Title: Therapeutic effects of Nrf2 activation by Bardoxolone methyl in Chronic Heart Failure

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Nonstandard abbreviations: CDDO-Me, Bardoxolone methyl; DH404, Dihydro-CDDO-trifluoroethyl amide; CDDO-Im, 1-[2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl] imidazole
Abstract

Oxidative stress plays an important role in the pathogenesis of chronic heart failure (CHF) in many tissues. Increasing evidence suggests that systemic activation of Nrf2 signaling can protect against post-infarct cardiac remodeling by reducing oxidative stress. However, it remains to be elucidated if Nrf2 activation exerts therapeutic effects in the CHF state. Here, we investigated the beneficial hemodynamic effects of bardoxolone methyl (CDDO-Me), a pharmacological activator of Nrf2, in a rodent model of CHF. Based on echocardiographic analysis, rats at 12 weeks post-myocardial infarction (MI) were randomly split into four groups. CDDO-Me (5 mg/kg, ip) was administered daily for another two weeks in sham and CHF rats and compared to vehicle treatment. Echocardiographic and hemodynamic analysis suggest that short-term CDDO-Me administration increased stroke volume and cardiac output in CHF rats, and decreased left ventricle end-diastolic pressure (LVEDP). Molecular studies revealed that CDDO-Me-induced cardiac functional improvement was attributed to an increase of both Nrf2 transcription and translation, and a decrease of oxidative stress in the non-infarcted areas of the heart. Furthermore, CDDO-Me reduced NF-kB binding and increased Nrf2 binding to the CREB-Binding Protein (CBP), respectively, which may contribute to the selective increase of Nrf2 downstream targets, including NQO1, HO-1, Catalase, and GCLC, and the attenuation of myocardial inflammation in CHF rats. Our findings suggest that Nrf2 activation may provide beneficial cardiac effects in MI-mediated CHF.
Significance Statement

CHF is the leading cause of death among the aged worldwide. The imbalance between pro- and anti-oxidant pathways is a determinant in the pathogenesis of CHF. Systemic activation of Nrf2 and antioxidant protein signaling by CDDO-Me may have beneficial effects on cardiac function and result in improvements by enhancing antioxidant enzyme expression and attenuating myocardial inflammation.
Introduction

The imbalance between pro- and anti-oxidant pathways are known to be important in the progression of CHF in the post-myocardial infarction (MI) state (Bhimaraj and Tang, 2012; Farias et al., 2017). Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) as a master transcription factor is expressed in most tissues to act as an amplifier of anti-oxidant pathways, and has been associated with the expression of antioxidant enzymes in the myocardium (Tan et al., 2011; Ma, 2013; Tian et al., 2018). Our previous work has shown that myocardial Nrf2 protein is decreased in the CHF state (Tian et al., 2018), thereby potentially disrupting redox balance and contributing to myocardial as well as remote organ dysfunction.

Nrf2 protein is repressed by Kelch-like ECH-associated protein 1 (Keap1), which is a redox-sensitive substrate adaptor for the Cullin-3 E3 ubiquitin ligase. Modification of cysteine residues in Keap1 by electrophilic compounds prevents ubiquitylation of Nrf2, allowing de novo synthesized Nrf2 to accumulate in the nucleus, where it transactivates genes that contain antioxidant response element (ARE) sequences and competes with NF-kB for binding to CBP (Kwak et al., 2002; Li et al., 2008; Liu et al., 2008; Kim et al., 2013; Tebay et al., 2015). Increasing evidence has demonstrated the potential beneficial effects of Nrf2 activation in a variety of animal models of cardiovascular disease (Appendino et al., 2011; Bai et al., 2015; Qian et al., 2015; Bai et al., 2017; Xin et al., 2018; Wafi et al., 2019).

Using a cardiac-specific mouse Nrf2 transgenic model, cardiac-specific Nrf2 expression was observed to be important in maintaining redox homeostasis and preventing oxidative stress and subsequent pathological remodeling in a model of isoproterenol-
induced heart failure (Shanmugam et al., 2019). Global deletion of Nrf2 in mice promoted the pathogenesis of heart failure by increasing oxidative damage in the heart (Li et al., 2009). Our laboratory has shown that when Nrf2 was selectively deleted in the rostral ventrolateral medulla (RVLM) of the brain stem, antioxidant enzyme expression was reduced and oxidative stress was significantly increased, thus potentially contributing to sympatho-excitation in CHF (Gao et al., 2017). On the other hand in the post myocardial infarction heart failure state selectively upregulating Nrf2 in this region reduced sympathetic outflow (Ma et al., 2019).

Currently, several Nrf2 activators have been examined in disease states and are now being actively promoted in clinical trials. Many of these studies focus on renal disease, skeletal muscle abnormalities and multiple sclerosis (Cuadrado et al., 2019). There have been few studies to investigate the effects of Nrf2 activators on cardiac functions in a model of CHF. In the current study our objective was to determine if the small molecule Nrf2 activator, bardoxalone methyl (CDDO-Me) altered hemodynamics and antioxidant enzyme expression in the myocardium of rats with post-MI CHF. CDDO-Me, a prototype of a class of triterpenoid compounds has been used to treat chronic kidney disease in a Phase 3 clinical trial (Pergola et al., 2011) and pulmonary hypertension in a Phase 2 clinical trial (Hu et al., 2015). Unfortunately, because CDDO-Me did not prevent the progression of renal disease and increased mortality from cardiovascular events in patients with type 2 diabetes mellitus and stage 4 chronic kidney disease, these clinical trials were terminated (de Zeeuw et al., 2013; Chertow and de Zeeuw, 2014). However, CDDO-Me still demonstrated a potential to reduce oxidative stress, decrease inflammatory mediators (Ruiz et al., 2013; Zoja et al., 2014; Pickering et al., 2018) and
prevent the cardiac and renal pathophysiology induced by high-fat diet in mice (Camer et al., 2016).

