Original Contribution

Redox proteomic identification of oxidized cardiac proteins in Adriamycin-treated mice

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Abstract

Adriamycin (ADR) is a potent anticancer drug, but its use is limited by a dose-dependent cardiotoxicity. Oxidative stress is regarded as the mediating mechanism of ADR cardiotoxicity. However, cardiac proteins that are oxidatively modified have not been well characterized. We took a redox proteomics approach to identify increasingly oxidized murine cardiac proteins after a single injection of ADR (ip, 20 mg/kg body wt). The specific carbonyl levels of three proteins were significantly increased, and these proteins were identified as triose phosphate isomerase (TPI), β-enolase, and electron transfer flavoprotein–ubiquinone oxidoreductase (ETF-QO). TPI and enolase are key enzymes in the glycolytic pathway, and ETF-QO serves as the transporter for electrons derived from a variety of oxidative processes to the mitochondria respiratory chain. Cardiac enolase activity in ADR-treated mice was reduced by 25%, whereas the cardiac TPI activity remained unchanged. Oxidation of purified enolase or TPI via Fenton chemistry led to a 17 or 23% loss of activity, respectively, confirming that a loss of activity was the consequence of oxidation. The observation that these cardiac enzymes involved in energy production are more oxidized resulting from ADR treatment indicates that the bioenergetic pathway is an important target in ADR-initiated oxidative stress.

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Keywords: Adriamycin; β-Enolase; Triose phosphate isomerase; Electron transfer flavoprotein–ubiquinone oxidoreductase; Oxidative stress; Redox proteomics; Free radicals

Adriamycin (ADR) (doxorubicin) is a potent anticancer drug that has been widely applied in treating cancers, including pediatric and adult patients. Severe cardiomyopathy and heart failure have been observed in ADR-treated cancer patients [1]. ADR-associated cardiotoxicity greatly limits the clinical dosage of ADR for cancer patients [1,2]. Many mechanisms that may contribute to ADR cardiotoxicity have been proposed, including tissue-specific mitochondrial DNA damage [3] and disturbance of calcium or iron homeostasis [4–7]. Oxidative stress is generally regarded as the mediating factor in ADR cardiotoxicity. The structure of ADR contains a quinone functionality that can be transformed to a semiquinone via one-electron reduction catalyzed by several endogenous reductases [8] and the endothelial isoform of nitric oxide synthase [9,10]. Oxygen can oxidize the semiquinone back to the quinone form with the production of one superoxide radical (O2·−). The cycling of ADR between the quinone and the semiquinone form can generate a large amount of O2·−, which further gives rise to a variety of other more active reactive oxygen species (ROS), including H2O2 and ·OH [11,12]. ROS can oxidize biomolecules like lipids, proteins, and DNA, triggering further cellular injury processes [3,13,14].
Current research has focused on the elucidation of proteins that are involved in the process of ADR-induced cardiac injury [9,15]. Two-dimensional electrophoresis (2-DE) is a powerful technique to detect quantitative or qualitative proteome changes in various physical or pathological processes [16]. Redox proteomics, combining the power of 2-DE and redox immunochemistry, is a well-established methodology to identify specifically oxidized proteins [17]. It has been successfully applied in identifying the protein targets of oxidative modification in neuropathological disorders, leading to a new understanding of what and how cellular processes are redox-modulated in these disease states [18,19]. In this study, we used the redox proteomics method and identified that cardiac triose phosphate isomerase (TPI or TIM), β-enolase, and electron transfer flavoprotein–ubiquinone oxidoreductase (ETF-QO) manifested increased oxidation after an acute ADR treatment (ip, 20 mg/kg body wt; saline used as the control) in C57BL/6 mice. We also examined how oxidation affected the activities of these enzymes. Our results reveal the targets of oxidative modification in ADR treatment and bring more insight into what cellular processes are affected in ADR-induced oxidative stress and cardiotoxicity.

