Original Article
ALDH2 attenuates Dox-induced cardiotoxicity by inhibiting cardiac apoptosis and oxidative stress

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Abstract: The anthracycline chemotherapy drug doxorubicin (DOX) is cardiotoxic. This study aimed to explore the effect of acetaldehyde dehydrogenase 2 (ALDH2), a detoxifying protein, on DOX-induced cardiotoxicity and unveil the underlying mechanisms. BALB/c mice were randomly divided in four groups: control group (no treatment), DOX group (DOX administration for myocardial damage induction), DOX + Daidzin group (DOX administration + Daidzin, an ALDH2 antagonist) and DOX + Alda-1 group (DOX administration + Alda-1, an ALDH2 agonist). Then, survival, haemodynamic parameters, expression of pro- and anti-apoptosis markers, reactive oxygen species (ROS) and 4-Hydroxynonenal (4-HNE) levels, expression and localization of NADPH oxidase 2 (NOX2) and its cytoplasmic subunit p47^{PHOX}, and ALDH2 expression and activity were assessed. Mortality rates of 0, 35, 5, and 70% were obtained in the control, DOX, DOX + Alda-1, and DOX + Daidzin groups, respectively, at the ninth weekend. Compared with control animals, DOX treatment resulted in significantly reduced left ventricular systolic pressure (LVSP) and ± dp/dt, and overtly increased left ventricular end-diastolic pressure (LVEDP); increased Bax expression and caspase-3/7 activity, and reduced Bcl-2 expression in the myocardium; increased ROS (about 2 fold) and 4-HNE adduct (3 fold) levels in the myocardium; increased NOX2 protein expression and membrane translocation of P47^{PHOX}. These effects were aggravated in the DOX + Daidzin group, DOX + Alda-1 treated animals showed partial or complete alleviation. Finally, Daidzin further reduced the DOX-repressed ALDH2 activity, which was partially rescued by Alda-1. These results indicated that ALDH2 attenuates DOX-induced cardiotoxicity by inhibiting oxidative stress, NOX2 expression and activity, and reducing myocardial apoptosis.

Keywords: DOX, ALDH2, myocardial apoptosis, oxidative stress, NOX2

Introduction

Doxorubicin (DOX) is an anthracycline antibiotic used for tumor treatment. The two major mechanisms underlying DOX-induced tumor death are: (1) DOX inhibits the synthesis, replication and transcription of DNA to impair the cell cycle [1, 2]; (2) DOX promotes the production of ROS (superoxide anion, hydroxyl radical and hydrogen peroxide) in cells, which is dependent upon the mitochondrial respiratory chain and irons [3-7]. Though DOX can delay the development and progression of multiple malignant tumors because of its broad-spectrum anti-tumor effects, its use as medicine would result in serious side effects such as cardiotoxicity because of non-specific anti-tumor effects. DOX-induced

cardiotoxicity is dose-dependent and non-reversible, especially severe in subchronic and delayed cardiotoxicity [8-15]. Currently, the dominant hypothesis for the physiopathological mechanisms underlying DOX-induced cardiotoxicity is that it increases the iron-catalyzed oxidative stress in myocardial cells and impairs DNA transcription/replication [1-5].

Numerous studies have shown that mitochondrial ROS production occurs mainly through the NADPH-dependent respiratory burst. NADPH oxidase (NOX) activation is critical to ROS production in this pathway. At least 7 isoforms of NADPH oxidase (NOX 1-5, Duox 1 and Duox 2) have been described, with myocardial cells mainly expressing NOX2 and NOX4. NOX is a

multicomponent enzyme containing the membrane-bound cytochrome b558, a heterodimer composed of two subunits (p22^{PHOX} and gp91^{PHOX}), and other cytosolic cofactors, including p67 ^{PHOX}, p47^{PHOX}, p40^{PHOX}, and the Rac2 GTPase. The first step of NOX activation depends on the cytoplasmic regulatory subunit and especially the phosphorylation activation of p47^{PHOX}. Then, p47^{PHOX} is translocated into the cell membrane and interacts with the membrane binding component to further recruit cytoplasmic components such as p67^{PHOX}, p40^{PHOX} and the small G protein Rac2, finally assembling the functional NOX oxidase complex to mediate ROS production [4, 16-21].

