Identification of protein targets underlying dietary nitrate-induced protection against doxorubicin cardiotoxicity

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Abstract

We recently demonstrated protective effect of chronic oral nitrate supplementation against cardiomyopathy caused by doxorubicin (DOX), a highly effective anticancer drug. The present study was designed to identify novel protein targets related to nitrate-induced cardioprotection. Adult male CF-1 mice received cardioprotective regimen of nitrate (1 g NaNO₃ per litre of drinking water) for 7 days before DOX injection (15 mg/kg, i.p.) and continued for 5 days after DOX treatment. Subsequently the heart samples were collected for proteomic analysis with two-dimensional differential in-gel electrophoresis with 3 CyDye labelling. Using 1.5 cut-off ratio, we identified 36 proteins that were up-regulated by DOX in which 32 were completely reversed by nitrate supplementation (89%). Among 19 proteins down-regulated by DOX, 9 were fully normalized by nitrate (47%). The protein spots were further identified with Matrix Assisted Laser Desorption/Ionization-Time-of-Flight (MALDI-TOF)/TOF tandem mass spectrometry. Three mitochondrial antioxidant enzymes were altered by DOX, i.e. up-regulation of manganese superoxide dismutase and peroxiredoxin 3 (Prx3), and down-regulation of Prx5, which were reversed by nitrate. These results were further confirmed by Western blots. Nitrate supplementation also significantly improved animal survival rate from 80% in DOX alone group to 93% in Nitrate + DOX group 5 days after the DOX treatment. In conclusion, the proteomic analysis has identified novel protein targets underlying nitrate-induced cardioprotection. Up-regulation of Prx5 by nitrate may explain the observed enhancement of cardiac antioxidant defence by nitrate supplementation.

Keywords: nitrate • cardioprotection • doxorubicin • cardiotoxicity • proteomics • 2D-DIGE • antioxidant

Introduction

Doxorubicin (DOX) is a highly effective anthracycline anticancer drug that has been used for more than four decades to treat a variety of human neoplasms [1]. The well-recognized cardiotoxic side effect of DOX, which may lead to irreversible dilated cardiomyopathy and heart failure [2, 3] has been a dilemma for both oncologists and cardiologists around the world and seriously limited its use in the treatment of many cancer patients. Despite intensive research in the past decades, an optimal therapeutic intervention for protecting heart against DOX-induced cytotoxicity has not been identified. Dexrazoxane is the only compound currently approved by U.S. Food and Drug Administration in September 2007 for protection against anthracycline-induced tissue injuries such as extravasation. However, due to the cytotoxic side effect and limited efficacy of dexrazoxane, there is an ongoing need for a better drug for protecting heart against DOX-induced cardiotoxicity [4].

The cardioprotective role of nitric oxide has been demonstrated in numerous animal models of myocardial damage such as ischemia-reperfusion injury [5–8]. The nitrate/nitrite/nitric oxide pathway is a crucial complementary or alternative system to the nitric oxide synthase dependent pathway for ensuring sufficient nitric oxide production, especially under pathophysiological conditions such as hypoxia and acidosis when the oxygen-dependent NOS activities are compromised [9, 10]. As a substantial portion of nitrate/nitrite in the body is derived from dietary sources, dietary supplementation of nitrate or nitrite was shown to attenuate myocardial ischemia-reperfusion injury [11, 12] and hypertension [13, 14]. We recently showed the protective effects of chronic oral nitrate intake against DOX-induced cardiac contractile dysfunction, myocardial necrosis/apoptosis and mitochondrial respiratory chain damages [15, 16]. To further explore the molecular mechanisms underlying nitrate-induced protection against DOX cardiotoxicity,
we designed this study to identify novel protein targets that are altered by treatment with DOX and chronic nitrate supplementation using a state-of-the-art proteomic approach with two-dimensional differential in-gel electrophoresis (2D-DIGE) and MALDI TOF/TOF tandem mass spectrometry. Our results show a number of novel protein targets that are potentially responsible for the protection of DOX-induced cardiomyopathy with nitrate supplementation.

Materials and methods

Animals

Adult male CF-1 outbred mice (body weight ~35 g) were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, IN, USA). The animal experimental protocols were approved by the Institutional Animal Care and Use Committee of the Virginia Commonwealth University. All animal experiments were conducted under the guidelines on humane use and care of laboratory animals for biomedical research published by the National Institutes of Health (No. 85–23, Revised 1996).

Protocol for animal experiments

The mice were administered a single dose of DOX intraperitoneally (15 mg/kg, dissolved in saline; purchased from Sigma-Aldrich, St. Louis, MO, USA; DOX group) or volume-matched saline (0.2 ml; Control group). Another group of mice (Nitrate + DOX group) received chronic nitrate supplementation with NaNO3 added into the drinking water at the concentration of 1 g/L (~12 mM) for 7 days before the DOX injection on day 8 and continued through the end of 5-day post-DOX period as described previously [15, 16]. Such nitrate supplementation protocol has been shown to protect against DOX-induced ventricular dysfunction [15] and mitochondrial respiratory chain damage [16]. Under surgical anaesthesia with pentobarbitone sodium (70 mg/kg, i.p.), the heart samples were collected via thoracotomy, rinsed off blood with saline and immediately frozen in liquid nitrogen and stored at –70°C until further proteomic experiments or Western blot analysis.