Materials and methods

Materials

Chemical, biochemical, and immunological reagents were obtained from Sigma (St. Louis, MO, USA) unless otherwise specified. Adriamycin (doxorubicin hydrochloride) was purchased from Pharmacia, Inc. (Kalamazoo, MI, USA). Urea, dithiothreitol (DTT), iodoacetamide, SYPRO Ruby gel stain, immobilized pH gradient (IPG) strips (11 cm, pH 3–10), and Criterion precast gels (8–16%) were purchased from Bio-Rad (Hercules, CA, USA). BCA protein assay reagents and in-gel tryptic digestion kit were purchased from Pierce (Rockford, IL, USA). OxyBlot protein oxidation detection kit was purchased from Chemicon International (Temecula, CA, USA). Proteinase inhibitor cocktail (Set III) was purchased from Calbiochem (Darmstadt, Germany). Rabbit anti-enolase polyclonal IgG and goat anti-TPI polyclonal IgG were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Animals

Male wild-type mice of inbred strain C57BL/6, 8–12 weeks of age, were used in this study. Mice (n = 6) were treated with ADR (2 mg/ml in saline) at a dose of 20 mg/kg body wt or an equal volume of saline by ip injection. All animals were handled according to the policies set by the Division of Laboratory Animal Resources, Medical Center, University of Kentucky, and all procedures were approved by the Institutional Animal Care and Use Committee.

Sample preparation

Seventy-two hours after ADR injection, the mice were euthanized. The hearts were collected, homogenized in potassium phosphate buffer (50 mM, pH 7.8) containing 0.1% of a proteinase inhibitor cocktail, and stored at −80°C until further analysis. Protein concentration was determined by the BCA method [20]. To derivatize protein carbonyl groups [21,22], 150 μg of heart homogenate was incubated with 4 vol of 2,4-dinitrophenylhydrazine (DNPH; 20 mM in 2 M HCl) or 2 M HCl (for 2-D gel protein mapping and mass spectrometry analysis) at room temperature for 30 min and then mixed with ice-cold trichloroacetic acid (final concentration, 15%) and incubated on ice for 10 min. Precipitates were centrifuged at 14,000 g at 4°C for 2 min. The pellets were washed with 500 μl of ethyl acetate/ethanol (1/1, v/v) three times. The final pellet was dissolved in 200 μl of rehydration buffer containing 7 M urea, 2 M thiourea, 2% Chaps, 0.8% (v/v) Amphyolyte, pH 3–10, 1% zwittergent, 45 mM DTT, and a trace amount of bromophenol blue. Samples were then sonicated on ice for 20 s for three times.

Two-dimensional gel electrophoresis

IPG strips were actively rehydrated with 200 μl of samples at 50 V and 20°C for 16 h. Isoelectric focusing (IEF) was performed at 20°C in a Protean IEF cell (Bio-Rad) as follows: 800 V for 2 h linear gradient, 1200 V for 4 h slow gradient, 8000 V for 8 h linear gradient, and 8000 V for 10 h rapid gradient. After IEF, the strips were stored at −80°C until processing. Before the second-dimension separation, the strips were equilibrated in 0.375 M Tris–HCl (pH 8.8) containing 6 M urea, 2% sodium dodecyl sulfate, 20% (v/v) glycerol, and 0.5% DTT for 10 min, followed by a reequilibration in a similar buffer containing 4.5% iodoacetamide in place of DTT for 10 min. Strips were placed on the Criterion precast gels and electrophoresed at 200 V for 65 min. Gels of HCl-treated samples were fixed in a solution containing 10% (v/v) methanol, 7% (v/v) acetic acid for 30 min and stained in SYPRO Ruby gel stain (50 ml/gel) with agitation at room temperature overnight. The gels were rinsed in the fixing solution for 60 min to remove background stain and washed with deionized water. Images of the stained gels were obtained using a fluorescence imager, Storm 860 (λex = 470 nm, λem = 618 nm; Molecular Dynamics, Sunnyvale, CA, USA).