ROS induce lipid peroxidation, which results in the production of toxic aldehyde metabolites in cells such as acetaldehyde, malondialdehyde (MDA), and 4-HNE [4, 19-23]. Overproduced, these aldehydes can bind to bio-macromolecules such as proteins in cells to form aldehyde-protein adducts (e.g. 4-HNE-protein adducts), leading to the loss of normal biological functions of the modified proteins and cellular damage. Normally, aldehyde dehydrogenase (ALDH) in cells dehydrogenizes toxic aldehydes into low toxicity or non-toxic organic acids with high water solubility, reducing the accumulation of toxic aldehydes and subsequent cytotoxicity [24, 25].

ALDH are a group of NAD(P) +-dependent enzymes. Currently 19 types of ALDH have been identified, with ALDH2 showing the highest activity among the isoenzymes and playing a major role in the clearance of aldehydes *in vivo*. ALDH2 is a nuclear gene encoding a homotetrameric enzyme; its precursor Pre-ALDH2 is translocated from the cytoplasm into the mitochondria, where it localizes in the matrix. High ALDH2 expression levels have been described in the heart, liver, lung and brain [24, 26-28]. However, it is still unclear how the changes of ALDH2 expression and activity impact the DOX-induced myocardial damage.

Therefore, we established a BALB/c mouse model of DOX-induced myocardial damage and treated the animals with ALDH2 antagonist (Daidzin) or agonist (Alda-1) intravenously to explore the impact of ALDH2 expression and activity changes on DOX-induced myocardial damage.

While Daidzin aggravated the DOX-induced myocardial damage, treatment of model mice with Alda-1 resulted in partial or complete alleviation of the various myocardial damage parameters. In addition, Daidzin further reduced the DOX-repressed ALDH2 activity, which was partially rescued by Alda-1. Our findings indicate that ALDH2 attenuates DOX-induced cardiotoxicity by inhibiting oxidative stress, NOX2 expression and activity, and reducing myocardial apoptosis.

Materials and methods

Animals

Specific pathogen-free (SPF) male BALB/c mice (7-8 weeks) were purchased from Laboratory Animal Center, Central South University. The mice weighed 22.1 ± 2.5 g and were housed at room temperature (25°C) with 40~60% relative humidity in a clean environment, under 12/12 h light dark cycle, with access to standard diet and water *ad libitum*. Animal experiments were approved by the Ethics Committee of Center for scientific research with animal models, Central South University (China).

Generation of mouse model of DOX-induced myocardial damage

DOX (Sigma, USA) was dissolved in normal saline and administered at 24 mg/kg to mice by intraperitoneal injection at weeks 1, 4 and 7.

Animal grouping

A total of 80 BALB/c mice were randomly divided into 4 groups (n = 20): control group (no treatment), DOX group (DOX administration for myocardial damage induction), DOX + Daidzin group (DOX administration + Daidzin, an ALDH2 antagonist) and DOX + Alda-1 group (DOX administration + Alda-1, an ALDH2 agonist). Daidzin or Alda-1 was dissolved in normal saline and 100 µl at 200 nmol/ml•kg administered twice (the first days of 2nd and 3rd weeks, respectively) by caudal vein injection. Control and DOX-treatment group animals were given 100 µl normal saline, also by tail vein injection.

Determination of cardiac hemodynamic parameters

Cardiac hemodynamic parameters were assessed as described previously [29]. Mice
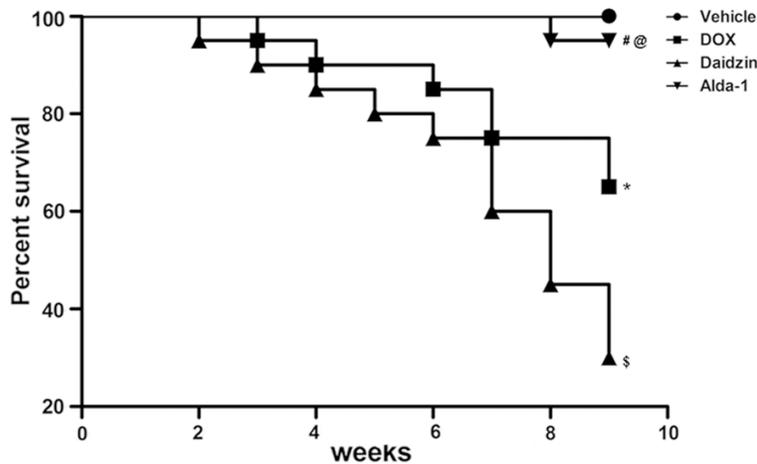
Figure 1. Impact of ALDH2 on the survival rate of DOX-treated mice. *P = 0.0157, compared with the control group; $P = 0.0347, #P = 0.0193, compared with the DOX group; @P < 0.0001, compared with the DOX + Daidzin group; P = 0.3173, comparison between the Dox + Alda-1 and control groups, no significance (n = 20).