Recently, a CDDO-Me derivative, DH404, has been shown to provide significant attenuation of pathological cardiac remodeling in CHF rats by decreasing the glutathionylation of endothelial nitric oxide synthase (eNOS) and increasing the physical interaction between eNOS and Glutaredoxin-1 (Grx1) (Bubb et al., 2017), suggesting that CDDO-Me may have clinical benefits for protecting the myocardium from pathophysiological remodeling. Therefore, we hypothesized that chronic low dose treatment with CDDO-Me would result in improved ventricular function by activating Nrf2/ARE signaling and increasing antioxidant enzyme levels in the myocardium of MI-induced CHF rats.
Materials and Methods

All animal studies were carried out with the approval of the University of Nebraska Medical Center Institutional Animal Care and Use Committee and followed the NIH Guide for the Care and Use of Animals.

Animals and Heart Failure Model

Animals were group housed in the Department of Comparative Medicine at the University of Nebraska Medical Center, and were subjected to a 12-hour light/dark cycle at approximately 25 °C. Myocardial infarction (MI) was created by ligating the left coronary artery following routine procedures in our laboratory and as described previously (Gao et al., 2005; Wang et al., 2010). In brief, male Sprague-Dawley rats (body weight: 180~200g) were anesthetized with 2% isoflurane in oxygen, intubated and placed on positive pressure ventilation. After opening the chest and removing the pericardium, the left anterior descending coronary artery was ligated with 5-0 silk suture in CHF animals, whereas the same procedures were followed except the coronary artery was not ligated in Sham animals. Following surgery, animals recovered in a heated cage until recumbent and moving. After 24 hours they were generally eating and drinking normally, and treated with 5 mg/kg carprofen and 0.1 mg/kg buprenorphine for three post-operative days. Twelve week post-MI, cardiac function was evaluated by echocardiography. Rats were considered to be in CHF when the ejection fraction (EF) was less than 40%.
Sham and CHF rats were given vehicle (DMSO; 0.1 ml, i.p) or CDDO-Me (5 mg/kg, 0.1 ml i.p) (APEBio, Houston, TX), respectively, daily starting at 12 weeks post-MI or Sham surgery as outlined in Figure 1 based on the first echo analysis.

**Infarct size**

To evaluate the infarct size, the CHF rats were euthanized with pentobarbital (150 mg/kg, ip). The left ventricle showing the transmural infarct was photographed, and the borders of the infarct and entire left ventricle were outlined. The scar borders were clearly visible in all infarcted animals. The areas were measured using Image J (NIH) software. The infarct size was calculated as the percentage of the entire left ventricle.

**Echocardiography**

Assessment of cardiac function was carried out using high-frequency echocardiography (Vevo 3100; 15MHz probe; Visual Sonics, Inc. Toronto, Canada). Under light isoflurane anesthesia rats were placed on a heated platform that recorded EKG, body temperature and respiratory rate. The thorax was shaved and treated with a depilatory to remove hair. Images were acquired in the 2D mode using the long axis view. An M-mode image was acquired using the 2D image to define a section at the level of the papillary muscles. In addition, an ECG-gated Kilohertz Visualization (EKV) image was acquired in the long axis to obtain high-fidelity averaged beat data. All cardiac parameters were analyzed using Visual Sonics software.

**Hemodynamics**
At the end of study, experimental rats were anesthetized with isoflurane. A 2F micromanometer-tipped pressure transducer catheter (SPR-407; Millar Instruments, Houston, TX) was inserted into the right carotid artery to measure arterial blood pressure (BP) and left ventricular (LV) pressure. Pressure signals were recorded on a Powerlab S16 using Lab Chart 7 software (ADInstruments, Inc., Colorado Springs, CO), from which heart rate (HR), left ventricular end-diastolic pressure (LVEDP), left ventricular peak-systolic pressure (LVPSP), the maximum rate of increase and decrease of left ventricular pressure (dp/dt\text{max} and dp/dt\text{min}) were derived.

**RNA extraction and qRT-PCR**

Myocardial tissues were subjected to RNA extraction using RNeasy Mini Kit (QIAGEN Inc., Valencia, CA) per the manufacturer’s recommendations. cDNA synthesis was carried out using reverse transcription MasterMix (Applied Biological Materials, Richmond, Canada). SYBR® Select Master Mix (Life Technologies, Los Angeles, CA) and the specified primer pairs (Integrated DNA Technologies, USA; See Table 2) were used to amplify samples with the StepOnePlus™ Real Time System (Applied Biosystems, Waltham, MA USA). The quantification of mRNA expression was performed with the $2^{-\Delta\Delta\text{Ct}}$ method.