Two-dimensional differential in-gel electrophoresis and minimal CyDye labelling

The 2D-DIGE and mass spectrometry protein identification were performed by Applied Biomics, Inc. (Hayward, CA, USA) following established protocols. In brief, the heart tissues were washed with a buffer containing: 10 mM Tris-HCl, 5 mM magnesium acetate (pH 8.0) three times to remove the contaminated blood. For 100 mg of tissues, 2 ml of the 2D cell lysis buffer (30 mM Tris-HCl, pH 8.8, containing 7 M urea, 2 M thiourea and 4%-[3-(Cholamidopropyl)-dimethylammonio]-1-propane sulfonate (CHAPS) was added and subjected to sonication at 4°C. After vigorous shaking for 30 min. at room temperature, the tissue lysates were centrifuged at 4°C for 30 min. at 14,000 rpm and the supernatant was collected. Following measurement of protein concentration using Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA), the lysate samples were diluted with 2D cell lysis buffer in order to obtain the equal protein concentration (5 mg/ml). Thirty micrograms of tissue lysate was labelled with 1 μl CyDye dilution (Cy2, Cy3 and Cy5, Amersham, Piscataway, NJ, USA). The CyDyes stock (1 nmol/μl) were diluted 1:5 with dimethylformamide, incubated on ice for 30 min. in dark, followed by addition of 1 μl of 10 mM lysis to stop the labelling reaction. The Cy2, Cy3 and Cy5 labelled samples were mixed with 2× 2D sample buffer [8 M urea, 4% CHAPS, 20 mg/ml dithiothreitol (DTT), 2% pharmalytes and trace amount of bromophenol blue], followed by addition of 100 μl destreak solution (GE Healthcare, Piscataway, NJ, USA) and rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 20 mg/ml DTT, 1% pharmalytes and trace amount of bromophenol blue) to make up the volume to 250 μl for loading into a 13 cm immobilized pH gradient (IPG) strip (pH 3–10, Linear, GE Healthcare/Amersham) and run the isoelectric focusing under dark. Upon finishing the isoelectric focusing, the IPG strips were incubated in 10 ml of equilibration buffer 1 (50 mM Tris-HCl, pH 8.8, containing 6 M urea, 30% glycerol, 2% SDS, trace amount of bromophenol blue and 10 mg/ml DTT) for 15 min., followed by incubation in 10 ml of equilibration buffer 2 (50 mM Tris-HCl, pH 8.8, containing 6 M urea, 30% glycerol, 2% SDS, trace amount of bromophenol blue and 45 mg/ml iodacetamide) for 10 min. The IPG strips were then rinsed in SDS-gel running buffer and transferred into 12% SDS-gel and sealed with 0.5% agarose solution in SDS-gel running buffer and electrophoresis was performed at 15°C.

Image scan and data analysis

Each gel was scanned immediately following SDS-PAGE using Typhoon Trio scanner (Amersham). The scanned images were then analysed by ImageQuantTL software (GE Healthcare), and then subjected to in-gel analysis and cross-gel analysis using DeCyder software version 6.5 (GE Healthcare). The change in ratio of differential protein expression was obtained from in-gel DeCyder software analysis. Quantitative comparisons were then made between two individual samples for each of the three possible combinations. The pairwise volume ratios (i.e. Control versus DOX, Control versus Nitrate + DOX and DOX versus Nitrate + DOX) were calculated for each protein spot and used to determine relative protein expression. To simplify the data presentation, only the ratios of comparisons against Control are presented.

Spot picking and digestion and MALDI-TOF/TOF

The selected spots were picked up by Ettan Spot Picker (GE Healthcare) following the DeCyder software analysis and spot picking design. The selected protein spots were subjected to in-gel trypsin digestion, peptides extraction, desalting and followed by MALDI-TOF/TOF to determine the protein identity. In brief, mass spectra (MS) of the peptides in each sample were obtained using Applied Biosystems (Carlsbad, CA, USA) Proteomics Analyzer. Ten to 20 of the most abundant peptides in each sample were further subjected to fragmentation and tandem mass spectrometry (MS/MS) analysis. Protein identification was based on peptide fingerprint mass mapping (using MS spectra) and peptide fragmentation mapping (using MS/MS spectra). Combined MS and MS/MS spectra were submitted for database search using GPS Explorer software equipped with the Mascot search engine to identify proteins from primary sequence databases.