were anesthetized by 10% chloral hydrate (2.5 ml/kg) by intraperitoneal injection at the 7th weekend and fixed in supine position on the operating table. Tracheal intubation was performed and linked to a rodent ventilator, with the respiratory rate maintained at 90~100/min (tidal volume, 7~8 ml/kg). Then, mouse chest skin was sterilized with alcohol and the chest opened near the left side of the sternum using an endotherm knife. A No. 6 blunt syringe needle pre-filled with normal saline containing 500 U/ml heparin was injected into the left ventricle from the apex of the heart following the vertical axis and linked to PowerLab System (AD instruments, Australia) after stabilization. A software for hemodynamics analysis was used to monitor the following parameters: left ventricular end-diastolic pressure (LVEDP), left ventricular systolic pressure (LVSP), and ± dp/dt max.

Assessment of ROS and 4-HNE adduct levels, and caspase-3/7 activity in mice myocardium

Fluorometric methods were employed to assess the levels of ROS and 4-HNE-protein adducts in the myocardium as well as Caspase-3/7 activity, according to the manufacturer's instructions.

For ROS levels, a part of left ventricular tissue was homogenized by glass homogenizer to produce myocyte suspensions. Cells were counted and adjusted to 1×10^7/mL. Then, 0.5 mL cells

were added into 1.5 ml EP tubes and mixed with the DHR123 dye (Sigma, USA) at a final concentration of 1 μmol/L. The mixture was incubated at 37°C with shaking water bath for 1 hour. Cells were collected by centrifugation at 1000 r/min for 10 minutes and washed 3 times with PBS. Cell lysis was performed with 0.5% SDS. Fluorescence was measured with excitation at 550 nm and emission at 590 nm on a Spectrophotometer.

Assessment of 4-HNE-protein adducts was performed as described previously [30] with ELISA kit for 4-HNE-protein adducts (Kemin biotech, Shanghai, China). Appropriate myocardial proteins were used to determine the level of 4-HNE-protein adducts on a multimode reader (Bio-Tek, USA) (excitation wavelength 355 nm/emission wavelength 460 nm, which is due to bonds between 4-HNE and protein amino acid residues). Final data are shown as arbitrary units (AU).

Caspase-3/7 activity was determined as described previously [31] using the Apo-One Caspase-3/7 kit (Promega, USA). Equivalent protein amounts were taken from myocardial protein lysates, diluted into 50 μl, and mixed with equivalent buffer containing Z-DEVD-R110 (specific substrate of caspase-3/7). The mixture was incubated at 37°C for 1 hour. 100 μl PBS buffer was used as blank control and the experiment was run in 3-5 replicates. Finally, fluorescence intensity was determined for each sample on a multimode reader (Bio-Tek, USA) (excitation wavelength 499 nm/emission wavelength 521 nm) (RFU: relative fluorescence units).