**Western blotting analysis**

Myocardial tissue was collected from non-infarcted regions in CHF rats, and the corresponding left ventricular regions in the Sham group. All the samples were directly subjected to protein extraction by homogenization in RIPA buffer supplemented with
Halt™ Protease Inhibitor Cocktail (100X) (ThermoFisher Scientific, Rockford, IL). The protein concentration of each fraction was determined by BCA Protein Assay (ThermoFisher Scientific). The protein samples were separated by SDS-PAGE gel and then transferred onto a PVDF membrane, which were blocked with 5% non-fat dry milk in PBS and then incubated overnight at 4°C with the primary antibodies: Nrf2 (1:1,000; Abcam, ab137550), GAPDH (1:10,000; MA5-15738, ThermoFisher Scientific), Heme Oxygenase 1 (1:1,000, ab68477), NQO-1 (1:1,000; ab80588), Catalase (1:1,000; AF3398, R&D Systems), SOD2 (FL-222) (1:1,000; sc-30080). After washing with PBST, the blots were then incubated with secondary antibodies (1:5,000, Thermo Scientific, Waltham, MA). The blots were washed with PBST and then subjected to chemiluminescent substrate and the UVP Bioimaging Systems (UVP LLC, Upland, CA) for imaging and quantification.

**Co-immunoprecipitation (Co-IP)**

Non-infarcted regions of left ventricles in CHF rats (14 weeks post-MI) and the corresponding left ventricular regions in the Sham group were collected, and subjected to homogenization with RIPA buffer supplemented with Halt™ Protease Inhibitor Cocktail (ThermoFisher Scientific) and serial centrifugation to clear the protein samples. After the normalization of protein concentration by BCA Protein Assay, the protein samples were incubated with CREB-binding protein antibody (CBP, sc-7300, Santa Cruz) overnight at 4°C. The immune complexes were incubated with protein A/G plus-Agarose (Santa Cruz Biotechnology, sc-2003, Dallas, Texas) at RT for 2 hours, and then subjected to western blotting analysis with the primary antibodies: Nrf2 (Abcam,
ab31163) and NF-κB (ab7970), respectively. An aliquot of the lysates were used as an input and loading control.

**Histological and Immunochemical Analysis**

Hearts were perfused with ice-cold PBS, and frozen on dry-ice. Frozen sections of the left ventricle were prepared (10μm, Leica CM 3050S Research Cryostat) and stored at -20°C until staining. After fixation with 4% PFA at RT for 30 min, the sections were permeabilized with 0.2% TritonX-100 for 20 min, and then blocked with 5% goat serum for 2 hours. After washing with PBS, the sections were incubated with primary antibodies: 4-HNE antibody (ab48506, Cambridge, MA) and 8-OHdG antibody (sc-393871, Santa Cruz, CA), respectively. The sections were then washed with PBS and subjected to fluorescence-conjugated secondary antibodies and nuclear staining with DAPI. Fluorescent images were taken by a Zeiss 710 confocal microscope (Carl Zeiss, Oberkochen, Germany). Quantification was done using Image J (NIH) software.

**Statistics**

All data are reported as the mean ± standard error of the mean (S.E.M). Statistical significance was considered when p values < 0.05 were determined by Student’s t-test for two different groups or one-way analysis of variance (ANOVA) followed by Tukey’s post-hoc test for multiple group comparisons, where appropriate, using GraphPad Prism 8.0 (GraphPad Software, San Diego, CA).
Results

Bardoxolone Methyl (CDDO-Me) promotes Nrf2 nuclear translocation and improves heart hemodynamics in CHF rats. Nrf2 activation was assessed by Nrf2 nuclear translocation after CDDO-Me treatment. Subcellular fractionation was performed with cardiac and skeletal muscle. Western blot results demonstrated that CDDO-Me systemically induced Nrf2 nuclear translocation in the heart and skeletal muscle (Supplemental Figure 1) and also induced the increase of Nrf2 and its target transcripts in a dose-dependent manner (Supplemental Figure 2).

Echocardiographic data are shown in Table 1. After two weeks of treatment, the infarct size in the CDDO-Me-treated CHF group showed a trend to decrease compared to vehicle-treated (from 36.2±8.3 to 29.6±8.1), although this was not significant. The left ventricular systolic volume in the CHF+Veh group was significantly higher than that in Sham+Veh group, however, there were no significant difference of left ventricular volumes between Sham or CHF group after CDDO-Me treatment. CDDO-Me treatment had no significant effects on echocardiographic parameters in the Sham group. On the other hand, CDDO-Me significantly increased stroke volume and cardiac output in the CHF group (Table 1). In addition, hemodynamic analyses done at 14 weeks post-MI demonstrated that there were a significant decreases in dp/dt max/min (Figure 2F and 2I), and a significant increase in LVEDP in the CHF+Veh group (Figure 2H), typical of the heart failure state. Although CDDO-Me had no effects on maximal and minimal dp/dt in Sham and CHF animals (Figure 2F and 2I), the CDDO-Me treated CHF group displayed a significantly lower LVEDP as compared with the Vehicle-treated CHF group (Figure 2H), suggesting some benefit of CDDO-Me to the failing heart. Moreover, CDDO-Me
had no significant effects on mean arterial pressure (MAP) (Figure 2A), heart rate (HR) (Figure 2D), systolic pressure (SBP) (Figure 2B), diastolic blood pressure (DBP) (Figure 2E), pulse pressure (PP) (Figure 2G) or left ventricular peak-systolic pressure (LVPSP) (Figure 2C).