Western blot analysis

To confirm the results of 2D-DIGE, we performed Western blots to assess expression levels of a few selected proteins. In brief, the mouse heart
samples were ground into fine powders under liquid nitrogen and homogenized with a Glas-Col® tissue homogenizer in 1 ml of ice-cold lysis buffer containing (in mM) 20 Tris•HCl pH 7.4, 150 NaCl, 1 ethylenediaminetetraacetic acid, 1 ethylene glycol tetraacetic acid (EGTA), 0.2 phenylmethylsulfonyl fluoride (PMSF), 0.2 Na3VO4, added with β-mercaptoethanol and a cocktail of protease and phosphatase inhibitors (Pierce, Rockford, IL, USA; product# 78440). After centrifugation at 12,000 × g for 15 min. (4°C), the supernatant was separated from the pellet and stored in −80°C until further analysis. Following measurement of the protein concentration with Bio-Rad protein assay kit, equal amount of protein for each sample were combined with an equal volume of a loading buffer solution made by mixing 25 μl of Laemmli sample buffer (Bio-Rad) and 475 μl β-mercaptoethanol (Bio-Rad). The samples were boiled for 5 min. before being loaded into each well on polyacrylamide gels (Bio-Rad). The gels were electrophoresed for 1 hr at 180 volts in running buffer [1× Tris/glycine/SDS]. The electrophoresed proteins were trans-blotted from the gel to nitrocellulose membranes at 400 mA for 90 min. (4°C). After placing in blocking solution containing 5% non-fat milk in 1× Tris-buffered saline and 0.05% Tween-20 (Bio-Rad) for 1 hr at room temperature, the membranes were incubated with primary antibodies overnight under 4°C at 1:1000 dilution for mitochondrial superoxide dismutase 2 (SOD2; Calbiochem, San Diego, CA, USA), myosin light chain 2 (MYL2), fatty acid binding protein 3 (FABP3), peroxiredoxin 3 (Prx3), Prx5 and brain glycogen phosphorylase (PYGB), which were supplied by Proteintech Group, Inc. (Chicago, IL, USA) and α-Tubulin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The blots were then washed and incubated with anti-rabbit (GE Healthcare) or anti-goat (Santa Cruz) secondary antibody conjugated to horseradish peroxidase at 1:2000 dilution in 5% milk in 1× TBST for 1 hr at room temperature. The membranes were then washed and the bands were visualized via enhanced chemiluminescence (PerkinElmer, Waltham, MA, USA) and subsequently exposed to a Kodak film. The optical density of the protein bands was quantified using Image J software. Expression for all the studied proteins was normalized to α-Tubulin expression.

Statistical analysis

The normalized densitometric results of protein expression by Western blots were analysed using one-way ANOVA followed by Student–Newman–Keuls post hoc test for pairwise comparison. The chi-square test was used to compare the animal survival rates between the four treatment groups. Probability value of P < 0.05 was considered significant.

Results

Detection of DOX-induced alterations in cardiac protein expression with 2D-DIGE

Immediately following the 2D-DIGE and SDS-PAGE procedures, the scanned images from each gel were subjected to in-gel analysis to determine the protein differential expression. Quantitative pairwise comparisons of the protein volume ratios for each protein spot were then made among the three treatment conditions (i.e. Control versus DOX, Control versus Nitrate + DOX, and DOX versus Nitrate + DOX). Figure 1A shows an overlay image for in-gel comparison between heart samples from control (labelled in green dye) and DOX-treated mouse (labelled in red dye). Similarly, Figure 1B depicts an overlay image for in-gel comparison between the heart samples from control (labelled in green dye) and heart sample from mouse that received nitrate supplementation plus DOX injection (Nitrate + DOX; labelled in red dye). The circled and numbered protein spots (82 in total with a cut-off ratio of 1.5 for alteration in protein expression among the three treatment conditions) are subsequently isolated from the gels and identified by MALDI TOF/TOF mass spectroscopy.

Fig. 1 Scanned images of the 2D gels. (A) Overlay image for in-gel comparison between hearts from Control (labelled in green dye) and DOX-treated (labelled in red dye) mice; (B) Overlay image for in-gel comparison between Control heart (labelled in green dye) and heart sample from mouse that received nitrate supplementation plus DOX injection (Nitrate + DOX; labelled in red dye). The circled and numbered protein spots (82 in total with a cut-off ratio of 1.5 for alteration in protein expression among the three treatment conditions) are subsequently isolated from the gels and identified by MALDI TOF/TOF mass spectroscopy.
DOX-induced modifications in protein expression (top panel), i.e. up-regulation at protein spot #21 and #25 versus down-regulation at spot #23. It is quite obvious that DOX-induced change in colour of these spots is normalized by nitrate supplementation (see the lower panel). A representative image of individual protein spot (#73) is shown in Figure 3, which includes the distinguishable difference based on dye colours (see the top panel) and the confirmation by black-and-white gel image as well as the integrated 3D DeCyder software analysis (see the bottom panel).

From these prominently identified spots we further selected 62 protein spots that were modified (greater than >1.5-fold) by DOX treatment for protein identification using MALDI TOF/TOF tandem mass spectrometry. We selected 41 protein spots that were up-regulated by DOX, among which three spots had no confidence on protein identification (i.e. #70, #71, #72) and 2 spots were matched with unnamed protein sequences (i.e. #56, #60). Table 1 shows results ranked from the largest to the smallest according to the fold changes of DOX/Control ratio. A total of 21 protein spots were down-regulated by DOX, in which only 1 spot had no confidence on protein identification (i.e. #63). Similarly, Table 2 summarizes protein spots down-regulated by DOX. Again, the rank order of the protein spots in this table is based on the DOX/control ratio fold changes, from the largest to the smallest.