Extraction of tissue proteins and western blot

For total tissue proteins, appropriate myocardial tissues were sheared into 1 mm³ using sterile eye scissors and washed 3 times with PBS at 4°C. Tissues were then transferred into an homogenizer containing the cell lysis buffer [RIPA: 1% Triton X-100, 150 mmol/L NaCl, 5
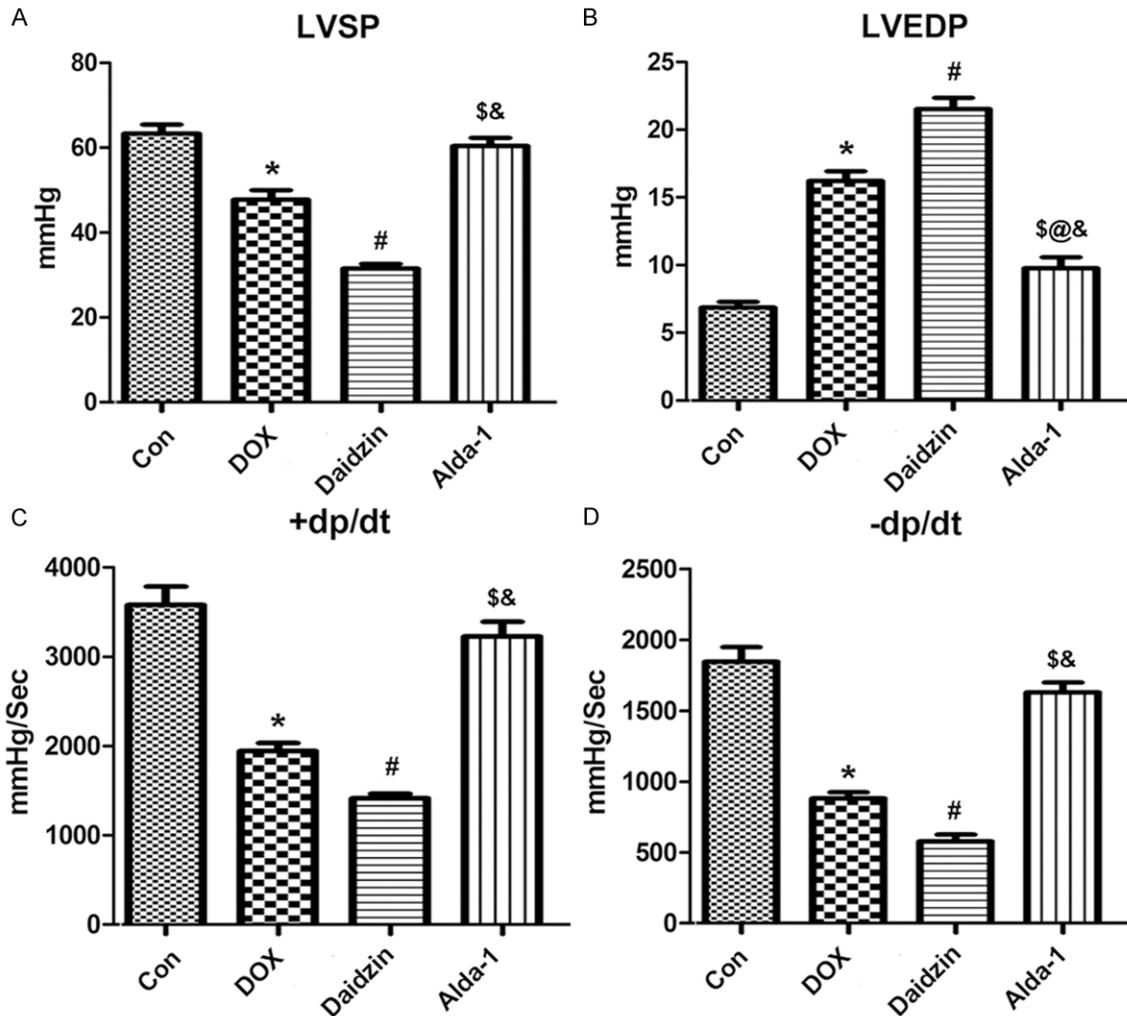
Figure 2. Effect of ALDH2 on heart parameters in DOX-treated mice. Data are mean ± SD. *$P < 0.01$, @$P < 0.05$, compared with the control group; $$P < 0.05$, #$P < 0.05$, compared with the DOX group; &$P < 0.01$, compared with the DOX + Daidzin group. n = 7.

mmol/L EDTA (ethylenediaminetetraacetic acid), 10 mmol/L Tris-HCl (pH 7.0)] with 1% proteinase inhibitor cocktail, and homogenized on ice. Lysates were then carefully transferred into 1.5 ml Eppendorf tubes and ultrasonicated on ice for 3 × 10 s at 15 s interval. Then, lysates were centrifuged at 12,000 g for 20 minutes at 4°C, and supernatants were transferred into sterile 1.5 ml tubes. Protein concentration was determined by the BCA method. Protein samples were aliquoted and stored at -80°C for subsequent experiments. Membrane-bound proteins were isolated using the membrane protein isolation kit (Pierce, USA).

Western blot was performed as follows: 30-40 µg protein were mixed thoroughly with equivalent 2 × SDS loading buffer (100 mmol/L Tris-

HCl, pH 6.8, 20% glycerin, 40 g/L SDS, 1 g/L bromophenol blue, 200 mmol/L DTT) and boiled at 98°C-100°C for 10 minutes. Samples were centrifuged at room temperature at 10000 rpm for 30 seconds. Proteins were separated by 10-15% SDS-PAGE and transferred onto a PVDF membrane (Millipore, USA). The membrane was blocked at room temperature for 2 hours in TBST containing 2% BSA, and incubated with specific primary antibodies raised against actin, ALDH2, Bax, Bcl-2, P47PHOX, NOX2, and TLR4 diluted at 1:500~ 1:2,000 (sigma, USA) at room temperature for 1-2 hours or at 4°C overnight. After 3 washes of 10 min with TBST, HRP-labeled secondary antibody (1:2000, Boster biotech, Wuhan, China) was added at room temperature for 30 minutes

Figure 3. Impact of ALDH2 on caspase-3/7 activity in the myocardium from DOX-treated mice. Data are mean ± SD. *$P < 0.01$, @$P < 0.05$, compared with the control group; $P < 0.01$, #$P < 0.01$, compared with the DOX group; &$P < 0.01$, compared with the DOX + Daidzin group. n = 5.

