induced by the above processes. As shown in Figure 1(D) (E), DJ-1 and Bcl-xL expressions levels were significantly down-regulated in diabetic rats compared with non-diabetic rats, I/R increased DJ-1 expression levels from non-diabetic rats, and IPO induced further increases in DJ-1 expression levels. However, these changes were not observed in diabetic groups. In addition, we investigated the myocardial I/R induced cardiomyocytes apoptosis and found that was alleviated by IPO treatment in non-diabetic rats but does not work in diabetic rats, Figure1 (F). These results suggest that IPO was able to increased DJ-1 and Bcl-xL expression in non-diabetic rats, as well as decreased Cleaved Caspase-3 expression, but this effect is lost in diabetes.

#### 3.2 Myocardial overexpression of DJ-1 during IPO induced cardioprotection

To further determine whether hyperglycaemia-induced DJ-1 inhibition compromises IPO induced cardioprotection in diabetic rats, we overexpressed the DJ-1 protein in myocardial tissue from diabetic rats via AAV9-CMV-DJ-1 injections. At 3 weeks after AAV9-CMV-DJ-1 infection, we determined that DJ-1 protein expression levels in treated rats were nearly 2.2-fold higher than those in control group. As shown in Figure 2 (A-C), DJ-1 overexpression alone slightly but not significantly reduced infarct sizes, CK-MB and LDH releases, whereas the combination of DJ-1 overexpression and IPO markedly decreased infarct sizes, CK-MB and LDH levels, indicating that DJ-1 overexpression restores IPO-induced cardioprotection in diabetic rats. We subsequently investigated the effects of the above treatments in cardiac mitochondrial DJ-1, Bcl-xL and cardiac cleaved caspase-3 levels. As shown in Figure 2 (D-E), the combination of cardiac AAV9-CMV-DJ-1 infection and IPO significantly activated cardiac mitochondrial DJ-1, Bcl-xL content and inhibited cardiac Cleaved Caspase-3 level. As shown in Table 1, DJ-1 overexpression alone slightly

increased heart rate, LVSP, +dp/dt or -dp/dt, but those did not reach statistical differences. However, in the presence of DJ-1 overexpression, IPO significantly improved heart rate, LVSP, +dp/dt and -dp/dt in diabetic rats.

### **3.4 IPO induces DJ-1 transfer into mitochondria in the diabetic rats**

To test whether hyperglycemia and IPO affect simultaneously the subcellular location of DJ-1 in cardiomyocytes, cytoplasmic and mitochondrial fractions were prepared, and mitochondrial and cytoplasmic DJ-1 levels were examined by Western blot. As presented in Figure 3(A), IPO treatment increased the levels of mitochondrial DJ-1 protein relative to the IR treated. Importantly, our results demonstrated that IPO treatment increased the mitochondrial/cytoplasmic ratio of DJ-1 in the diabetic rats subjected to IR, suggesting that IPO promotes DJ-1 translocation from the cytosol to the mitochondria, show in Figure 3(B). Overall, these results suggest a possible involvement of IPO modulation DJ-1 translocation from the cytosol to the mitochondria.

### **3.5 HPO preserved the protective effects of overexpress DJ-1**

Additional investigations were performed using embryonic rat cardiomyocyte-derived H9c2 cells. As shown in Figure 4(A-B), HG stimulation noticeably decreased cell viability and increased LDH release compared with the LG group. These effects were amplified by hypoxia-reoxygenation(H/R). HPO significantly reversed these effects under LG conditions, but not under HG conditions. However, DJ-1 significantly restored the protective effects of HPO, as demonstrated by increased cell viability and decreased LDH release. We subsequently investigated the effects of the above treatments on mitochondrial DJ-1, Bcl-xL and cellular Cleaved Caspase-3 levels. As shown in Figure 4(C-E), AAV9-CMV-DJ-1 mediated DJ-1 overexpression alone did not significantly affect the expression of DJ-1, Bcl-xL in mitochondria, nor did it affect Cleaved Caspase-3 expression in H9c2 cells exposed to HG. However, the combination of AAV9-CMV-DJ-1 infection and IPO significantly increased the mitochondrial DJ-1, Bcl-xL levels, reduced cardiomyocytes apoptosis.

