Free Radical Biology and Medicine 72 (2014) 55–65

Contents lists available at ScienceDirect

Free Radical Biology and Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Original Contribution

Redox proteomic identification of HNE-bound mitochondrial proteins in cardiac tissues reveals a systemic effect on energy metabolism after doxorubicin treatment

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ARTICLE INFO

Article history:
Received 16 September 2013
Received in revised form
17 February 2014
Accepted 4 March 2014
Available online 12 March 2014

Keywords:
Doxorubicin
Cardiac injury
ATP synthase
Dihydrolipoyl dehydrogenase
Succinate dehydrogenase [ubiquinone] flavoprotein
NADH dehydrogenase [ubiquinone] iron–sulfur protein 2
Oxidative stress
Redox proteomics
Free radicals
Metabolism

ABSTRACT

Doxorubicin (DOX), one of the most effective anticancer drugs, is known to generate progressive cardiac damage, which is due, in part, to DOX-induced reactive oxygen species (ROS). The elevated ROS often induce oxidative protein modifications that result in alteration of protein functions. This study demonstrates that the level of proteins adducted by 4-hydroxy-2-nonenal (HNE), a lipid peroxidation product, is significantly increased in mouse heart mitochondria after DOX treatment. A redox proteomics method involving two-dimensional electrophoresis followed by mass spectrometry and investigation of protein databases identified several HNE-modified mitochondrial proteins, which were verified by HNE-specific immunoprecipitation in cardiac mitochondria from the DOX-treated mice. The majority of the identified proteins are related to mitochondrial energy metabolism. These include proteins in the citric acid cycle and electron transport chain. The enzymatic activities of the HNE-adducted proteins were significantly reduced in DOX-treated mice. Consistent with the decline in the function of the HNE-adducted proteins, the respiratory function of cardiac mitochondria as determined by oxygen consumption rate was also significantly reduced after DOX treatment. Treatment with Mn(III) *meso*-tetrakis(*N*-*n*-butoxyethylpyridinium-2-yl)porphyrin, an SOD mimic, averted the doxorubicin-induced mitochondrial dysfunctions as well as the HNE–protein adductions. Together, the results demonstrate that free radical-mediated alteration of energy metabolism is an important mechanism mediating DOX-induced cardiac injury, suggesting that metabolic intervention may represent a novel approach to preventing cardiac injury after chemotherapy.

Doxorubicin (DOX), one of the most effective anticancer drugs, has been a therapeutic for a broad spectrum of human cancers for almost half a century and remains the first choice for many aggressive tumors, such as acute myeloid leukemia [1]. Its clinical application is highly limited owing to the dose-related, progressive, and irreversible cardiac damage it causes. The mortality of patients who develop congestive heart failure after DOX treatments can be as high as 50% [2], increasing significantly when cumulative doses are more than 500 mg/m^2 [3].

Numerous studies have focused on the mechanisms behind DOX-induced cardiotoxic effects and demonstrated multifactorial causes. However, there is consensus that oxidative stress is a primary mechanism of DOX-induced cardiotoxicity and that the stress is attributed to the formation of reactive oxygen species (ROS). DOX generates ROS by various means, mainly through redox cycling. The quinone moiety of DOX can be converted one-electronically to semiquinone by several cellular oxidoreductases [4]. One-electron

Abbreviations: DOX, doxorubicin; ATP5B, ATP synthase subunit β; DLD, dihydrolipoyl dehydrogenase; SDHA, succinate dehydrogenase [ubiquinone] flavoprotein subunit; NDUFS2, NADH dehydrogenase [ubiquinone] iron–sulfur protein 2; ROS, reactive oxygen species; HNE, 4-hydroxy-2-nonenal; TCA cycle, citric acid cycle; ETC, electron transport chain; OCR, oxygen consumption rate; ECAR, extracellular acidification rate; FH, fumarate hydratase; HADHA, trifunctional enzyme subunit α; CKMT2, creatine kinase S-type; Oxct1, 3-oxoacid–CoA transferase 1; MnP, Mn(III) *meso*-tetrakis(*N*-*n*-butxyethylpyridinum-2-yl) porphyrin or MnTnBuOE-2-PyP^{5+}; NAC, *N*-acetylcysteine