Figure 4. Effect of ALDH2 on Bax and Bcl-2 protein expression levels in the myocardium of DOX-treated mice.

to 1 hour. The DAB kit (Boster biotech, Wuhan, China) was used for immunodetection.

Tissue ALDH2 activity quantitation

The enzymatic activity of ALDH2 was determined as described previously [32], by the changes of absorbance at 340 nm using ultraviolet spectrometry during the transformation of NAD + into NADH +. Tissue proteins were isolated as described above. Then, 0.1 ml total protein (50 µg) was added onto reaction buffer [phosphate buffer (pH 8.5) at 20 mmol/L, 5 mmol/L substrate acetaldehyde and 1.25 mmol/L NAD +]. Finally, absorbance at 340 nm was read on a microplate reader (Bio-Tek, USA). Activity unit of dehydrogenase was defined as the amount of enzyme producing 1 µmol of NADH per minute.

Statistical analysis

Statistical analysis was performed using the SPSS 15.0 software (SPSS, USA) and measurement data are presented as mean ± standard deviation (mean ± SD). Pairwise comparison was performed with SNK method, while comparison among groups was carried out by One-Way ANOVA. Survival rate was analyzed using the log-rank test. $P < 0.05$ was considered statistically significant.

Results

ALDH2 reduces the high mortality rate in DOX-treated mice

Mortality rates in the four groups were examined carefully at the ninth weekend, and were 0 and 35% in the control and DOX groups, indicating a significant increase after DOX treatment ($P = 0.0157$). The mortality rate was 5% in the Dox + Alda-1 group, significantly lower compared with the DOX group ($P = 0.0193$) but similar to control animals ($P = 0.3173$). However, a mortality rate of 70% was obtained for the DOX + Daidzin group, a value significantly higher compared with the DOX group ($P = 0.0347$) (**Figure 1**).

ALDH2 improves the DOX-induced cardiac function impairment

In the control group, LVSP and LVEDP were 63.39 ± 5.58 mmHg and 6.87 ± 1.07 mmHg, respectively; meanwhile, +dp/dt and -dp/dt values of 3580.43 ± 546.34 mmHg/s and 1844.43 ± 279.23 mmHg/s, respectively, were obtained. DOX-treated animals showed significantly reduced LVSP and ± dp/dt, while LVEDP was overtly enhanced compared with the control group ($P < 0.01$). Interestingly, LVSP (31.6 ± 2.9), +dp/dt (1416.4 ± 126.4) and -dp/dt (578.3 ± 125.6) in the DOX + Daidzin group were significantly lower (LVSP: 47.8 ± 5.8; +dp/dt: 1944.7 ± 238.0; -dp/dt: 880.1 ± 116.0) compared with the values obtained for the DOX group. LVEDP (21.5 ± 2.2) was significantly higher in the DOX + Daidzin group compared with the DOX group (LVEDP: 16.2 ± 1.9) ($P < 0.05$) (**Figure 2**).

ALDH2 attenuates DOX induced myocardial apoptosis

Myocardial apoptosis and necrosis are the major causes of myocardial damage. So the activity of caspase-3/7 and the expression of apoptosis-associated proteins such as Bcl-2 and BAX were examined. In the control group caspase-3/7 activity was about 88.2 ± 8.6, while DOX treatment significantly increased this value to 196.2 ± 14.7 ($P < 0.01$). In the DOX + Daidzin group, caspase-3/7 activity was