### **3.6 HPO induced DJ-1 translocate in mitochondria and promoted binding to Bcl-xL**

To further confirm the effects of HPO combine with DJ-1 can alleviate high glucose-induced H9c2 H/R injury, we overexpressed DJ-1 using pEX-2-EGFP-DJ-1 transfected and examined its effects on Bcl-xL. The overexpress efficiency of pEX-2-EGFP-DJ-1 is shown in Figure 5 (A). As show in Figure 5 (B-E), overexpress of DJ-1 did not significantly change mitochondrial Bcl-xL protein levels. However, with HPO, Bcl-xL protein levels were significantly higher in the mitochondria. Therefore, we presume that DJ-1 may play a major role as molecular chaperone in affecting some important protein during HPO. We next examined the binding of mitochondrial DJ-1 to Bcl-xL in H9c2 cell subjected to HPO. The binding of DJ-1 to Bcl-xL was assessed by co-immunoprecipitation. Mitochondrial lysates were immunoprecipitated with anti-DJ-1 antibody. Precipitates were immunoblotted for DJ-1, and Bcl-xL respectively. After co-immunoprecipitation analysis, a novel and intriguing interaction between DJ-1 and Bcl-xL was discovered

(Figure 5F). Furthermore, members of other apoptosis families, such as Bax, Bcl-2, and Mcl-1, coincidentally have no detected interaction with DJ-1 (Figure 5G).

### 3.7 Cys-106 of DJ-1 is required for its binding to Bcl-xL

Although our data implicate a functional relationship between DJ-1 and Bcl-xL, the mechanistic basis underlying this relationship is not well understood. The ability of DJ-1 to protect against oxidative stress is dependent on C<sup>106</sup> oxidation and oxidation-driven mitochondrial localization. To examine if the interaction between DJ-1 and Bcl-xL is mediated by the oxidative state of DJ-1, we created a C<sup>106A</sup> mutant to examine whether C<sup>106</sup> is required for DJ-1 to bind to Bcl-xL. DJ-1(C106A), which cannot be oxidized, bound to Bcl-xL much less than DJ-1 did in co-immunoprecipitation assays and could not bind to Bcl-xL without HPO treatment in cells. We performed co-immunoprecipitation experiments. As expected, C<sup>106A</sup> interacted with Bcl-xL but not C<sup>106A</sup> mutant as shown in Figure 6 (A), and lower bind to Flag-Bcl-xL without HPO in H9c2 cells, Figure 6 (B). These results suggest that the interactions between DJ-1 and Bcl-xL are oxidation-dependent.

## 3 Discussion

In the present study, we demonstrate that IPO can alleviate diabetic myocardial ischemic reperfusion injury by promoting DJ-1 transfer to mitochondria. (1) AAV9-mediated DJ-1 overexpression, restored the IPO induced cardioprotection in diabetic heart, and (2) DJ-1 translocates to mitochondria under IPO treatment. The precise localization of DJ-1 in mitochondria has been confirmed by several groups to be in the outer mitochondrial membrane, although it has also been reported to be inserted into the inter-membrane space or in the mitochondrial matrix. In our observations, in diabetic heart under IPO treatment, DJ-1 binds to Bcl-xL (a typical Bcl-2 family protein that primarily localizes in the outer mitochondrial membrane) in the mitochondria. (3) DJ-1 directly bound to Bcl-xL in a C<sup>106</sup>-dependent manner, the interactions between DJ-1 and Bcl-xL are oxidation-dependent.

Recent studies have shown that IPO significantly protects cardiomyocytes against I/R injury in diabetes[2, 3]. IPO maintains mitochondrial homeostasis, attenuates oxidative stress, regulates autophagy and alleviates apoptosis[21-23]. Our present study demonstrated that IPO confers protection against myocardial I/R injury in non-diabetic rats, but not in diabetic rats, a finding consistent with those of previous studies[2, 3], demonstrating that hyperglycaemia-induced DJ-1 inhibition may be responsible for the loss of IPO-induced cardioprotection in diabetes.

Under basal conditions, endogenous DJ-1 is present in the cytosol and nucleus. Upon oxidation, DJ-1 translocates from the cytosol to the outer mitochondrial membrane, a process which is required for its cardioprotective effect. It has been suggested that DJ-1 binds to electron transport chain complexes and is required for the latter's normal function such that knockdown of DJ-1 inhibits complex I activity[24]. Experimental studies have demonstrated that the activation of DJ-1 in response to myocardial I/R injury protects the heart by regulating the SUMOylation status of Drp1 and attenuating excessive mitochondrial

fission[12]. A recent study has also shown that mouse embryonic lacking DJ-1 had impaired mitochondrial respiration due to complex I inhibition, increased mitochondrial oxidative stress, reduced mitochondrial membrane potential, more fragmented mitochondria, and impaired mitophagy, findings which confirmed that DJ-1 is required for normal mitochondrial function[25, 26]. Furthermore, we observed that IPO was able to increase DJ-1 expression and modulation DJ-1 translocate from the cytosol to the mitochondria in non-diabetic rats, but not in diabetic rats. We, therefore, overexpressed the DJ-1 protein in myocardial tissue from diabetic rats via AAV9-CMV-DJ-1 injections, found that DJ-1 translocates to mitochondria restored the sensitivity of IPO induced cardioprotection in diabetic heart. Importantly, overexpression of DJ-1 activation the protection associated with preconditioning, suggesting that ischemic preconditioning mediates its protective effect through the IPO-dependent activation of DJ-1. More recent studies have suggested that DJ-1 may inhibit oxidative stress-induced apoptotic cell death by interacting with ASK1 directly and preventing its dissociation with Thioredoxin 1, a factor which inhibits ASK1 activity under basal conditions and reducing JNK activity[27]. Furthermore, DJ-1 has been reported to prevent apoptotic cell death by activating and phosphorylating the anti-apoptotic protein kinase, Akt, by suppressing PTEN activity[28]. A previous study showed that the protective effect of DJ-1 in the mitochondria and the interaction between DJ-1 and Bcl-xL are dependent on the oxidation state of Cys106[29].