Western blot

After the electrophoresis, proteins of the DNPH-treated samples were electrotransferred from gels to nitrocellulose membranes using a semidyed electrotransfter apparatus (Bio-Rad) at 15 V for 2 h. The membranes were blocked with 3% bovine serum albumin in phosphate-buffered saline containing 0.2% (v/v) Tween 20 (PBST) at 4°C overnight. The membranes were incubated with agitation with rabbit polyclonal anti-dinitrophenyl hydrazine antibody diluted 1:100 in PBST, at room temperature for 2 h, and were washed three times for 5 min in
The membranes were then incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG diluted 1:3000 in PBST, at room temperature for 2 h, washed 3 × 5 min in PBST, and developed using Sigmafast Tablets (BCIP/NBT). Background due to nonspecific binding of the secondary antibody was negligible. Membranes were dried and scanned with a Microtek Scan 4900 (Microtek, Carson, CA, USA).

To immunochemically detect TPI and \( \beta \)-enolase, proteins were separated by 2-DE and electroblotted onto a nitrocellulose membrane. To detect \( \beta \)-enolase, a rabbit anti-enolase polyclonal IgG was used at 1: 500 in PBST, and other treatments were the same. The same membrane was stripped and reprobed with a goat anti-TPI polyclonal IgG at 1:500 in PBST, and the secondary antibody used was an alkaline phosphatase-conjugated rabbit anti-goat IgG at 1:2000 in PBS, and all other treatments were the same.

**Image analysis**

PDQuest software (Bio-Rad) was used to analyze the gels and the membranes and to compare protein and carbonyl contents in cardiac samples of saline- and ADR-treated mice.

**In-gel digestion**

Samples were prepared according to the method described by Thongboonkerd et al. [23]. Briefly, the gel piece containing the protein of interest was cut out from the gel with a clean razor blade and transferred into a 1.5-ml microcentrifuge tube, which could be stored at \(-20^\circ\)C overnight. The gel piece was incubated with 30 \( \mu \)l of 0.1 M \( \text{NH}_4\text{HCO}_3 \) at room temperature for 15 min, then for another 15 min after the addition of 30 \( \mu \)l of acetonitrile, and was air dried for 30 min after the removal of the liquid. The gel piece was rehydrated with 20 \( \mu \)l of 20 mM DTT in 0.1 M \( \text{NH}_4\text{HCO}_3 \) at 56\(^\circ\)C for 45 min. The DTT solution was removed, and the gel piece was incubated with 20 \( \mu \)l of 55 mM iodoacetamide in 0.1 M \( \text{NH}_4\text{HCO}_3 \) in the dark at room temperature for 30 min. The liquid was drawn off, and the gel piece was incubated with 30 \( \mu \)l of 50 mM \( \text{NH}_4\text{HCO}_3 \) at room temperature for 15 min, then for another 15 min after the addition of 30 \( \mu \)l of acetonitrile, and was air dried for 30 min after the removal of the liquid. The gel piece was rehydrated with the addition of a minimal volume of 20 \( \mu \)g/\( \mu \)l modified trypsin in 50 mM \( \text{NH}_4\text{HCO}_3 \) and was incubated with shaking at 37\(^\circ\)C overnight (\(< 18\) h).

**Mass spectrometry and protein identification**

Peptides resulting from in-gel trypsin digestion were spotted on a 384-position, 600-\( \mu \)m AnchorChip Target (Bruker Daltonics, Bremen, Germany) and prepared according to AnchorChip recommendations (AnchorChip Technology, Rev. 2; Bruker Daltonics). Briefly, 1 \( \mu \)l of sample was mixed with 1 \( \mu \)l of \( \alpha \)-cyano-4-hydroxycinnamic acid (0.3 mg/ml in ethanol: acetone, 2:1) directly on the target and dried at room temperature. The sample spot was washed with 1 \( \mu \)l of a 1% trifluoroacetic acid (TFA) solution for approximately 60 s. The TFA droplet was gently blown off the sample spot with compressed air. The resulting diffuse sample spot was recrystallized (refocused) using 1 \( \mu \)l of a solution of ethanol: acetonitrile:0.1% TFA (6:3:1). Spectra were recorded on a Spec 2E matrix-assisted laser desorption ionization–time of flight mass spectrometer in the reflectron mode as summations of 100 laser shots. External calibration of the mass axis was used for acquisition, and internal calibration using either trypsin autolysis ions or matrix clusters was applied postacquisition for accurate mass determination. Peptide mass fingerprinting was used to identify proteins from tryptic peptide fragments by utilizing the MASCOT search engine (http://www.matrixscience.com) based on the entire SwissProt protein database. Database searches were conducted allowing for up to one missed trypsin cleavage and using the assumption that the peptides were monoisotopic, oxidized at methionine residues, and carbamidomethylated at cysteine residues. Mass tolerance of 100 ppm was the window of error allowed for matching the peptide mass values. Probability-based MOWSE scores were estimated by comparison of search results against estimated random match population and were reported as \( -10 \times \log_{10}(P) \), where \( P \) is the probability that the identification of the protein is not correct. MOWSE scores greater than 54 were considered to be significant (\( p < 0.05 \)). All proteins identified were within the expected size and pI range based on the positions in the gel.