Figure 5. Impact of ALDH2 on the production of ROS (A) and 4-HNE adducts (B) in the myocardium from DOX-treated mice. Data are mean ± SD. *$P < 0.05$, @$P < 0.05$, compared with the control group; $$P < 0.05$, #$P < 0.05$, compared with the DOX group; &$P < 0.01$, compared with the DOX + Daidzin group (n = 5).
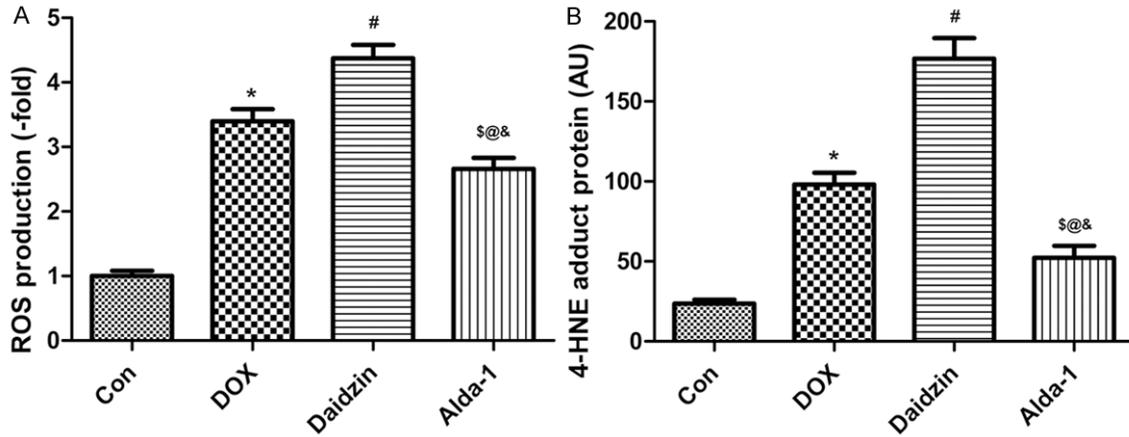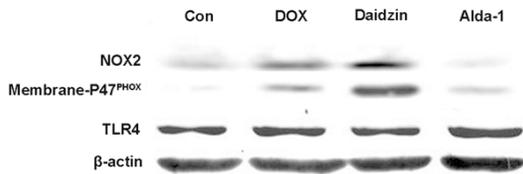
Figure 6. Effect of ALDH2 on protein expression of NOX2 and membrane-located p47PHOX in the myocardium from DOX-treated mice.

further increased to 248.8 ± 27.9, which was significantly higher than the value in the DOX group ($P < 0.01$). However, caspase-3/7 activity was about 142.5 ± 20.8 in the DOX + Alda-1 group, which was significantly lower compared with values obtained for the DOX + Daidzin and DOX groups ($P < 0.01$), but still significantly higher than control values ($P < 0.05$) (**Figure 3**). Western blot data demonstrated that DOX treatment inhibited the expression of the anti-apoptotic protein Bcl-2 but increased that of the pro-apoptotic protein BAX in the myocardium. In the DOX + Daidzin group, Bax expression was higher while Bcl-2 levels were lower than those of the DOX group; in the Alda-1 + DOX group, Bax expression was higher and that of Bcl-2 lower compared with DOX and DOX + Daidzin group values, in mouse myocardium (**Figure 4**).

ALDH2 alters DOX-induced ROS and 4-HNE-protein adducts levels

Upregulation of ROS production might be an important contributor to DOX induced myocar-

dial apoptosis and damage. Therefore, we examined ROS production in the myocardium using ROS detection kits and analyzed the status of ROS-mediated lipid peroxidation using the 4-HNE adduct assessment method. We found that DOX increased ROS production by about 2 folds in the myocardium compared to control values (**Figure 5A**). What's more, ROS production in the DOX + Daidzin group was increased by 3.4 fold in mouse myocardium. These findings demonstrated that ROS production in DOX + Daidzin treated animals was significantly higher than that in the DOX group ($P < 0.05$). In the Alda-1 + DOX group, ROS production in mouse myocardium was increased by only 1.2 fold compared to the control group ($P < 0.05$), which was significantly lower than values obtained for the DOX + Daidzin and DOX groups ($P < 0.05$) (**Figure 5A**). Furthermore, 4-HNE-protein adduct levels were increased in the DOX group by about 4 folds compared to control values. Consistent with ROS results, DOX + Daidzin significantly increased the levels of 4-HNE-protein adducts induced by DOX ($P < 0.05$), while DOX + Alda-1 treatment inhibited the induction of 4-HNE-protein adducts by DOX ($P < 0.01$) (**Figure 5B**).