Bcl-2 family proteins play important roles in the control of mitochondrial cell death. Bcl-2 family proteins include both anti-apoptotic and pro-apoptotic molecules[9]. The anti-apoptotic protein Bcl-xL (primarily localized to the outer mitochondrial membrane) plays a role in keeping mitochondria intact to inhibit cytochrome C release[30]. In our present study, we found that DJ-1 binds to Bcl-xL in mitochondria by IPO treatment in diabetic heart. Upon IPO treatment, the interaction between DJ-1 and Bcl-xL is enhanced. It has been reported that DJ-1 inhibits the ubiquitination of NF-E2-related factor 2 by preventing its association with its E3 ligase Keap1[31]. It is therefore possible that the interaction of DJ-1 with Bcl-xL blocks the relevant E3 ligases from interacting with and ubiquitinating Bcl-xL. Interestingly, DJ-1's translocation to mitochondria under oxidative stress is dependent on oxidation of its Cys-106 site. However, DJ-1(C106A), a loss of oxidized form of DJ-1, does not bind to Bcl-xL in the mitochondria and fails to perform its protective function under IPO treatment in diabetic heart. We found that IPO promotes the translocation of DJ-1 to the mitochondria and its binding to Bcl-xL, and the binding of DJ-1 and Bcl-xL is dependent on the oxidative state of DJ-1. However, DJ-1(Cys-106A) exhibits very low binding affinity to Bcl-xL in co-immunoprecipitation assays. This means oxidation of DJ-1 at Cys-106 is important for its protective effects. For instance, the oxidation of Cys-106 is required for DJ-1 protection against mitochondrial fragmentation and cell death, and DJ-1 fails to protect cells when Cys-106 is in reduced state. Taken together with our findings, we propose that the protective effects of IPO in diabetic I/R injury heart are mediated by the enhanced interactions between DJ-1 and Bcl-xL in mitochondrial.

## 4 Conclusions

In conclusion, the findings of our present study provide compelling evidence that DJ-1 binds to Bcl-xL in mitochondria contributes to impairments in IPO-induced cardioprotection in STZ-induced Type 1 diabetic

rat. Overexpression of DJ-1 could restore IPO-induced cardioprotective effects in diabetic rats; the underlying mechanisms are implicated in DJ-1 and Bcl-xL signalling activation and its interactions between DJ-1 and Bcl-xL are Cys-106-oxidation dependent manner. Therefore, targeting DJ-1 may be critical with respect to restoring myocardial responsiveness to IPO in diabetes.

## **Abbreviations**

Ischemic post-conditioning (IPO)

Ischemic-reperfusion (I/R)

B-cell lymphoma-extra large (Bcl-xL)

Streptozotocin (STZ)

Triphenyl tetrazolium chloride (TTC)

Dulbecco's modified Eagle's medium (DMEM)

Fetal bovine serum (FBS)

Left anterior descending (LAD)

Left ventricular systolic pressure (LVSP)

Maximal rates of left ventricular-developed pressure increases and decreases ( $\pm dp/dt$ )

Lactate dehydrogenase (LDH)

Creatine kinase-MB (CK-MB)

## **Declarations**

### **Data Availability**

The data used to support the findings of this study are available from the corresponding author upon request.

### **Conflicts of Interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

### **Author contributions**

Wei Li and Zhong-yuan Xia designed research; Yan Leng and Yong-hong Xiong analyzed data; Wen-yuan Li, Rui Xue, Rong Chen performed research; Yan Leng, Yong-hong Xiong, and Zhong-yuan Xia wrote the paper; Qing-tao Meng contributed new reagents and analytic tools.