**Effect of nitrate supplementation on DOX-induced modification of proteins**

As summarized in Table 1, among the 36 protein spots up-regulated by DOX, 32 were completely reversed by nitrate supplementation (89%), 1 was partially reversed, and only 3 were not affected (8%; i.e. spot #2a – myosin binding protein C, cardiac; spot #45 –
| 2D-DIGE spot no. | Top-ranked protein name | Accession no. | Protein MW (kDa) | Confidence of protein ID | DOX/control ratio | Nitrate + DOX/control ratio | Reversed by nitrate |
|-----------------|-------------------------|---------------|-----------------|-------------------------|-----------------|----------------------------|-----------------|
| 73              | FABP3, muscle and heart | gi|6753810        | 14.8          | High                    | 8.04            | −1.65                      | Yes (completely) |
| 71              | Nuclear receptor subfamily 1, group I, member 2 isoform | gi|148636879       | 44.9          | (fragments?)            | 4.82            | −1.01                      | Yes (completely) |
| 60              | Unnamed protein product | gi|74146841       | 17.9          | High                    | 4.58            | 1.39                       | Yes (completely) |
| 58              | Nucleoside-diphosphate kinase 2 | gi|6679078        | 17.4          | High                    | 4.18            | 5.27                       | No              |
| 64              | Myoglobin                | gi|21359820       | 17.1          | High                    | 3.73            | −1.15                      | Yes (completely) |
| 65              | Myoglobin                | gi|21359820       | 17.1          | High                    | 3.72            | −1.9                       | Yes (completely) |
| 50              | Crystallin, αβ           | gi|6753930        | 20.1          | High                    | 3.61            | −1.23                      | Yes (completely) |
| 49              | SOD2, mitochondrial      | gi|31980762       | 24.6          | High                    | 3.58            | −1.4                       | Yes (completely) |
| 46              | Esterase 1               | gi|20070420       | 28.1          | High                    | 3.53            | 1.14                       | Yes (completely) |
| 62              | Myoglobin                | gi|21359820       | 17.1          | High                    | 2.86            | 1.62                       | (partially)     |
| 55              | Myosin, light polypeptide 2, regulatory, cardiac, slow | gi|153791853     | 18.9          | High                    | 2.72            | −4.76                      | Yes (completely) |
| 12a             | α1 antitrypsin 1–3       | gi|205371731      | 45.8          | High                    | 2.69            | 1.06                       | Yes (completely) |
| 40              | Myosin, light polypeptide 3 | gi|33563284       | 22.4          | High                    | 2.66            | 1                          | Yes (completely) |
| 69              | mCG18900                 | gi|148705042      | 15.6          | High                    | 2.48            | −1.11                      | Yes (completely) |
| 18              | ATP synthase, H+ transporting, mitochondrial F1 complex, β polypeptide | gi|23272966       | 56.6          | High                    | 2.43            | 1                         | Yes (completely) |
| 61              | Myoglobin                | gi|21359820       | 17.1          | High                    | 2.35            | −1.14                      | Yes (completely) |
| 43              | Prx3                     | gi|6680969       | 28.1          | High                    | 2.34            | 1.14                       | Yes (completely) |
| 71              | Ran/T4C binding protein  | gi|431422         | 23.6          | No                      | 2.23            | −1.08                      | Yes (completely) |
| 46              | Esterase 1               | gi|20070420       | 28.1          | High                    | 2.2             | 1.13                       | Yes (completely) |
| 70              | Iron/zinc purple acid phosphatase-like protein | gi|166979753     | 50.6          | No                      | 2.12            | 1                         | Yes (completely) |
| 82              | ATP synthase, H+ transporting, mitochondrial F0 complex, subunit d | gi|16741459       | 18.8          | High                    | 2.11            | −1.15                      | Yes (completely) |
| 21              | Ubiquinol-cytochrome c reductase core protein 1 | gi|46593021       | 52.8          | High                    | 2.01            | −1.08                      | Yes (completely) |
| 72              | Centromere protein A, isoform CRA_b | gi|148705337     | 13.0          | No                      | 1.92            | −1.07                      | Yes (completely) |
| 30              | Ubiquinol-cytochrome c reductase core protein 1 | gi|3342500       | 35.6          | High                    | 1.91            | −1.09                      | Yes (completely) |
| 66              | Cytochrome c oxidase, subunit IV, isoform 1 | gi|6753498       | 19.5          | High                    | 1.9             | −1.24                      | Yes (completely) |
| 53              | ATP synthase, H+ transporting, mitochondrial F0 complex, subunit d | gi|21313679       | 18.7          | High                    | 1.84            | −1.17                      | Yes (completely) |
| 45              | mCG115348, isoform CRA_b | gi|148668894      | 25.4          | High                    | 1.83            | 1.6                        | No              |
| 22              | α-cardiac actin          | gi|387090         | 41.8          | High                    | 1.82            | −1.03                      | Yes (completely) |
| 25              | Long-chain acyl-CoA dehydrogenase | gi|726095        | 48.1          | High                    | 1.81            | −1.07                      | Yes (completely) |
| 2a              | Myosin binding protein C, cardiac | gi|161611787     | 140.5         | High                    | 1.76            | 1.52                       | No              |
| 12b             | α1 antitrypsin 1–3       | gi|205371731      | 45.8          | High                    | 1.75            | −1.05                      | Yes (completely) |
| 33              | Myozin 2                 | gi|10946916      | 29.7          | High                    | 1.74            | 1.01                       | Yes (completely) |
| 19              | Sarcalumenin            | gi|34328417       | 99.1          | High                    | 1.72            | 1                          | Yes (completely) |
| 29              | Isocitrate dehydrogenase 3 (NAD+) α | gi|148693875     | 34.6          | High                    | 1.68            | −1.05                      | Yes (completely) |
| 5               | Muscle glycogen phosphorylase | gi|6755256       | 97.2          | High                    | 1.67            | 1.04                       | Yes (completely) |
| 56              | Unnamed protein product  | gi|74198777       | 56.0          | High                    | 1.65            | −1.13                      | Yes (completely) |
| 20              | Sarcalumenin, isoform CRA_a | gi|148664814     | 54.4          | High                    | 1.64            | −1.07                      | Yes (completely) |
| 36              | mCG199938, isoform CRA_b | gi|148691000     | 39.7          | High                    | 1.6             | −1.26                      | Yes (completely) |
| 28              | Aspartate aminotransferase | gi|871422         | 46.2          | High                    | 1.58            | −1.04                      | Yes (completely) |
| 51              | mCG10592, isoform CRA_c | gi|148703873      | 16.5          | High                    | 1.58            | −1.21                      | Yes (completely) |
| 16              | Pyruvate kinase, muscle  | gi|31981562       | 57.8          | High                    | 1.54            | −1.02                      | Yes (completely) |