ALDH2 inhibits the DOX-mediated upregulation of NOX2 expression and membrane translocation of p47PHOX

The expression and activation of NOX2 in myocardial membranes is an important molecular mechanism for ROS production, and NOX2 activation depends on the phosphorylation and
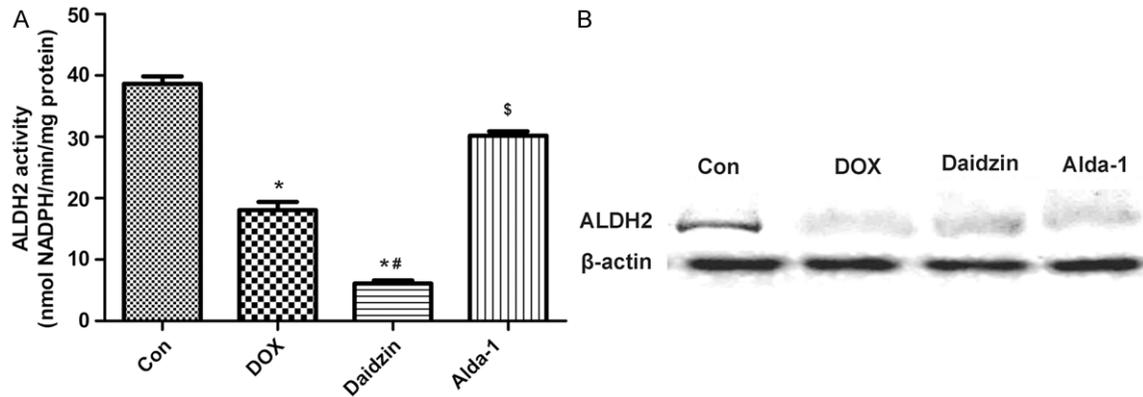
Figure 7. Impact of Daidzin and Alda-1 on ALDH2 activity (A) and expression (B) in the myocardium of DOX-treated mice. *P < 0.01, compared with the control group; #P < 0.05, P < 0.05, compared with the DOX group (A: n = 5; B, n = 3).

translocation into the cell membrane of P47[PHOX], its cytoplasmic subunit [5, 7, 33]. Here, the expression of NOX2 and P47[PHOX] in the myocardium and expression of membrane-bound P47[PHOX] were examined by western blot. Results showed that DOX treatment increased the total expression of NOX2 and P47[PHOX] in the myocardium and enhanced the level of membrane-bound P47[PHOX]. Furthermore, treatment with DOX + Daidzin increased the expression levels of NOX2 and the membrane-bound p47[PHOX]; meanwhile, DOX + Alda-1 treatment resulted in overtly reduced expression of NOX2 and the membrane-bound p47[PHOX] (**Figure 6**).

DOX inhibits ALDH2 expression and activity

4-HNE is a substrate to ALDH2, and can be transformed into nonenoic acid to attenuate its cytotoxicity to myocardial cells [30]. In turn, ROS and 4-HNE can cause ALDH2 denaturation to reduce its activity. It was shown by western blot that DOX treatment reduced ALDH2 protein expression in the myocardium (**Figure 7A**) and significantly inhibited its activity (**Figure 7B**). While further reduction was observed in the DOX + Daidzin group for ALDH2 activity, Alda-1 treatment overtly rescued the impairment of ALDH2 activity induced by DOX2. However, the antagonist Daidzin and agonist Alda-1 did not affect ALDH2 protein expression.

Discussion

As shown above, ALDH2 reduced the increased mortality rate, improved the impairment of the cardiac function, attenuated myocardial apoptosis, inhibited the upregulation of ROS and 4-HNE adducts, and blocked the increased NOX2 expression and p47[PHOX] membrane translocation, which were all induced by DOX in mice. In addition, DOX inhibited the activity and expression of ALDH2.

DOX-induced cardiotoxicity has a cumulative effect, and underlying mechanisms are closely associated with DOX-mediated lethal oxidative stress, necrosis, and apoptosis in myocardial cells. Oxidative stress is directly associated with ROS overproduction. During evolution intrinsic antioxidant system emerged in cells to reduce the level of ROS and oxidative stress and improve cell survival ability. Important antioxidant enzymes include glutathione peroxidase, superoxide dismutase (SOD) and catalase. In addition, aldehyde dehydrogenases such as ALDH2 exist in eukaryotic mitochondria, and could dehydrogenize toxic aldehyde materials into carboxylic acids to reduce toxicity [18, 19, 21, 22, 26].

Previous studies have shown that ALDH2 activity could be modulated by Daidzin and Alda-1, with the former being an antagonist and the latter an agonist. Various studies in animals, in isolated hearts and in cells have shown that modulation of ALDH2 activity by Daidzin or Alda-1 could regulate myocardial apoptosis induced by ischemia-reperfusion injury or hypoxia/reoxygenation [33, 34]. So here we used Daidzin and Alda-1 to modulate ALDH2 activity and examine the impact of ALDH2 in DOX-induced myocardial damage.