**CDDO-Me-induced the transcriptional and translational activation of Nrf2 in CHF.**

To confirm the activation of Nrf2/ARE signaling by CDDO-Me in CHF rats, we determined myocardial Nrf2 mRNA levels in four different groups. CDDO-Me treatment increased Nrf2 mRNA levels in both Sham and CHF groups (Figure 3A). However, there were no differences between CHF rats treated with vehicle vs CDDO-Me. Although there was also a significant increase in Nrf2 mRNA in the CHF group with vehicle treatment, Nrf2 protein was significantly decreased in vehicle-treated CHF rats which was partially reversed by CDDO-Me treatment (Figures 3 B and C). To further confirm the activation of Nrf2/ARE signaling after CDDO-Me treatment, we evaluated the transcriptional and translational levels of some downstream targets of Nrf2. The results demonstrated that there were significant increases of several antioxidant enzyme mRNA levels in CDDO-Me-treated CHF group compared to Vehicle-treated CHF group, including Heme Oxygenase 1 (HO1), NADPH Oxidase Quinone 1 (NQO1), catalase and glutamate-cysteine ligase (GCLC). The effects of CDDO-Me on the transcription of antioxidant enzymes in the CHF group are markedly greater than that in the Sham group (Figure 4 A to D). However, other potential targets of Nrf2, including SOD1, SOD2, Grx1 and Gpx1 showed no significant differences (Figure 4 E to F). Consistently, western blot analysis of some antioxidant enzymes also demonstrated that CDDO-Me
selectively stimulated an increase of some antioxidant proteins including HO1, NQO1, and Catalase but not SOD2 (Figure 5A and 5B) in animals with CHF.

**CDDO-Me enhances Nrf2 binding to CREB Binding Protein (CBP) and reduces the binding of NF-κB to CBP.** In addition to activating antioxidant enzyme expression by binding to the AREs on antioxidant promoters, Nrf2 has been shown to reduce inflammatory mediators by competing with NF-κB (Li et al., 2008; Liu et al., 2008; Kim et al., 2013). To determine if CDDO-Me reduced the NF-κB binding to the CREB Binding Protein (CBP), we carried out co-immunoprecipitation experiments with the CBP antibody using myocardial tissues, and then blotted with either NF-κB or Nrf2 antibodies, respectively. As shown in Figure 6, the binding of NF-κB to the CBP was significantly increased in CHF rats whereas it was markedly reduced by CDDO-Me treatment. In contrast, CDDO-Me promoted a marked increase of Nrf2 binding to CBP in the CHF group corresponding to the reduction of NF-κB binding to CBP. However, CDDO-Me had no significant effects on NF-κB or Nrf2 binding to the CBP in sham rats (Figure 6A and 6B). Consistent with these findings, the enhanced binding of NF-κB to CBP significantly promoted the production of the pro-inflammatory cytokine TNFα in the myocardium of left ventricle in Vehicle-treated CHF group, which was attenuated by CDDO-Me treatment (Figure 6C). In addition, myocardial oxidative stress, including lipid peroxidation indicated by 4-HNE staining (Figure 7A and 7B) and DNA/RNA oxidation indicated by 8-OHdG staining (Figure 7C and 7D) were significantly increased in the Vehicle-treated CHF group, which was attenuated by CDDO-Me treatment.
Discussion

In the current study, we clearly show that systemic administration of CDDO-Me improved cardiac functions, including stroke volume and cardiac output in CHF rats and resulted in a decrease in LVEDP. The latter finding is of extreme importance since elevated LVEDP leads to pulmonary congestion and ultimately death in the CHF state. Moreover, CDDO-Me not only upregulated the expression of antioxidant enzymes to reduce myocardial oxidative stress in the myocardium of CHF rats by promoting the transcription and translation and subsequent nuclear translocation of Nrf2, but also attenuated cardiac inflammation by preventing the physical binding of NF-kB p65 to CBP. Our findings strongly suggest that low dose CDDO-Me has therapeutic potentials for improving cardiac function in the CHF state.