**Enzyme activity assay**

Enolase activity assay was performed as described previously [24]. Heart homogenate (10 \( \mu \)g/\( \mu \)l) was mixed with 190 \( \mu \)l of assay solution (20 mM sodium phosphate buffer, pH 7.4, 400 mM KCl, 1 mM MgCl\(_2\), 0.01 mM EDTA, 2 mM 2-phospho-D-glycerate) in a UV-transparent microtiter plate (Corning, MA, USA). Enolase activity was assessed by the change in \( A_{240} \) at 25\(^\circ\)C for 5 min. TPI activity was assayed as described previously [25]. Heart homogenate (10 \( \mu \)l, 0.1 \( \mu \)g/\( \mu \)l) was mixed with 190 \( \mu \)l of assay solution (20 mM triethanolamine-HCl buffer, pH 7.9, 300 \( \mu \)M NADH, 0.4 mM D-glyceraldehyde 3-phosphate, 40 \( \mu \)g/ml glycophosphatase dehydrogenase) in a UV-transparent microtiter plate. TPI activity was assessed by the decrease in \( A_{240} \) at 25\(^\circ\)C for 3 min. Three measurements were taken for each sample.

**In vitro oxidation of proteins**

Enolase (100 \( \mu \)g) or TPI (50 \( \mu \)g) was incubated in 100 \( \mu \)l of 400 mM sodium phosphate buffer containing Fe\(^{2+} \) (30 \( \mu \)M), ascorbic acid (3 mM), and \( \text{H}_2\text{O}_2 \) (1 mM) at 25\(^\circ\)C for 3 h. The reaction mixture was then incubated with 5 \( \mu \)l of catalase (1 mg/ml) and 25 \( \mu \)l of Chelex 100/\( \text{H}_2\text{O}_2 \) slurry (1/1, w/v) at 4\(^\circ\)C for 30 min to remove \( \text{H}_2\text{O}_2 \) and iron ions. Control samples were similarly treated except that Fe\(^{2+} \) and \( \text{H}_2\text{O}_2 \) were omitted. After proper dilutions, 10 \( \mu \)l of enolase (77 ng/\( \mu \)l) or TPI (0.04 ng/\( \mu \)l) was assayed for enzyme activity as mentioned above. Samples were oxidized in triplets and three activity measurements were taken for each sample.
**Statistical analysis**

A two-tailed Student t test was used to assess statistical significance of data. $p < 0.05$ was considered significant for comparison between control and experimental data sets [26].

**Results**

This paper reports the effects of ADR on the oxidation of cardiac proteins, following a well-established redox proteomics methodology [18,19]. Two parallel sets of cardiac protein samples (control or DNPH-treated) were separated by 2-D gel electrophoresis. Proteins (control) in one set of gels were stained for visualization and quantification (Fig. 1), whereas proteins (DNPH-treated) in the other set of gels were blotted onto nitrocellulose membranes and the carbonyl level of each protein spot was detected immunochemically (Fig. 1). The specific carbonyl level for each protein was calculated as the ratio of the carbonyl level on the membrane to the protein level on the gel, depicting the carbonyl level per unit of protein. Three murine cardiac proteins exhibited significantly higher specific carbonyl levels after ADR treatment compared to that after saline treatment (Table 1): β-enolase, TPI, and ETF-QO (Table 2). Identification of these proteins can also be performed via an immunochemical approach, which could be used as a supplement to the MS characterization. After 2-D separation, electroblotting onto a nitrocellulose membrane, and Ponceau S staining, the proteins of interests were pinpointed on the membrane (Fig. 2A), which could be referred back to the protein spots on the 2-D gel through image analysis (Fig. 1). TPI or β-enolase was recognized by a TPI or an enolase antibody, respectively (Figs. 2B and 2C). ETF-QO was omitted from the immunochemical characterization, because its antibody was not commercially available.