The data are listed according to the high-to-low fold change ratio of protein abundance between DOX and control hearts. The cut-off ratio was +1.5. The italic spot numbers (70, 71, and 72) in the first column indicate the low or no confidence on the protein spot identity as marked in the fifth column. MW: molecular weight; DOX: doxorubicin; FABP3: fatty acid binding protein 3; SOD: superoxide dismutase; Prx: peroxiredoxin.
Furthermore, among the 20 identified protein spots down-regulated by DOX, 9 of them were completely reversed by nitrate supplementation (47%), 2 were partially reversed, and 8 were not affected (42%), as shown in Table 2. Figure 4 shows DeCyder 3D images highlighting the substantial differences in protein abundance between three treatment groups in five representative protein spots. The DOX-induced changes in these proteins and the normalizing effects of nitrate supplementation are easily visualized.

**Classification of DOX-modified protein expression based on biological function**

To further understand the functional significance of DOX-modified proteins in the heart, we grouped positively identified proteins according to their known biological functions (Table 3) under the following seven categories. The first group included three mitochondria-localized antioxidant proteins, i.e. SOD2, Prx3 and Prx5. The second group comprised 12 cytoskeletal/contractile proteins, in which 8 proteins were up-regulated and 4 were down-regulated by DOX. The myoglobin, identified at four different spots (#61, #62, #64, #65) in the 2D-DIGE gels, was consistently up-regulated by DOX, but completely reversed by nitrate (Tables 2 and 3). The third group included seven proteins that are involved in fatty acid or glucose metabolism. DOX treatment caused an increase in five of these metabolic proteins and a decrease in the remaining two. Most notably, an 8-fold enhancement in FABP3 (spot #73) was observed after DOX administration (Tables 1 and 3; Fig. 3), which was completely normalized by nitrate. Conversely, there was a 6-fold down-regulation of PYGB (spot #4) caused by DOX and nitrate supplementation partially reversed this trend.

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**Table 2** Cardiac proteins down-regulated by DOX: Effects of nitrate supplementation

| 2D-DIGE spot no. | Top-ranked protein name | Accession No. | Protein MW (kD) | Confidence of protein ID | DOX/control ratio | Nitrate + DOX/control ratio | Reversed by nitrate |
|----------------|-------------------------|---------------|----------------|------------------------|-----------------|-----------------------------|------------------|
| 4              | PYGB                    | gi|24418919         | 96.7             | High                     | −6.24           | −1.84                       | Yes (partially)  |
| 63             | Unnamed protein product | gi|26350173         | 12.0             | No                       | −3.42           | 2.07                        | Yes (completely) |
| 42             | mCG9061, isoform CRA_c  | gi|148706375        | 28.4             | High                     | −3.2            | −2.2                        | Yes (partially)  |
| 15             | Aldehyde dehydrogenase 4 family, member A1 | gi|225543103        | 61.8             | High                     | −3.11           | −3.67                       | No               |
| 59             | Peroxisomal membrane protein 20 (Prx5) | gi|6746357          | 17.0             | High                     | −2.73           | 2.07                        | Yes (completely) |
| 48             | es1 protein             | gi|20070420         | 28.1             | High                     | −2.64           | −3.27                       | No               |
| 68             | ATP synthase, H+ transporting, mitochondrial F1 complex, δ subunit, isoform CRA_a | gi|148699643        | 14.3             | High                     | −2.34           | −2.75                       | No               |
| 39             | Tyrosine 3-monooxygenase/tryptophan | gi|148676868       | 29.1             | High                     | −2.3            | −1.43                       | Yes (completely) |
| 13b            | α1-antitrypsin 1–3      | gi|205371731        | 45.8             | High                     | −2.25           | −1.17                       | Yes (completely) |
| 23             | Succinate-coenzyme A ligase, GDP-forming, β subunit | gi|165972309        | 46.8             | High                     | −2.06           | −1.29                       | Yes (completely) |
| 52             | MYL2                    | gi|199985           | 18.9             | High                     | −2.03           | 2.79                        | Yes (completely) |
| 54             | Ferritin heavy chain 1  | gi|6753912          | 21.1             | High                     | −1.94           | 1.25                        | Yes (completely) |
| 11             | α-fetoprotein           | gi|191765           | 47.2             | High                     | −1.93           | −1.3                        | Yes (completely) |
| 67             | ATP synthase, H+ transporting, mitochondrial F1 complex, δ subunit, isoform CRA_a | gi|148699643        | 14.3             | High                     | −1.87           | −1.97                       | No               |
| 57             | Nucleoside-diphosphate kinase 2 | gi|6679078          | 17.4             | High                     | −1.7            | −1.72                       | No               |
| 74             | β2-globin               | gi|4760594          | 15.7             | High                     | −1.68           | −3.82                       | No               |
| 1a             | Myosin, heavy polypeptide 6, cardiac muscle, α | gi|83405899         | 223.4            | High                     | −1.67           | −1.2                        | Yes (completely) |
| 1b             | Myosin, heavy polypeptide 6, cardiac muscle, α | gi|83405899         | 223.4            | High                     | −1.56           | −1.14                       | Yes (completely) |
| 35             | Four and a half LIM domains 2 | gi|18204139         | 32.0             | High                     | −1.54           | −1.57                       | No               |
| 14             | Fibrinogen, α-polypeptide isoform 2 | gi|33563252         | 61.3             | High                     | −1.51           | −1.66                       | No               |
| 13a            | α1-antitrypsin 1–3      | gi|205371731        | 45.8             | High                     | −1.51           | −1.14                       | Yes (completely) |