Cardiovascular (CV) dysfunction in the CHF state is multifactorial, and involves almost every organ system. There is an increased oxidative stress in the progression of chronic heart failure, which is major contributors during the development of CHF (Gutierrez et al., 2006; Santos et al., 2011). The dysregulation of antioxidant defense systems results in oxidative stress (Tsutsui et al., 2006; Canton et al., 2011), thus affecting cardiac structure and function (Siwik et al., 1999; Siwik et al., 2001; Costa et al., 2016). Nrf2 as a highly conserved transcription factor regulates the expression of many target genes involving in cellular defense systems by binding to the AREs in their promoter regions (Zhang et al., 2013). These target genes are involved in the pathogenesis of cardiovascular diseases through oxidative stress-mediated cardiac pathological remodeling (Lu et al., 2008; Ashrafian et al., 2012). Nrf2 activation has resulted in beneficial effects in a variety of cardiovascular diseases (Satta et al., 2017). Although...
significant improvement of renal function was observed in chronic kidney disease in patients with type 2 diabetes, this clinical trial was prematurely terminated due to adverse effects (de Zeeuw et al., 2013; Chertow and de Zeeuw, 2014). In spite of these adverse effects, we hypothesized that low dose CDDO-Me (5 mg/kg/day) would be beneficial in experimental CHF. CDDO-Me evoked Nrf2 nuclear translocation and downstream target activation (Supplemental Figures 1 and 2; Figures 4 and 5). Although the maximal and minimal LV dp/dt and several other hemodynamic parameters did not significantly change in the CHF animals treated with CDDO-Me LVEDP, a critical index of chronic heart failure (Nagendran et al., 2014), was significantly reduced as compared with the Vehicle-treated CHF group (Figure 2H), suggesting a beneficial effects of CDDO-Me on the left ventricle. While the underlying mechanism is not clear, it appeared that CDDO-Me did not directly improve myocardial contractility or the rate of relaxation. It is possible that the reduced LVEDP is due to the CDDO-Me-induced peripheral vasodilation (Wang et al., 2014) and/or a reduction in preload. Importantly, CDDO-Me in rats with CHF also showed a significant increase in stroke volume and cardiac output. Recently, using a derivative of CDDO-Me (DH404), a similar study in a rat model of myocardial ischemia-reperfusion injury where DH404 was administrated at an early time point for 28 days resulted in structural and functional benefits, in part, by decreasing glutathionylation of eNOS and increasing the physical interaction between eNOS and Glutaredoxin-1 (Grx1) (Bubb et al., 2017). Our data are supportive of a recent study where enhanced cardiac-specific Nrf2 expression protected myocardial structure and function from pathological remodeling through an increased expression of
antioxidant enzymes and an improved redox environment (Shanmugam et al., 2019) in an isoproterenol-induced heart failure model.

The current study suggests that systemic administration of CDDO-Me may improve cardiac function through upregulation of antioxidant enzymes by a CDDO-Me-mediated Nrf2 activation, thus contributing to the attenuation of oxidative stress in the left ventricle (Figures 3, 4, 5 and 7). In addition, Nrf2 has also been shown to reduce inflammatory mediators by competing with NF-κB (Li et al., 2008; Liu et al., 2008; Kim et al., 2013). Consistently, we observed enhanced binding of Nrf2 to CBP in the heart in response to systemic administration of CDDO-Me in the CHF group, which prevented binding of NF-κB to CBP and subsequent inhibition of pro-inflammatory cytokine production (Figure 6).

In addition, it has been reported that synthetic triterpenoid CDDO derivatives have multiple molecular targets including STATs, IκB kinase (IKK) β and mTOR (Liby et al., 2006; Yore et al., 2006; Yore et al., 2011). In particular, CDDO imidazolide (CDDO-Im) inhibits the binding of NF-κB to DNA and subsequent transcriptional activation through the direct interaction with IKKβ to prevent NF-κB p65 nuclear translocation (Yore et al., 2006). To determine this possibility, we down-regulated Nrf2 expression in cultured cardiomyocytes using Nrf2 siRNA, and then treated cells with CDDO-Me. The co-immunoprecipitation assay with CBP also demonstrated that CDDO-Me inhibits the binding of NF-κB to CBP in the absence of Nrf2 (Supplemental Figure 3). These previous studies and our current findings suggested that these two potential mechanisms may be involved in the beneficial effects of CDDO-Me on cardiac function in CHF. Although we observed that CDDO-Me increased Nrf2 transcription in Sham rat hearts (Figure 3), Nrf2 transcription was also increased in the CHF group treated with
vehicle alone (Figure 3A). While the finding of increased transcription and reduced protein may seem paradoxical, we attribute this to the translational inhibition of Nrf2 mRNA by microRNAs, which is supported by our recent work (Tian et al., 2018), and also supported by the expression of downstream targets, such as NQO-1 and Catalase, which were lower in the Vehicle-treated CHF group than in vehicle-treated Sham group (Figure 5). In this regard CDDO-Me treatment may provide some compensation for the Nrf2 translational inhibition and thus partly restore Nrf2 protein.

The premature termination of CDDO-Me in Phase 3 clinical trials was due to side effects for patients with type 2 diabetes mellitus and stage 4 chronic kidney disease. These patients were also observed to show characteristics of heart failure and hospitalization history (Chin et al., 2014b). In the present study, our data clearly demonstrated that low dose of CDDO-Me administration still exert therapeutic effects on cardiac function in the CHF setting, consistent with other pre-clinical studies (Chin et al., 2014a).

We must however, acknowledge some limitations of the current study. First, we only evaluated a relatively short-term effect (2-week administration) of CDDO-Me on cardiac function. The long-term, more chronic effects of CDDO-Me therapy remain to be investigated. Second, it is unclear why CDDO-Me selectively upregulated the expression of some Nrf2 target genes including HO-1, NQO-1, Catalase, and GCLC rather than other putative targets, such as SOD1, SOD2, Grx1 and Gpx1. Third, we initiated CDDO-Me treatment at 12 weeks post-MI. It is unclear if the therapeutic effects of CDDO-Me on MI-induced CHF will be determined by different mechanisms when the administration is initiated at different time points.
In conclusion, short-term treatment (two weeks) of CDDO-Me improved cardiac function in CHF rats when administrated at 12 weeks post-MI. The significant improvements of oxidative and inflammatory environments in CDDO-Me-treated CHF animals suggest that CDDO-Me can not only modulate the imbalance of free radical generation and elimination by antioxidant defense systems through the upregulation of downstream targets by the enhanced Nrf2 transcription and translation resulting in the increased binding of Nrf2 to CBP, but also attenuate the production of pro-inflammatory cytokines through the decreased binding of NF-κB to CBP. The two potential mechanisms by which CDDO-Me improves heart function are summarized in Figure 8. This study provides proof of principle that Nrf2 activation may be beneficial in the setting of CHF.
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Authorship Contributions:

Participated in research design: Tian and Zucker.