Oxidative modification of enzymes often leads to decreased enzymatic activities [24,27,28]. To examine the biological consequences of the elevated specific carbonyl levels, we compared the enzymatic activities of enolase and TPI in the heart homogenates of the saline- and ADR-treated mice ($n = 6$). We did not examine the activity of ETF-QO, because ETF and acyl-CoA dehydrogenase, two indispensable auxiliary enzymes in the ETF-QO assay, were neither commercially available nor particularly easy to purify [29]. Total enolase activity in the heart homogenate of ADR-treated mice demonstrated a 25% loss in comparison to that of saline-treated mice (Fig. 3). However, TPI activity in ADR-treated samples was slightly lower but without statistical significance (Fig. 3). To further examine the effects of oxidation on the enzymatic activities, we carried out in vitro metal-catalyzed oxidation of commercially available purified enolase and TPI [28,30].

<table>
<thead>
<tr>
<th>Identified protein</th>
<th>Saline treated ($n = 6$)</th>
<th>ADR treated ($n = 6$)</th>
<th>$p$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Enolase</td>
<td>1.08 ± 1.07</td>
<td>3.43 ± 1.37</td>
<td>0.017</td>
</tr>
<tr>
<td>TPI</td>
<td>0.21 ± 0.15</td>
<td>2.78 ± 1.04</td>
<td>0.013</td>
</tr>
<tr>
<td>ETF-QO</td>
<td>0.41 ± 0.19</td>
<td>1.27 ± 0.39</td>
<td>0.004</td>
</tr>
</tbody>
</table>

* Specific carbonyl level = (intensity of spot on blot)/(intensity of spot on gel).

\[
\text{Table 1}
\]

**Fig. 1.** (A) A typical 2-D gel and 2-D Western blot of mouse cardiac proteins. (B) A zoom-in view of the identified proteins on both 2-D gels and 2-D Western blots of samples of saline- or ADR-treated mice.
incubated in a solution of Fe^{2+} (30 μM), ascorbic acid (3 mM), and H₂O₂ (1 mM) at 25°C, a system that produces hydroxyl radicals. After an incubation of 3 h, the activity of enolase decreased by 17% and that of TPI decreased by 23%, in comparison to the controls (p < 0.05) (Fig. 4).

**Discussion**

Normal cardiac function demands a high amount of energy supplied as ATP. In human hearts, the amount of high energy phosphate is correlated with the systolic function [31]. ADR treatment reduces cardiac systolic function [32], suggesting a defective bioenergetic process. We found that TPI, β-enolase, and ETF-QO exhibited higher specific carbonyl levels in cardiac samples of ADR-treated mice, which revealed ADR-induced oxidative stress in the heart tissue (Table 1). All three enzymes are involved in energy metabolic pathways. TPI and β-enolase are key enzymes in the glycolytic pathway. ETF-QO is the electron transporter for electrons derived from a variety of oxidative processes to the mitochondria respiration chain [33].

TPI (EC 5.3.1.1) catalyzes the interconversion of glycer-aldehyde 3-phosphate and dihydroxyacetone phosphate at the diffusion rate limit in the glycolytic pathway, active only in the form of a homodimer [34]. Surprisingly, we noticed that, in cardiac samples of ADR-treated mice, which revealed ADR-induced oxidative stress in the heart tissue (Table 1). All three enzymes are involved in energy metabolic pathways. TPI and β-enolase are key enzymes in the glycolytic pathway. ETF-QO is the electron transporter for electrons derived from a variety of oxidative processes to the mitochondria respiration chain [33].