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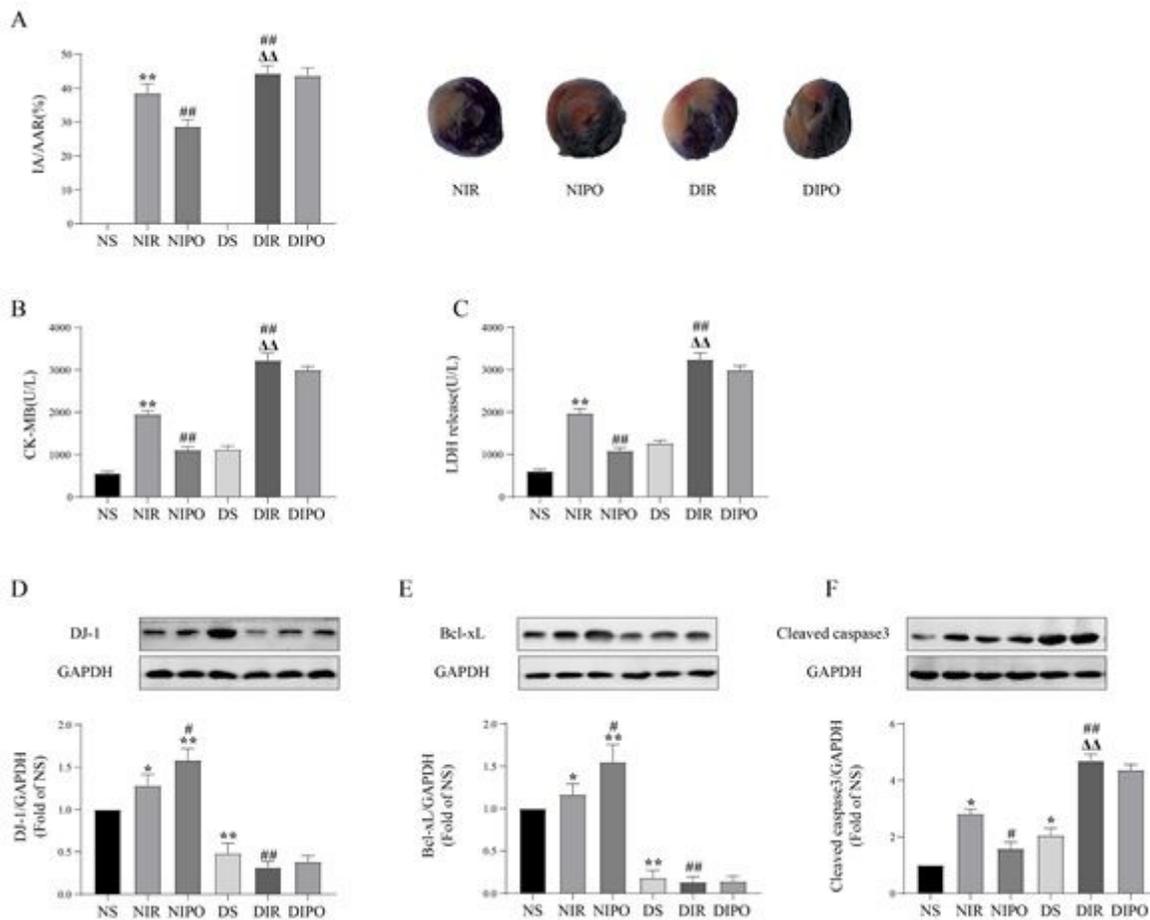
## Tables

Table 1: Hemodynamic parameters reflecting left ventricular function in diabetic rats after 2h of reperfusion

Groups/Parameters	baseline	IR	IPO
HR (beats/min)			
NS	401±23	260±21*	310±25+
DM	298±17*	204±19#+	186±15
DM+DJ-1 <sup>+</sup>	301±20	212±14	250±17▲
LVSP (mmHg)			
NS	131±19	82±8*	112±12+
DM	102±12*	62±8#+	51±6
DM+DJ-1 <sup>+</sup>	98±11	66±7	92±10▲
+dp/dt (mmHg/s)			
NS	6213±512	4812±315*	5642±587+
DM	4651±342*	2718±235#+	2654±314
DM+DJ-1 <sup>+</sup>	4668±421	3124±413	4809±615▲
-dp/dt (mmHg/s)			
NS	5121±345	3331±422*	4098±513+
DM	3421±413*	2135±354#+	1944±342
DM+DJ-1 <sup>+</sup>	3765±365	2213±312	3231±416▲