Conducted experiments: Tian, Gao, Zhang and Hackfort

Contributed new reagents or analysis tools: Tian, Gao and Hackfort

Performed data analysis: Tian, Gao and Hackfort

Wrote or contributed to the writing of the manuscript: Tian and Zucker
References


Liu GH, Qu J and Shen X (2008) NF-kappaB/p65 antagonizes Nrf2-ARE pathway by depriving CBP from Nrf2 and facilitating recruitment of HDAC3 to MafK. *Biochim Biophys Acta* **1783**:713-727.


Footnotes

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Figure legends

Figure 1. Outline of the experimental protocol. Rats were allowed to recover for 12 weeks after thoracic surgery. At this time an echocardiogram was taken and treatment with either vehicle or CDDO-Me was begun for 2 weeks. At 14 weeks post thoracic surgery a second echocardiogram was taken and hemodynamic measurements were determined and tissues taken for biochemical analyses.

Figure 2. Hemodynamic data from CHF rats treated with CDDO-Me. Blood pressure (A); Systolic pressure (SBP) (B); left ventricular peak-systolic pressure (LVPSP) (C); Heart rate (D); Diastolic blood pressure (DBP) and Pulse pressure (PP) (G), no significant difference was observed between different groups; Left-ventricular maximum rate of pressure rise (dp/dt$_{max}$) and decrease (dp/dt$_{min}$) were significantly decreased in CHF rats treated with Vehicle compared to that in Sham rats treated with vehicle (Veh) (F and I); and Left ventricular end-diastolic pressure (LVEDP) was significantly increased in Veh-treated CHF rats, and partially restored after CDDO-Me treatment (H). Sham-Veh (n=7), Sham-CDDO-Me (n=6), CHF-Veh (n=8), CHF-CDDO-Me (n=8) (±SEM).

Figure 3. CDDO-Me elevated both mRNA and protein levels of Nrf2 in the infarcted heart. (A) qRT-PCR data shows CDDO-Me increases the Nrf2 transcription in the heart (n=6, ±SEM). Typical Immunoblots (B) and pooled data (C) showing CDDO-Me-induced
the translational increase of Nrf2 in the non-infarcted LV two weeks post CDDO-Me administration (n=7, ±SEM).

Figure 4. Cardiac tissues from LVs were collected and then subjected to qRT-PCR analysis with specific primers for HO-1 (A); NQO-1 (B); Catalase (C); GCLC (D); SOD1(E); SOD2 (F); Grx1 (G); Gpx1 (H). GAPDH was used as an internal control (n=5-6, ±SEM).

Figure 5. Sham and CHF rats were treated with vehicle or CDDO-Me respectively, for two weeks. The non-infarcted area of the left ventricles were collected and subjected to western blotting analysis for HO-1, NQO-1, Catalase and SOD2. GAPDH was used as a loading control (A); Mean data are shown in B (± SEM) as the ratio of each protein to GAPDH.

Figure 6. Left ventricular tissue was subjected to Co-Immunoprecipitation with CBP antibody, and then western blotting analysis with Nrf2 and NF-kB (p65) antibody, respectively. Immunoblots (A) and pooled data (B) showing the enhanced binding of Nrf2 protein to CBP and reduced binding of NF-kB to CBP in the non-infarcted LV two weeks post CDDO-Me administration (n=4, ± SEM), & denotes p< 0.05 vs CHF+Veh; # denotes p<0.0001 vs Sham+Veh and ## denotes p<0.0001 vs CHF+Veh ; (C) The non-infarcted region of the left ventricle was collected and then subjected to RNA extraction and qRT-PCR analysis with specific primers for TNF-α. qRT-PCR data show CDDO-Me inhibits the transcription of TNF-α in the infarcted heart compared with that in Veh-
treated CHF group (n=6, ± SEM), # denotes p<0.05 vs Sham+Veh and ## denotes p<0.05 vs CHF+Veh.

**Figure 7.** CDDO-Me decreases oxidative stress of heart. Oxidative stress in the left ventricles of hearts were determined in Sham and CHF rats (12 weeks post MI) that were treated with Vehicle or CDDO-Me for an additional two weeks, respectively. Representative confocal microscopic images of LV with 4-HNE staining (A). 4-HNE-positive is shown green. The relative fluorescence intensities were quantified by Image J software (NIH) (B), (Sham+Veh: n=3; Sham+CDDO-Me: n=4; CHF+Veh and CHF+CDDO-Me: n=6). # denotes p<0.0001 vs Sham+Veh and ## denotes p<0.0001 vs CHF+Veh. Representative confocal microscopic images of LV 8-OHdG staining (C). 8-OHdG-positive is shown red. The percentage of 8-OHdG+ cells were quantified (D) (Sham+Veh and Sham+CDDO-Me: n=4; CHF+Veh and CHF+CDDO-Me: n=6). # denotes p<0.0001 vs Sham+Veh, and ## denotes p<0.0001 vs CHF+Veh. Nuclei are shown in blue (DAPI). Magnification, x400. All images were processed with the same confocal settings.