Table 2

<table>
<thead>
<tr>
<th>Identified protein</th>
<th>SwissProt Accession No.</th>
<th>No. peptides matched</th>
<th>% Sequence coverage</th>
<th>pI</th>
<th>MW (kDa)</th>
<th>MOWSE score</th>
<th>Probability of a random hit</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-enolase</td>
<td>P21550</td>
<td>12</td>
<td>33</td>
<td></td>
<td>6.81; 47.2</td>
<td>3.5 × 10⁻¹¹</td>
<td></td>
</tr>
<tr>
<td>TPI</td>
<td>P17751</td>
<td>7</td>
<td>39</td>
<td></td>
<td>7.08; 95</td>
<td>4.1 × 10⁻⁶</td>
<td></td>
</tr>
<tr>
<td>ETF-QO</td>
<td>Q921G7</td>
<td>11</td>
<td>24</td>
<td></td>
<td>7.34; 119</td>
<td>1.8 × 10⁻⁸</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 3. The activities of enolase and TPI in the heart homogenates of saline- or ADR-treated mice (n = 6). Data are expressed as means ± SD. *p < 0.05 (Student’s t test).

Fig. 2. Immunochemical characterization of β-enolase and TPI. (A) Ponceau S staining of proteins on a 2-D blot. (B) Immunochemical detection of enolase with a rabbit polyclonal IgG being raised against human α-enolase that reacts with α-, β-, and γ-enolase of mouse, rat, and human origin. (C) The membrane in (B) was reprobed with a goat polyclonal IgG being raised against human TPI that reacts with TPI of mouse, rat, and human origin. Both antibodies were purchased from Santa Cruz Biotechnology. Arrows point to the protein spots that underwent MS identification (also see Fig. 1).
ADR-treated samples, even though TPI exhibited elevated carbonyl levels (Table 1) its activity remained unaffected (Fig. 3). This result is, however, consistent with what has been found in the brain tissue in Alzheimer disease and in a senescence-accelerated mouse model; the brain tissue is under oxidative stress, but the activity of TPI is not altered [35,36]. TPI has a slow turnover rate and a decreased activity offered a rationale for this observed phenomenon.

ETF-QO (EC 1.5.5.1) is an iron–sulfur ([4Fe–4S]) flavoprotein located in the inner mitochondrial membrane, catalyzing electron transfer from ETF to coenzyme Q. The reducing equivalents derived from nine acyl-CoA dehydrogenases and two N-methyl dehydrogenases, which are involved in fatty acid β-oxidation and amino acid oxidative degradation, use ETF-QO as the entry into the respiratory chain [33,50]. Although direct examination of ETF-QO activity after ADR treatment was hampered by the lack of two auxiliary enzymes, ETF and acyl-CoA dehydrogenase, it has been reported that superoxide radical, which is the primary by-product of ADR recycling, inactivates many [4Fe–4S]-containing enzymes [51]. Furthermore, both superoxide and ADR semiquinone can release iron from the [4Fe–4S] cluster of aconitate [7]. These previous findings suggested a reduced ETF-QO activity in ADR-treated mice, due to the susceptibility of the [4Fe–4S] cluster to ROS. It is possible that iron released from oxidized iron–sulfur clusters could promote metal-catalyzed oxidation of proteins [11,28], leading to the increased carbonyl level in ETF-QO (Table 1).