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http://dx.doi.org/10.1016/j.freeradbiomed.2014.03.001
0891-5849/

oxidation of the DOX-semiquinone radical to the DOX-quinone form leads to the generation of a highly reactive superoxide, which can be further involved in the production of a variety of ROS, including $OH^{\bullet}$, $ROO^{\bullet}$, $ONOO^{-}$, ROOH, and H_2O_2 [5].

ROS are highly reactive with biomolecules, including lipids, proteins, carbohydrates, DNA, and RNA, and lead to cellular dysfunction [6]. A major source of ROS-mediated injury is lipid peroxidation, the reaction of ROS with the polyunsaturated fatty acids of lipid membranes. Lipid peroxidation generates a number of cytotoxic and highly reactive by-products such as aldehydes, alkenals, and hydroxyalkenals. Among lipid peroxidation products, 4-hydroxy-2-nonenal (HNE) is the most abundant [7]. HNE readily reacts with proteins and, at higher concentrations, with DNA [8,9]. HNE has a high affinity for and covalently attaches to Cys, His, and Lys residues by the Michael addition [10], which leads to dysfunction in the target proteins linked to intracellular signal transduction, aging, and many human diseases.

Cardiomyocytes have more mitochondria, in both number and volume, than other cells, and they are the most active cells with regard to the oxidative phosphorylation that is required for their energy needs [11]. The complex that DOX forms with cardiolipin, which resides in the mitochondrial inner membrane [12], places DOX in close proximity to the mitochondrial electron transport chain. The redox cycling of DOX is mediated through its interaction with NADH dehydrogenase (complex I) of the mitochondrial electron transport chain (ETC) [13]. Thus, active mitochondria are both important sources and primary targets of DOX-induced ROS. Multiple studies indicate that mitochondrial dysfunction is a key factor in the process of DOX-induced pathogenicity [14–16]. Studies utilizing tissue samples from patients receiving DOX reveal histopathological evidence that suggests disruption of mitochondrial structure and membranes [17]; similar observations were made in animal models and in cells in vitro [8,18].

HNE–protein adductions play important roles in regulating the function of proteins that may lead to cellular dysfunction. In this study, we used two-dimensional (2D) electrophoresis and redox immunochemistry analysis followed by mass spectrometry and interrogation of protein databases to identify eight HNE-adducted proteins in mouse heart mitochondria after DOX treatment. We then verified the consequences of HNE adduction on the functions of those proteins via enzymatic activity assays as well as on mitochondrial function.

Materials and methods

Materials

Doxorubicin was obtained from Bedford Laboratories (Bedford, OH, USA). Succinate dehydrogenase [ubiquinone] flavoprotein subunit (SDHA), dihydrolipoyl dehydrogenase (DLD), and fumarate hydratase (FH) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). MnTnBuOE-2-PyP^{5+} (MnP) was produced by Dr. Ines Batinic-Haberle of the Department of Radiation Oncology, Duke University. *N*-acetylcysteine (NAC) and ATP synthase antibody were purchased from Sigma (St. Louis, MO, USA). NADH dehydrogenase [ubiquinone] iron–sulfur protein 2 (NDUFS2) and HNE antibodies and complex I and ATP synthase activity assay kits were purchased from Abcam (Cambridge, MA, USA); H9C2, a neonatal rat heart cell line, was from the ATCC (Manassas, VA, USA).