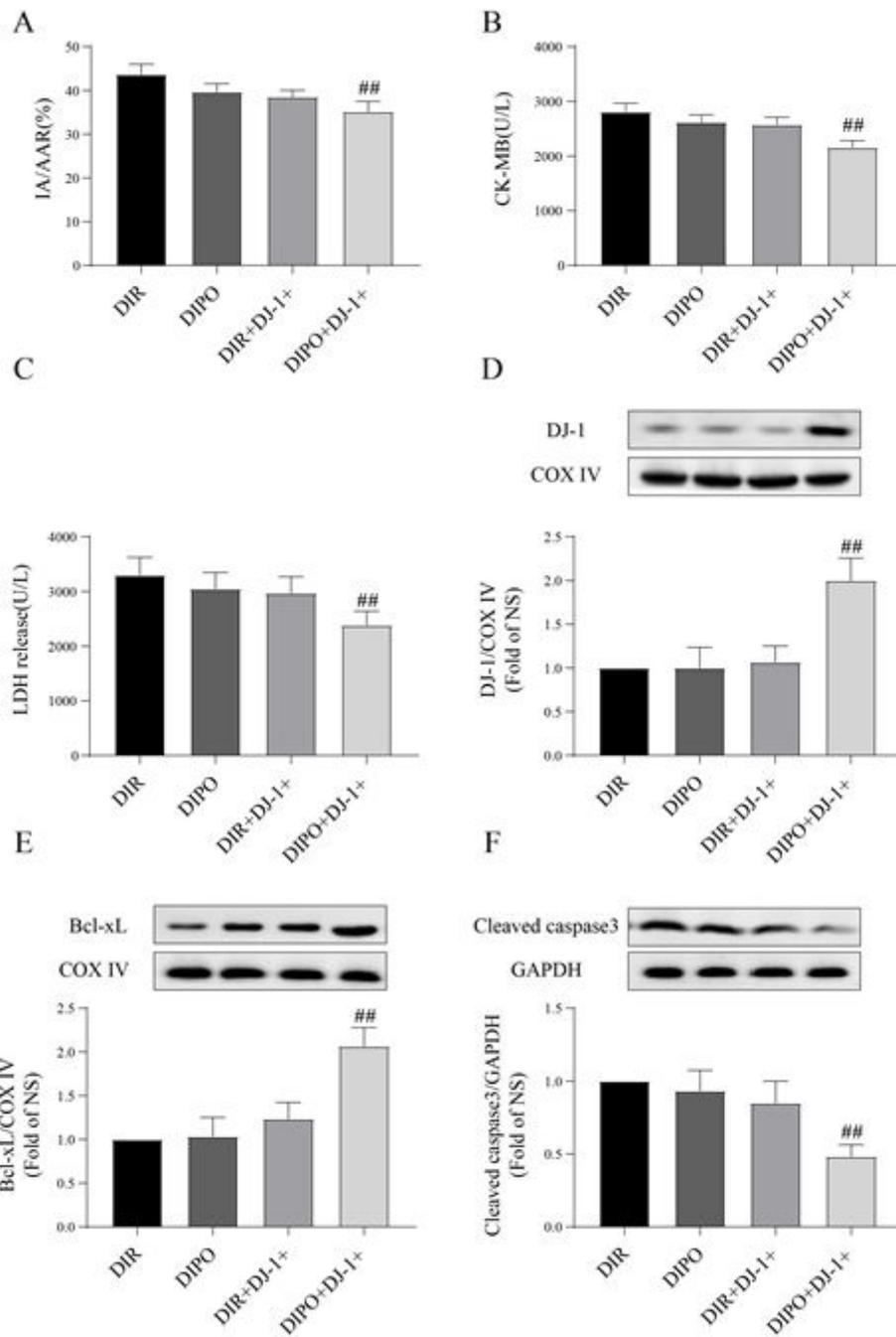
Values are presented as the means ± SEM (n=8 per group). \**P*<0.01 versus NS group at baseline, #*P*<0.01 versus DM at baseline, +*P*<0.01 versus NS + IR group; ▲*P*<0.01 versus DM + IR group.