We have found that three metabolic enzymes are more oxidized in murine hearts after the mice were given a single high dose of ADR (20 mg/kg). Currently, there exist at least two dosage models for the study of ADR cardiotoxicity: the single-high-dose model [52–55] and the low-dose chronic model [56,57]. For the single-high-dose model, the dosage that is usually used, 20 mg/kg, is equivalent to a high-dose single injection in cancer patients [58], such as patients with small-cell lung cancer [59]. After a single dose of ADR is injected into experimental animals, biochemical, functional, and pathological changes are characterized at certain later time points [53–55]. For the low-dose chronic model, a small dose of ADR (e.g., 2 mg/kg) is periodically given to experimental animals over several weeks until the total dosage reaches an equivalent dosage received by patients (e.g., 2520 mg/70-kg man) [56], and biochemical, functional, and pathological changes are studied as the accumulative toxicity is manifested. We have previously observed mitochondrial ultrastructural injury 5 days after a single high dose of ADR (20 mg/kg) [53]. Rosenoff et al. reported subacute cardiomyopathy 4 days after a single dosage of ADR, which is similar to the delayed ADR-induced cardiomyopathy noted in human [52]. These facts indicate the single-high-dose model is biologically relevant and can serve as a valuable tool to study fatal ADR cardiotoxicity.

Fig. 4. In vitro oxidation decreases the activities of purified enolase and TPI. Purified enolase (100 μg) or TPI (50 μg), purchased from Sigma, was oxidized in triplets in 100 μl of a mixture of 400 mM sodium phosphate buffer, Fe²⁺ (30 μM), ascorbic acid (3 mM), and H₂O₂ (1 mM) at 25°C for 3 h. The reaction mixture was then incubated with 5 μl of catalase (1 mg/ml) and 25 μl of Chelex 100/H₂O slurry (1/1, w/v) at 4°C for 30 min to remove H₂O₂ and iron ions. Control samples were similarly treated except that Fe²⁺ and H₂O₂ were omitted. After proper dilutions, 10 μl of enolase (77 ng/μl) or TPI (0.04 ng/μl) was assayed for enzyme activity. Data are expressed as means ± SD. *p < 0.05 (Student’s t test).

Enolase (EC 4.2.1.11) catalyzes the conversion of 2-phosphoglycerate to phosphoenolpyruvate in the glycolytic pathway. In mammals, enolase exits as a dimer consisting of three subunits, α, β, and γ, each coded by a distinct gene. α-Enolase is universally found in all tissues, whereas αβ-/ββ- or αγ-/γγ-enolase are cell-type specific, existing in striated muscle cells or neurons, respectively [42]. Enolase has been identified as a target for oxidation in yeast [43] and Escherichia coli [44]. Under oxidative stress conditions, α-enolase is the target of carbonylation in the brain [36,45], whereas β-enolase is a target of carbonylation in muscle tissue [46], which is consistent with our results (Table 1). In the heart of adult mouse, αα-, αγγ-, and ββ-enolase account for 7, 37, and 56% of the total enolase activity, respectively [47]. This fact explains why increased carbonylation of β-enolase could lead to a substantial decrease in the total enolase activity in the heart of ADR-treated mice (Fig. 3). It has been reported that expression of β-enolase is decreased in heart hypertrophy [48], and animals with heart hypertrophy are more susceptible to ADR [49]. Our results that ADR treatment led to β-enolase oxidation and a decreased activity offered a rationale for this observed phenomenon.
Using the single-high-dose model, our group has reported that ADR treatment significantly decreases the activities of complexes I and II in mouse heart tissue [60], indicating that the energy metabolic pathway is a target in ADR-induced oxidative damage. Our present study provides further evidence by identifying individual metabolic enzymes that are more oxidatively modified in ADR treatment. In addition to the inhibitory effects on enzymatic activities, it has been shown ADR-induced oxidative modification of mitochondria proteins accompanies the ultrastructural alterations of cardiac mitochondria [61], suggesting that oxidative modification of proteins might also play a direct role in the process of structural damage in ADR-induced oxidative stress. Mechanistic insights into ADR cardiotoxicity can reveal targets for potential therapeutic interventions. Previous research has shown that it is possible to attenuate ADR cardiotoxicity by modulation of key proteins related to ADR-induced oxidative stress [6,55,60,62]. For example, overexpression of antioxidant enzymes, such as manganese superoxide dismutase [53] and catalase [62], has been shown to protect heart from ADR cardiotoxicity in transgenic mice. The present study identifies additional potential targets for intervention. It will be interesting to investigate whether modulation of oxidative products of TPI, β-enolase, and ETF-QO will mitigate ADR cardiotoxicity.

Acknowledgment

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