The data are listed according to the high-to-low fold change ratio of protein abundance between DOX and control hearts. The cut-off ratio was −1.5. The italic spot number (63) in the first column indicates the low or no confidence on the protein spot identity as marked in the fifth column. MW: molecular weight; DOX: doxorubicin; PYGB: brain glycogen phosphorylase; Prx: peroxiredoxin; MYL: myosin light chain.
Fig. 4 Representative 3D DeCyder software integrated graphs showing differences in protein abundance. Five protein spots are selected from three treatment groups. Note that DOX-induced changes are normalized by nitrate supplementation. PYGB: brain glycogen phosphorylase; Prx3: peroxiredoxin 3; SOD2: mitochondrial superoxide dismutase 2; MYL2: myosin light chain 2; FABP3: fatty acid binding protein 3.
Table 3 DOX-modified cardiac proteins grouped based on biological function and the effects of nitrate supplementation on the modifications

| Protein name | 2D-DIGE spot | MW (kD) | DOX/control ratio | Nitrate effect |
|--------------|--------------|---------|-------------------|---------------|
| **Antioxidant proteins** | | | | |
| SOD2, mitochondrial | 49 | 24.6 | 3.58 ↑ | Fully reversed |
| Prx3 | 43 | 28.1 | 2.34 ↑ | Fully reversed |
| Peroxisomal membrane protein 20 (Prx5) | 59 | 17.0 | 2.73 ↓ | Fully reversed |
| **Cytoskeletal/contractile proteins** | | | | |
| Myoglobin | 61, 62, 64, 65 | 17.1 | 2.35–3.73 ↑ | Fully reversed |
| Myosin, light polypeptide 2, regulatory, cardiac, slow | 55 | 18.9 | 2.72 ↑ | Fully reversed |
| Myosin, light polypeptide 3 | 40 | 22.4 | 2.66 ↑ | Fully reversed |
| α-cardiac actin | 22 | 41.8 | 1.82 ↑ | Fully reversed |
| Myosin binding protein C, cardiac | 2a | 140.5 | 1.76 ↑ | No effect |
| Myozin 2 | 33 | 29.7 | 1.74 ↑ | Fully reversed |
| Sarcalumenin | 19 | 99.1 | 1.72 ↑ | Fully reversed |
| Sarcalumenin, isoform CRA_a | 20 | 54.4 | 1.64 ↑ | Fully reversed |
| MYL2 | 52 | 18.9 | 2.03 ↓ | Fully reversed |
| Ferritin heavy chain 1 | 54 | 21.1 | 1.94 ↓ | Fully reversed |
| β2-globin | 74 | 15.7 | 1.68 ↓ | No effect |
| Myosin, heavy polypeptide 6, cardiac muscle, α | 1a, 1b | 223.4 | 1.56–1.67 ↓ | Fully reversed |
| **Fatty acid/glucose metabolism proteins** | | | | |
| FABP3, muscle and heart | 73 | 14.8 | 8.04 ↑ | Fully reversed |
| Long-chain acyl-CoA dehydrogenase | 25 | 48.1 | 1.81 ↑ | Fully reversed |
| Isocitrate dehydrogenase 3 (NAD+) α | 29 | 34.6 | 1.68 ↑ | Fully reversed |
| Muscle glycogen phosphorylase | 5 | 97.2 | 1.67 ↑ | Fully reversed |
| Aspartate aminotransferase | 28 | 46.2 | 1.58 ↑ | Fully reversed |
| PYGB | 4 | 96.7 | 6.24 ↓ | Partially reversed |
| Succinate-Coenzyme A ligase, GDP-forming, β subunit | 23 | 46.8 | 2.06 ↓ | Fully reversed |
| **Kinases** | | | | |
| Nucleoside-diphosphate kinase 2 | 58 | 17.4 | 4.18 ↑ | No effect |
| Nucleoside-diphosphate kinase 2 | 57 | 17.4 | 1.70 ↓ | No effect |
| Pyruvate kinase, muscle | 16 | 57.8 | 1.54 ↑ | Fully reversed |
| **Mitochondrial respiratory chain** | | | | |
| ATP synthase, H+ transporting, mitochondrial F1 complex, β polypeptide | 18 | 56.6 | 2.43 ↑ | Fully reversed |
| ATP synthase, H+ transporting, mitochondrial F0 complex, subunit d | 53, 82 | 18.8 | 1.84–2.11 ↑ | Fully reversed |
| Ubiquinol-cytochrome c reductase core protein 1 | 21, 30 | 35.6, 52.8 | 1.91–2.01 ↑ | Fully reversed |
| Cytochrome c oxidase, subunit IV, isoform 1 | 66 | 19.5 | 1.90 ↑ | Fully reversed |
| ATP synthase, H+ transporting, mitochondrial F1 complex, δ subunit, isoform CRA_a | 67, 68 | 14.3 | 1.87–2.34 ↓ | No effect |