A large number of studies have demonstrated that ALDH2 plays important roles in myocardial endogenous protection [35-37]. It was reported previously that point mutations in a specific population leads to reduced ALDH2 activity and expression, which is an important cause of cardiomyopathy [28]. Recently, multiple studies have shown that upregulation of ALDH2 expression in myocardial cells via gene transfection to improve ALDH2 activity could attenuate apoptosis and cardiac hypertrophy, and diminish myocardial infarct size induced by alcohol, aldehyde, ischemia, hypoxia and ischemia-reperfusion injury to improve the heart function [38]. These studies are consistent with our finding that ALDH2 improves DOX-induced cardiac function impairment and inhibits DOX-induced myocardia apoptosis.

Currently it is considered that ALDH2-mediated myocardial endogenous protection might be associated with the following mechanisms: firstly, ALDH2 promotes the dehydrogenation of acetaldehyde and 4-HNE to reduce their toxicity as well as the production of aldehyde-protein adducts in myocardial, cells to alleviate myocardial damage [33]; secondly, ALDH2 modulates multiple signaling pathways such as MAPK and PI3K/AKT pathways to improve the anti-injury ability of myocardial cells [38]; thirdly, ALDH2 could also regulate some endogenous protective genes or transcriptional factors such as hypoxia inducible factor 1 (HIF-1), heat shock factor 1 (HSF1) and heat shock protein (HSP) to exert anti-injury effects [39]; finally, ALDH2 could inhibit/promote the expression of specific genes such as the pro-apoptotic genes p53 and Bax or the activity of Sirt1 [29, 39]. The first mechanism is in accordance with our results showing that ALDH2 inhibits the increased ROS production and 4-HNE-protein adduct formation induced by DOX.

Increased ROS levels induce oxidative stress, lipid peroxidation and accumulation of aldehyde materials, all of which contribute significantly to myocardial apoptosis [18, 20, 40]. NOX2 expression and activation in myocardial membranes is an important molecular event in ROS production, and NOX2 activation depends on the phosphorylation of P47[PHOX] and its translocation to cellular membrane [5, 7, 33]. As shown above, DOX significantly increased membrane-bound NOX2 and P47[PHOX] expression levels, indicating that P47[PHOX] translocation into the membrane and NOX2 activation might be the mechanisms underlying DOX-induced upregulation of oxidative stress and enhanced lipid peroxidation in the myocardium, while ALDH2 could inhibit DOX-induced upregulation of NOX2 expression and p47[PHOX] membrane translocation.

Though numerous studies at the cellular level have reported the inhibition by DOX of ALDH2 activity [41], it is not known whether DOX influences the expression of the ALDH2 protein. Here we employed western blot and enzymatic activity analysis to explore the impact of DOX on ALDH2 activity and expression at the protein level *in vivo*. Results showed that during DOX-induced myocardial damage and cardiac functional impairment, DOX inhibits ALDH2 activity and its expression at the protein level. It is speculated that DOX-mediated ALDH2 inhibition might be associated with DOX-induced ROS production and accumulation of toxic aldehyde materials in myocardial cells. But it remains elusive how DOX impairs ALDH2 protein expression. Protein expression level might be attributed to gene transcription and mRNA stability, so it is necessary to investigate the mechanisms underlying DOX-mediated ALDH2 expression regulation from several angles such as post-transcriptional regulation mediated by miRNAs, long non-coding RNAs and RNA binding proteins or transcriptional regulation mediated by transcriptional factors [42-45].

Taken together, maintenance of ALDH2 activity contributes to the alleviation of DOX-induced myocardial damage. On the one hand, ALDH2 dehydrogenizes aldehyde metabolites to reduce the formation of aldehyde-protein adducts and attenuate damage to myocardial proteins, therefore protecting myocardial cell functions; on the other hand, ALDH2 inhibits P47[PHOX] membrane translocation, blocks NOX2 activation, reduces NOX2-mediated ROS production and decreases cellular oxidative stress and lipid peroxidation to inhibit apoptosis and protect the myocardium. Furthermore, ALDH2 regulates the expression of the anti-apoptotic protein Bcl2 and inhibits the pro-apoptotic protein BAX to block the apoptosis-associated mitochondrial pathway and inhibit myocardial apoptosis.

Acknowledgements

Disclosure of conflict of interest

None.

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