Animal treatment and mitochondrial isolation

All animals were housed in the University of Kentucky Animal Facility and experiments were conducted using procedures approved by Institutional Animal Care in accordance with the NIH *Guide for the Care and Use of Laboratory Animals.* In-house bred, 9-week-old, male, C57BL/6 mice were treated with a single dose of 20 mg/kg DOX or saline via intraperitoneal injection. Seventy-two hours after injection, mice were euthanized.

Heart mitochondria were isolated as described previously [19]. Briefly, freshly isolated hearts were washed in ice-cold isolation buffer (0.225 M mannitol, 0.075 M sucrose, 1 mM EGTA, pH 7.4) and homogenized at 500 rpm with a chilled Teflon pestle for 10 strokes in isolation buffer. The homogenate was centrifuged at 480 *g* at 4 °C for 5 min in a Sorval SS 34 rotor. The resulting supernatant was filtered through double-layered cheesecloth and centrifuged at 7700 *g* at 4 °C for 10 min. The pellet was washed twice by gentle resuspension in 3 ml isolation buffer and centrifuged at 7700 *g* at 4 °C for 10 min. The resulting mitochondria were either used or frozen in liquid nitrogen for further analysis. The purity of mitochondria was examined using lamin A (nuclear protein) and IκB-α (cytoskeletal protein) on Western blot.

Total protein-bound HNE detection

The levels of total protein-bound HNE were determined in the Free Radical Biology in Cancer Shared Resource Facility (FRBC SRF) at the University of Kentucky. The mitochondrial samples (pellets) were thawed and resuspended in a small volume (75–150 μl) of ice-cold homogenization buffer (0.32 M sucrose, 10 mM Hepes, pH 7.4, 2 mM EDTA, protease inhibitors). Five microliters of the homogenized sample was mixed and diluted with an equal volume of 12% SDS. Samples were further denatured with 10 μl of modified Laemmli buffer (0.125 M Trizma base, 4% SDS, and 20% glycerol) for 20 min at room temperature. Next, 250 ng of the derivatized protein was loaded in each slot (48-well slot format Bio-Dot SF apparatus with nitrocellulose membranes, pore size 0.2 μm, Bio-Rad, Hercules, CA, USA). The antibody reaction was developed using 5-bromo-4-chloro-3-indolyl phosphate in conjunction with nitroblue tetrazolium. The immunodetection was performed by using 1:5000 anti-4-hydroxynonenal antiserum (Alpha Diagnostic International, San Antonio, TX, USA) and goat 1:7500 anti-rabbit IgG (Sigma–Aldrich) antibody for the secondary detection. The nitrocellulose membranes were scanned by photo scanner (Epson Perfection V600, Long Beach, CA, USA), and slot-blot line densities were quantified by the ImageQuant TL software package (GE Healthcare Bio-Sciences, Piscataway, NJ, USA).

Two-dimensional gel electrophoresis and protein mass spectrometry studies

Two-dimensional gel electrophoresis was performed in the core facility of FRBC SRF using a protocol similar to that previously described [20]. Briefly, a duplicate amount (150 μg protein) of each isolated mitochondrial sample underwent trichloroacetic acid precipitation and rehydration in 2D gel rehydration medium (8 M urea, 2 M thiourea, 2% Chaps, 0.2% biolytes, 50 mM dithiothreitol, bromophenol blue dissolved in deionized water and made fresh before use). Samples were separated according to their isoelectric point using 11-cm, pH 3–10, immobilized pH gradient (IPG) strips, using an isoelectric focusing cell system (Bio-Rad) for the first-dimension separations. For each sample we produced two identical IPG strips with an equal amount of initial protein. After the proteins were separated in the first dimension, every pair of IPG strips was separated according to its molecular migration rate in one gel running box (each box ran two gels simultaneously) with precast Criterion XT 8–16% Bis–Tris gel w/Mops for the second-dimension separations. All gels ran under constant voltage (200 V) for 65 min. The "twin" gels from each sample were handled as follows: one twin gel was stained with Sypro ruby stain (Bio-Rad) for total protein and the second twin gel was