Continued
DOX treatment caused alterations in two kinases (i.e. nucleoside-diphosphate kinase 2 and pyruvate kinase). The changes for nucleoside-diphosphate kinase 2 were identified in two separate spots (#57, #58) and neither of these was reversed by nitrate. However, DOX-induced mild increase in pyruvate kinase abundance was fully abolished by nitrate treatment. The fifth group included five proteins related to mitochondrial respiratory chain. There are three isoforms of ATP synthase (i.e. complex V), ubiquinol-cytochrome c reductase core protein 1 and cytochrome c oxidase (subunit IV). Majority of these mitochondrial proteins were increased by DOX treatment, which were reversed by nitrate supplementation. Interestingly, the α subunit of F1 complex of ATP synthase was detected in two different spots (#67, #68) with opposing effects by DOX. Nevertheless, nitrate treatment had no effect on the DOX-induced changes.

In the sixth category, only one low molecular weight stress protein (αB crystallin; spot #50) was increased ~3.6-fold by DOX, which was completely blunted by nitrate treatment.

Finally, there are eight positively identified proteins that have uncertain role in cardiac function. Among the six proteins down-regulated by DOX, four (i.e. aldehyde dehydrogenase 4 family, esterase 1, four and a half LIM domains 2, fibrinogen) were not affected by nitrate supplementation (Table 3).

**Western blot analysis for verification of 2D-DIGE results**

To confirm the 2D-DIGE results, DOX-induced six prominently modified proteins with known functional significance were probed by Western blots. These included antioxidant enzymes (SOD2, Prx3, Prx5), cytoskeletal/contractile protein (MYL2) and metabolic protein (FABP3, PYGB). To a larger extent, Western blot results confirmed most of the changes detected by 2D-DIGE for PYGB, SOD2, Prx3 and Prx5 (Figs 5 and 6). In contrast, two proteins (MYL2 and FABP3) showed opposite trend of the 2D-DIGE proteomic analysis (Fig. 5).

**Effect of nitrate supplementation on mortality**

As shown in Figure 7, 80% of the mice received DOX alone survived 5 days after the DOX injection. Nitrate supplemented mice in Nitrate + DOX group had a significantly higher survival rate (93%) as compared with DOX group (P < 0.05). There was no mortality in both Control and Nitrate alone groups during the entire 13-day experimental protocol.

**Discussion**

The cardiotoxicity of DOX remains an unsolved dilemma for both clinical and research communities. There is an ongoing call for a better therapeutic intervention to prevent and/or treat this devastating pathological condition of the heart. The present study was an extended endeavour to identify novel protein targets in protection against DOX-induced cardiomyopathy by inorganic nitrate supplementation [15, 16]. We utilized a globally non-biased proteomic approach with 2D-DIGE and MALDI TOF/TOF tandem mass spectrometry. The advantages of this method over conventional 2D gels in detecting global protein expression and post-translational modification are evident in our study, as it enables the identification of multiple proteins with altered expression, including those with uncertain functions.

In conclusion, our study provides new insights into the molecular mechanisms underlying the cardiotoxicity of DOX and supports the potential of nitrate supplementation as a promising therapeutic strategy for the prevention and treatment of DOX-induced cardiotoxicity.

| Protein name | 2D-DIGE spot | MW (kD) | DOX/control ratio | Nitrate effect |
|--------------|--------------|---------|-------------------|----------------|
| Crystallin, αB | 50           | 20.1    | 3.61 ↑            | Fully reversed |
| Esterase 1     | 46, 47       | 28.1    | 2.20–3.53 ↑       | Fully reversed |
| α1 antitrypsin 1–3 | 12a, 12b    | 45.8    | 1.75–2.69 ↑       | Fully reversed |
| Aldehyde dehydrogenase 4 family, member A1 | 15 | 61.8 | 3.11 ↓ | No effect |
| Esterase 1     | 48           | 28.1    | 2.64 ↓            | No effect |
| α-fetoprotein  | 11           | 47.2    | 1.93 ↓            | Fully reversed |
| Tyrosine 3-monoxygenase/ tryptophan | 39 | 29.1 | 2.30 ↓ | Fully reversed |
| Four and a half LIM domains 2 | 35 | 32.0 | 1.54 ↓ | No effect |
| Fibrinogen, α polypeptide isoform 2 | 14 | 61.3 | 1.51 ↓ | No effect |

Note: The data are listed according to the high-to-low fold change ratio of protein abundance between DOX and control hearts. The cut-off ratio of change was ≥1.5. MW: molecular weight; DOX: doxorubicin; Prx: peroxiredoxin; SOD2: mitochondrial superoxide dismutase 2; MYL2: myosin light chain 2; FABP3: fatty acid binding protein 3; PYGB: brain glycogen phosphorylase.
modifications have been increasingly appreciated in recent years [17, 18]. In particular, the conventional 2D gels methods are subject to inherent gel-to-gel variability and errors, which can be eliminated by multiplex, high-resolution 2D-DIGE technique. This technique uses both size- and charge-matched, spectrally resolvable fluorophores (CyDye) to simultaneously separate up to three samples on a single 2D gel. As a result, every protein spot on the gel has its own internal standard and direct comparisons between the samples can be made with high sensitivity without any gel-to-gel variability [17, 18].