**Figure 8.** A schematic diagram of the potential mechanisms for CDDO-Me-mediated Nrf2 activation in CHF. On the one hand, CDDO-Me promotes the dissociation of Nrf2 from Keap1 (a natural Nrf2 inhibitor) in the cytosol thus translocating to the nucleus where enhanced binding of Nrf2 to CBP promotes pro-antioxidant pathways, and reduced binding of NF-κB to CBP results in the inhibition of pro-oxidant pathway in CHF. At the same time, Nrf2 binds to its own promoter at AREL1 and AREL2 sites to
amplify its anti-oxidative effects by increasing its own transcription. On the other hand, CDDO-Me may inhibit the binding of NF-κB to DNA and subsequent transcriptional activation through the direct interaction with IKKβ to prevent NF-κB p65 nuclear translocation. \textit{AREL1 and AREL2}: Antioxidant Response Element (ARE)-Like Sequence 1 and 2.
Table 1: Echocardiographic analysis of CHF rats treated with CDDO-Me at 14 wk post thoracic surgery

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Sham+Veh (n=7)</th>
<th>Sham+ CDDO-Me (n=7)</th>
<th>CHF+Veh (n=8)</th>
<th>CHF+ CDDO-Me (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume; s (µl)</td>
<td>189.4±83.9</td>
<td>213.6±77.0</td>
<td>537.0±136.9*</td>
<td>570.0±279.5</td>
</tr>
<tr>
<td>Volume; d (µl)</td>
<td>596.3±231.3</td>
<td>546.4±188.3</td>
<td>820.0±226.0</td>
<td>953.1±391.5</td>
</tr>
<tr>
<td>Stroke Volume (µl)</td>
<td>406.9±155.5</td>
<td>332.8±116.1</td>
<td>232.1±52.4**</td>
<td>425.2±249***</td>
</tr>
<tr>
<td>Ejection Fraction (%)</td>
<td>68.5±4.1</td>
<td>60.7±3.8</td>
<td>34.2±8.5ξ</td>
<td>39.2±15.9</td>
</tr>
<tr>
<td>Fractional Shortening (%)</td>
<td>29.7±11.4</td>
<td>31.8±12.6</td>
<td>13.8±7.2Ψ</td>
<td>18.2±7.0</td>
</tr>
<tr>
<td>Cardiac Output (ml/min)</td>
<td>142.7±58.3</td>
<td>152.5±15.7</td>
<td>30.7±85.7ω</td>
<td>174.3±81.6Δ</td>
</tr>
<tr>
<td>Infarct size (%)</td>
<td>—</td>
<td>—</td>
<td>36.2±8.3</td>
<td>29.6±8.1</td>
</tr>
</tbody>
</table>

Note:*P<0.0001 vs Sham+Veh; **P<0.03 vs Sham+Veh; ***P<0.05 vs CHF+Veh; ξP<0.0001 vs Sham+Veh; ΨP<0.01 vs Sham+Veh; ωP<0.02 vs Sham+Veh; ΔP<0.01 vs CHF+CDDO-Me
Table 2: Primer pairs used for qRT-PCR analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
<th>Gene Bank number</th>
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<tbody>
<tr>
<td>Nrf2</td>
<td>Forward: 5′-GCCAGCTGAACCTCTTAGAC-3'</td>
<td>NM_031789.2</td>
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<tr>
<td></td>
<td>Reverse: 5′-GATTCGTGCACAGCACAGCA-3'</td>
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<tr>
<td>HO-1</td>
<td>Forward: 5′-CGACAGCATGTCCAGGATT-3'</td>
<td>NM_012580.2</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5′-TCGCTCTATCTCCTCTTTCCAGG-3'</td>
<td></td>
</tr>
<tr>
<td>NQO-1</td>
<td>Forward: 5′-CATTCTGAAAGGCTGTTTGA-3'</td>
<td>NM_017000.3</td>
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<tr>
<td></td>
<td>Reverse: 5′-CTAGCTTTGACTGTTGTCG-3'</td>
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<tr>
<td>SOD1</td>
<td>Forward: 5′-GCCGTTGAACCAGTTGCTG-3'</td>
<td>NM_017050.1</td>
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<td></td>
<td>Reverse: 5′-GCTGGACCAGCCATGTTTCT-3'</td>
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<tr>
<td>SOD2</td>
<td>Forward: 5′-AGGAGAGTGGTGGAGGCTA-3'</td>
<td>NM_017051.2</td>
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<td>Reverse: 5′-AGCGGAATAAGGCCTTGTGTT-3'</td>
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<tr>
<td>CAT</td>
<td>Forward: 5′-CGACCAGGGGATCCAGATG-3'</td>
<td>NM_012520.2</td>
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<tr>
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<td>Reverse: 5′-CCTGCTCTTCAACAGGCAA-3'</td>
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<tr>
<td>GCLC</td>
<td>Forward: 5′-GTGGACACCCCGATGCAGTAT-3'</td>
<td>NM_012815.2</td>
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<td></td>
<td>Reverse: 5′-TCATCCACCTGGCAACAGTC-3'</td>
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<tr>
<td>Grx1</td>
<td>Forward: 5′-CTGTCAGCATGGCTCAGAGT-3'</td>
<td>NM_022278.1</td>
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<td>Reverse: 5′-CCACAAATTCCAGGAGACCAC-3'</td>
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<td>GPX1</td>
<td>Forward: 5′-CACAGTCCACCCTGTATGCC-3'</td>
<td>NM_030826.4</td>
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<td>Reverse: 5′-AAATGGGGCTGCCCTCTCATCAGTT-3'</td>
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<td>TNF-α</td>
<td>Forward: 5′-AAATGGGGCTCCCTCTCATCAGTTC-3'</td>
<td>X66539.1</td>
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<tr>
<td></td>
<td>Reverse: 5′-TCTGCTTGGTTGGTACGAC-3'</td>
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</tr>
</tbody>
</table>
Figure 2