## Figures



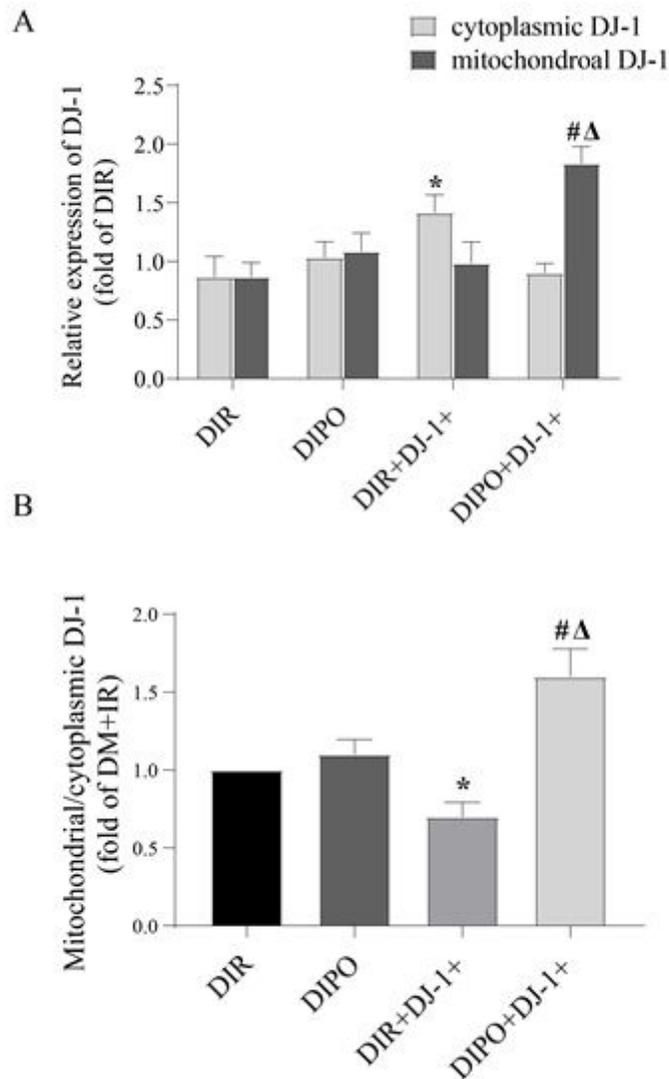
**Figure 1**

Effects of IPO on myocardial injury, DJ-1 and Bcl-xL expression levels, apoptosis levels after 30 min of ischemia followed by 2 h of reperfusion in non-diabetic and diabetic rats (A) Infarct area relative to the area at risk (IA/AAR  $\times$  100%). Blue-stained represent the nonischemic areas, red-stained represent the area at risk and pale areas indicate myocardial infarction. (B) Serum CK-MB level. (C) Myocardial LDH release. (D) DJ-1 expression level. (E) Bcl-xL expression level. (F) Cleaved caspase3 level in cardiac. Data are presented as the means  $\pm$  SEM. (n=6 in each group). \*P<0.05 and \*\*P<0.01 versus the NS group; #P<0.05 and ##P<0.01 versus the NIR group;  $\Delta$ P<0.05 and  $\Delta\Delta$ P<0.01 versus the DS group.