One of the salient findings of the present study is the reversal in DOX-induced modification of three mitochondria-localized antioxidant enzymes (SOD2, Prx3, Prx5) by nitrate supplementation. Induction of cardiac SOD2 expression by DOX treatment has been reported by us [19] and others [20]. Because the protective role of SOD2 against DOX-induced cardiac injury has been well established by transgenic mice overexpressing SOD2 [21, 22], the up-regulation of SOD2 in the hearts from DOX-treated mice appears to be an adaptive response to DOX. However, the observed adaptive induction of cardiac SOD2 by DOX [19, 20] was not sufficient to prevent the DOX-induced cardiac ROS generation [23] and dysfunction [15]. The normalization of cardiac SOD2 expression by nitrate supplementation is likely due to the reduced level of oxidative stress following nitrate treatment that we recently reported [23]. Similarly, an increase of Prx3 was previously reported by Merten et al. [20]. Nitrate treatment completely restored Prx3 expression to its control level. On the other hand, Prx5 was the only thiol-based antioxidant that was up-regulated by the cardioprotective nitrate supplementation. Peroxiredoxin represents a new type of peroxidase distinct from catalase and glutathione peroxidase, in terms of abundance in expression, broad intracellular distribution, and high affinity for hydrogen peroxide [24, 25]. Among the total of six identified Prx isoforms, only Prx3 and Prx5 localize in mitochondria. Currently the role of Prx5 in protection against DOX-induced cardiotoxicity is unknown, although its importance in protecting cells against injuries caused...
by reactive oxygen species (particularly hydrogen peroxide and peroxynitrite) has been suggested by several studies [26–29]. Further studies are needed to demonstrate a cause-and-effect relationship between induction of cardiac Prx5 and cardioprotection against DOX cardiotoxicity by nitrate supplementation.

It is noteworthy that Western blot results showed an opposite trend for the expression of MYL2 and FABP3 when compared with the 2D-DIGE results. We do not know the specific reason for these contrasting results. Nevertheless, it is possible that 2D-DIGE is a much more sensitive technique than the 1-D Western blots because it can identify changes occurring primarily in certain isoform(s) of a protein which may not be discernible by total protein expression that essentially detects all the isoforms with identical molecular weight.

Although a number of genomic studies have been reported to understand the mechanisms of DOX-induced cardiotoxicity [30, 31], only limited studies have used proteomic approaches which were specifically targeted toward comparing the protein
abundance between the DOX treatment with or without the cardio-protective intervention [20, 32–36], including transgenic overexpression of metallothionein [20], heat shock preconditioning [35] and co-treatment with docetaxel [33]. To our best knowledge, this is the first study using 2D-DIGE to determine alterations in cardiac proteins caused by DOX. Our results are particularly significant in terms of providing novel information on the reversal of DOX-induced modification of three mitochondrial antioxidant enzymes (SOD2, Prx3, Prx5) by nitrate supplementation, particularly the selective up-regulation of Prx5, which may play an important role in attenuating oxidative stress in the heart following nitrate supplementation [23].

Inorganic nitrate is a water soluble, very inexpensive chemical that appears ideal for long-term oral administration in preventing DOX-induced cardiomyopathy during its prolonged pathological process. On the other hand, there have been concerns on the potential toxic effects of high nitrate intake, particularly infant methemoglobinemia related to the high nitrate levels in drinking water and foods in certain areas such as Transylvania region of Romania [37] and the highly controversial issue of nitrate-induced carcinogenesis [38]. We cautiously believe that these potential side effects of nitrate are not present in the case of nitrate-induced protection against DOX cardiotoxicity. This is because methemoglobinemia rarely occurs in children or adults due to the physiological induction of methemoglobin reductase during the post-weaning developmental period [38]. Also, there should be no concern on the questionable carcinogenesis during the course of nitrate supplementation, because the targeted cancer patients would have already received anticancer therapy with DOX. Moreover, in the present study, the nitrate-supplemented mice had significantly higher survival rate (93%) than the controls (80%) 5 days after DOX treatment (P < 0.05), whereas the 13-day course of high nitrate intake alone did not cause any mortality or other apparent adverse effects in the mice.

The current upper limit of WHO acceptable intake (ADI) for nitrate is 222 mg per day for an adult with 60 kg body weight (i.e. 3.4 mg/kg) [39]. The oral nitrate dose used in the present study is about 180 mg/kg/day, which gives 14.6 mg/kg/day of human equivalent dose according to the FDA published conversion formula (i.e. mouse dose divided by 12.3). Therefore the nitrate dose is less than 400% of the WHO-ADI, which is within the physiological limits, considering particularly that just a cup of raw spinach contains 926 mg of nitrate [39]. Oral nitrate supplementation can be easily obtained from foods containing high levels of nitrate, e.g. leafy green vegetables [39] as well as from nitrate-enriched capsules and beverages that are already commercially available and safely used in human beings [40, 41]. Nitrate intake has been shown to exert a number of beneficial effects to attenuate myocardial ischemia-reperfusion injury [11, 12], hypertension [13, 14] and platelet aggregation [38]. In addition, nitrate supplementation has been shown to alleviate other degenerative chronic diseases such as type-2 diabetes [42], decreasing whole body oxygen cost during exercise and improving maximal performance [40, 41]. Taken together, it is conceivable that oral nitrate supplementation may have greater potential for clinical application in reducing cardiotoxicity, bodily suffering and financial burden of the thousands of cancer patients receiving DOX.

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Conflict of interest

The authors confirm that there are no conflicts of interest.

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