A: MAP (mmHg)

B: SBP (mmHg)

C: LVSP (mmHg)

D: HR (bpm)

E: DBP (mmHg)

F: dp/dtmax (mmHg)

G: PP (mmHg)

H: LVEDP (mmHg)

I: dp/dtmin (mmHg)

Legend:
- Sham+Veh
- Sham+CDDO-Me
- CHF+Veh
- CHF+CDDO-Me

Significance levels:
- p<0.0001
- p<0.003
- p<0.05
- p=0.0003
Figure 5

A

Sham + Veh  |  Sham  + CDDO-Me  |  CHF  + Veh  |  CHF  + CDDO-Me

HO-1 (33KD)

NQO-1 (34KD)

CAT (60KD)

SOD2 (25KD)

B

HO-1 protein in heart

NQO-1 protein in heart

Catalase protein in heart

SOD2 protein in heart

M (KD)
Figure 6

A. IP with CBP

- Sham+Veh
- Sham+CDDO-Me
- CHF+Veh
- CHF+CDDO-Me

IB: Nrf2 (110 KD)

IB: NF-κB (65 KD)

IB: CBP (265 KD)

B. NF-κB binding with CBP

C. TNF-α mRNA level in heart

Relative expression
Supplemental Data

Therapeutic effects of Nrf2 activation by Bardoxolone methyl in Chronic Heart Failure

Changhui Tian, Lie Gao, Andi Zhang, Bryan T. Hackfort, Irving H. Zucker

Journal of Pharmacology and Experimental Therapeutics
Supplemental figure 1 Sprague-Dawley rats were treated with two different doses of CDDO-Me as indicated for five days. The left ventricle of hearts (A) and gastrocnemius of legs (B) were freshly collected, and then subjected to subcellular fractionation using NE-PER™ Nuclear and Cytoplasmic Extraction Reagents supplemented with Halt™ Protease Inhibitor Cocktail (100X) (ThermoFisher Scientific, Rockford, IL) following the manufacturer’s instructions, and western blotting analysis with a primary Nrf2 antibody. Lamin B (C-20) (1:1,000, sc-6216) and Histone H1 (1:1,000, ab181977) were used as loading controls for the cardiac tissue and skeletal muscle nuclear fraction, respectively. Histone H1 was used for skeletal muscle nuclear fraction, and GAPDH was used for whole tissue lysates.
Supplemental figure 2. Sprague-Dawley rats were treated with two different doses of CDDO-Me as indicated for five days. The left ventricle of hearts and gastrocnemius were freshly collected, and then subjected to RNA extraction using RNeasy Mini Kit (QIAGEN Inc., Valencia, CA) per the manufacturer’s recommendations, and cDNA synthesis using reverse transcription MasterMix (Applied Biological Materials, Richmond, Canada). Nrf2 and NQO-1 mRNA level were determined by qRT-PCR using SYBR® Select Master Mix (Life Technologies, Los Angeles, CA) and the specified primer pairs (Integrated DNA Technologies, USA). GAPDH was used as an internal control (n=4, ±SEM). The quantification of mRNA expression was performed with the $2^{-\Delta\Delta Ct}$ method.
Supplemental figure 3, H9C2 rat cardiomyocytes were transfected with Control siRNA-A (sc-37007) and Nrf2 siRNA (r) (sc-156128), respectively, using Lipofectamine™ RNAiMAX Transfection Reagent per the manufacturer’s recommendations (ThermoFisher Scientific, Cat. 13778030). Cells were subjected to RNA extraction using RNeasy Mini Kit (QIAGEN Inc., Valencia, CA) and then cDNA synthesis using reverse transcription MasterMix (Applied Biological Materials, Richmond, Canada). qRT-PCR was carried out using SYBR® Select Master Mix (Life Technologies, Los Angeles, CA) and the specified primer pairs, including Nrf2 (A), NQO-1 (B) and GAPDH as an internal control; H9C2 cells cultured on 100 mm dish were transfected with Control siRNA and Nrf2 siRNA, respectively. After 24 hours, transfected cells were treated with vehicle
(DMSO) and 5 μM CDDO-Me, respectively, for another 24 hours. Cell lysates were subjected to Co-Immunoprecipitation with CBP antibody (sc-7300), and subsequent western blotting analysis with NF-kB (p65) antibody (ab16502) (C). Nrf2 and CBP levels in 10% input were determined by Nrf2 (ab31163) and CBP antibodies (D), and GAPDH (MA5-15738) was used as a loading control.