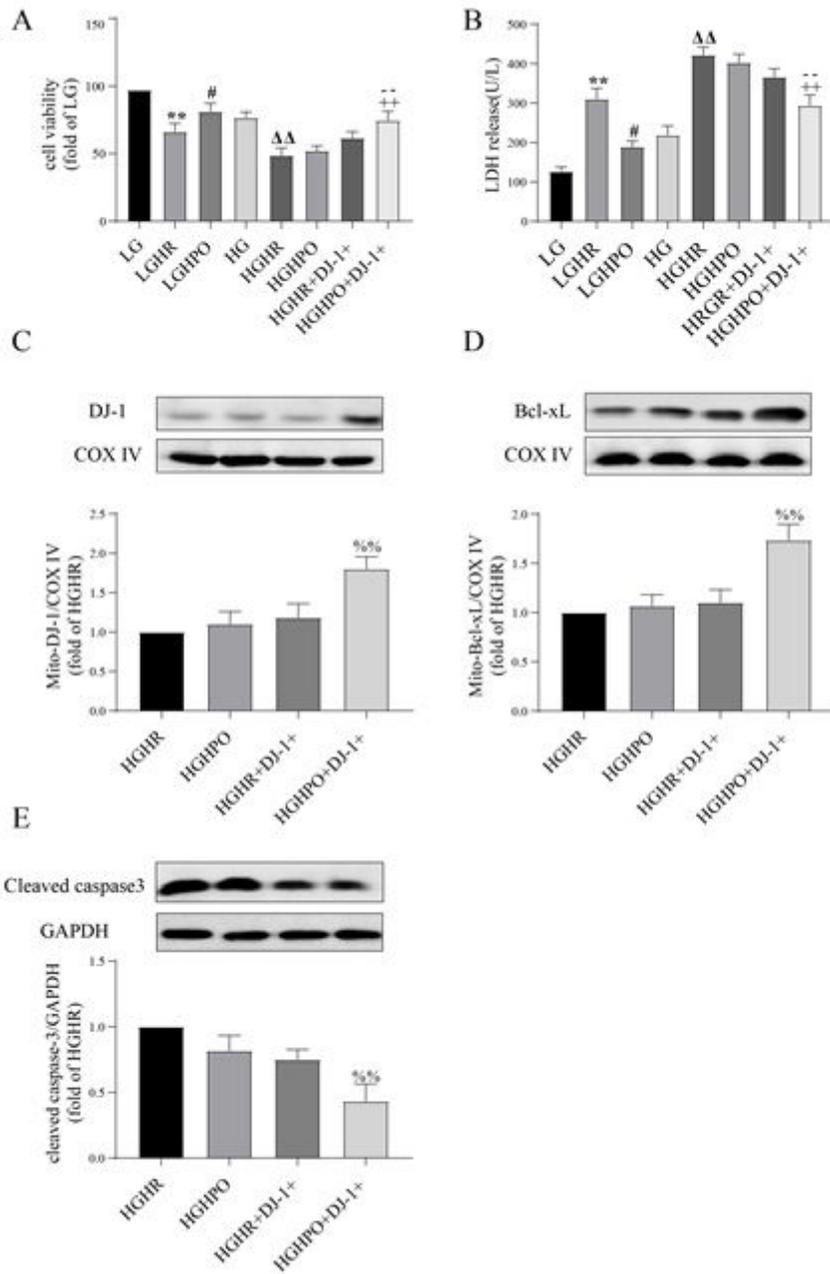
**Figure 2**

AAV9-CMV-DJ-1 mediated myocardial DJ-1 overexpression effects on IPO induced cardiac protection in diabetic rats (A) Infarct size. (B) Serum CK-MB level. (C) Myocardial LDH release. (D) DJ-1 expression level in cardiac mitochondria. (E) Cardiac mitochondrial Bcl-xL expression level. (F) Cleaved Caspase-3 level in cardiac. Data are presented as the means  $\pm$  SEM. (n=6 in each group). \*P<0.05 and \*\*P<0.01 versus the DIR group; #P<0.05 and ##P<0.01 versus the DIPO group;  $\Delta$ P<0.05 and  $\Delta\Delta$ P<0.05 versus the DIR+DJ-1+ group.



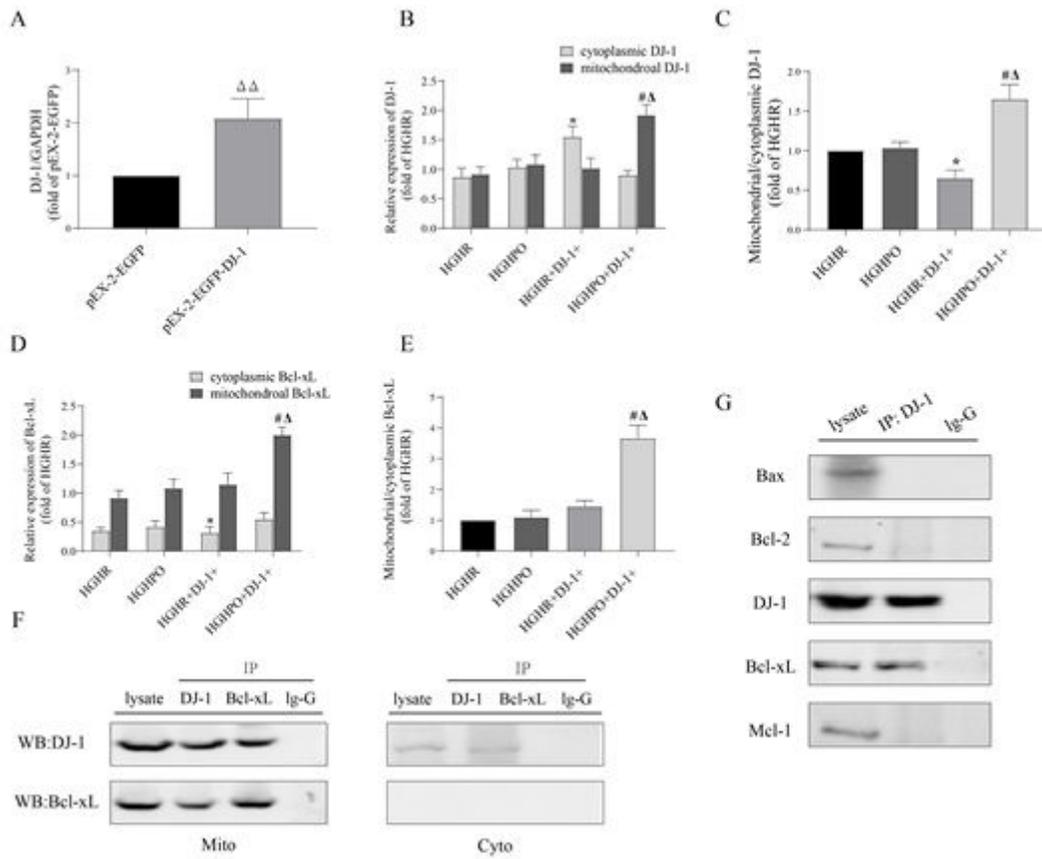
**Figure 3**

Effect of overexpress AAV9-CMV-DJ-1 on mitochondrial DJ-1 protein relative expression in diabetic rats. Cytoplasmic and mitochondrial fractions were prepared, and mitochondrial and cytoplasmic DJ-1 levels were examined by Western blot, as described in the Materials and methods section. Tubulin was used as cytoplasmic fraction purity marker and loading control, VDAC bands as mitochondrial fraction purity marker and loading control. (A) Relative expression of DJ-1. (B) Ratio of mitochondria to cytoplasmic DJ-1. Data are presented as the means  $\pm$  SEM. (n=6 in each group). \*P<0.05 versus the DIR group; #P<0.05 versus the DIPO group; ΔP<0.05 versus the DIR+DJ-1+ group.



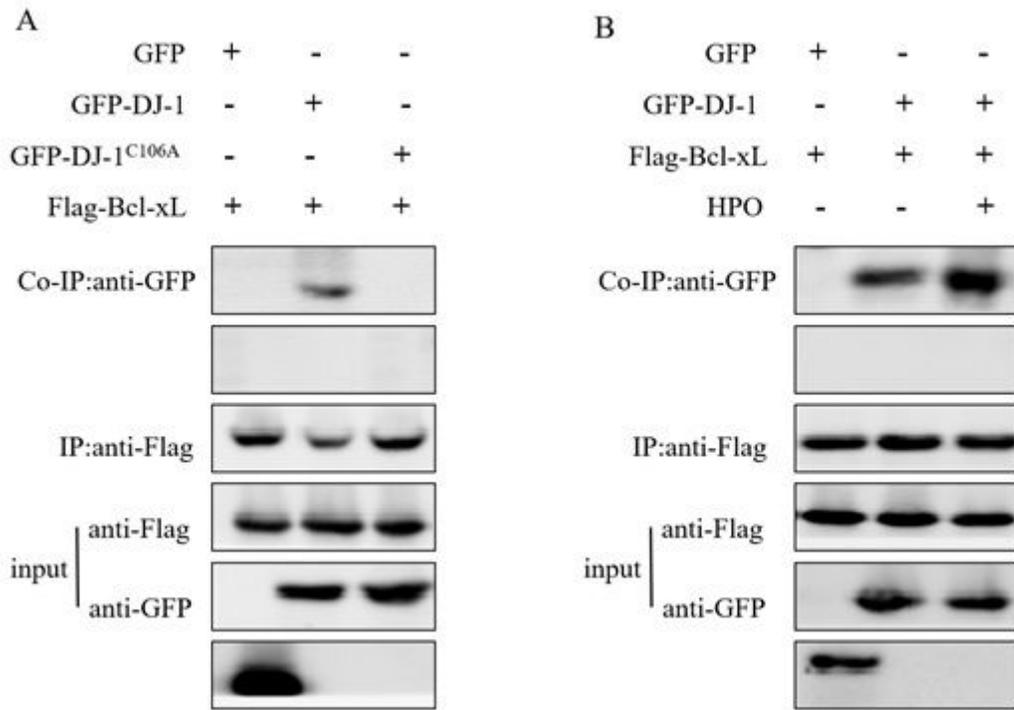
**Figure 4**

Effects of overexpress DJ-1 on HPO in H9c2 cells exposed in HG conditions (A) Cell viability. (B) LDH release. (C) DJ-1 expression level in mitochondria. (D) mitochondrial Bcl-xL expression level. (E) Cleaved Caspase-3 level. Values are presented as the means  $\pm$  SEM. (n=6 in each group). \*P<0.05 and \*\*P<0.01 versus the LG group; #P<0.05 and ##P<0.01 versus the LGHR group;  $\Delta$ P<0.05 and  $\Delta\Delta$ P<0.01 versus the HG group. +P<0.05 and ++P<0.01 versus the HGHR group, %P<0.05 and %%P<0.01 versus the HGHPO group, -P<0.05 and -P<0.01 versus the HGHPO+GJ-1+ group.



**Figure 5**

HPO induced the mitochondrial translocation of DJ-1 and promoted the binding of DJ-1 to Bcl-xL (A) Overexpress efficiency of pEX-2-EGFP-DJ-1. (B) Relative expression of DJ-1. (C) Ratio of mitochondria to cytoplasmic DJ-1. (D) Relative expression of Bcl-xL. (E) Ratio of mitochondria to cytoplasmic Bcl-xL. Data are presented as the means  $\pm$  SEM. (n=6 in each group).  $\blacktriangle\blacktriangle$  P<0.01 versus the pEX-2-GFP group; \*P<0.05 versus the HGHR group; #P<0.05 versus the HGHPO group,  $\Delta$ P<0.05 versus the HGHR+DJ-1+ group. (F) Interaction between DJ-1 and Bcl-xL in mitochondria. (G) Other apoptosis families have no detected interaction with DJ-1.



**Figure 6**

Oxidative DJ-1 is required for DJ-1 binding to Bcl-xL (A) H9c2 cells transfected with GFP, GFP-DJ-1 and GFP-DJ-1C106A along with Flag-Bcl-xL. The cells were collected and subjected to immunoprecipitation using anti-GFP or anti-Flag antibodies. (B) H9c2 cells transfected with GFP or GFP-DJ-1 along with Flag-Bcl-xL, were treated with or without HPO and treated. The cells were collected and subjected to immunoprecipitation using anti-GFP or anti-Flag antibodies.

## Supplementary Files

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