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Brief communication (Original)

Endoplasmic reticulum protein ERp29 and doxorubicin-induced toxicity in H9c2 cardiomyocytes: a comparative proteomics analysis

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Background: Doxorubicin has been widely used to treat many cancers. It also induces cumulative and delayed cardiomyopathy. New biological markers to predict cardiac toxicity is needed.
Objectives: We identified novel markers and potential therapeutic targets of doxorubicin (DOX)-induced cardiac toxicity by proteomics approach.
Methods: The protein profiling of H9c2 cells in response to DOX at an apoptosis-induced concentration (0.5 □M) were compared by two-dimensional electrophoresis (2-DE) and mass spectrometry.
Results: A total of nine differently expressed proteins were identified including six up-regulated and three down-regulated proteins. We further confirmed the expression of two down-regulated proteins, prohibitin and endoplasmic reticulum protein ERp29 (ERp29), decreased in response to DOX induction by Western-blot, and over-expression of ERp29 also partially recovered the MTT reduction.
Conclusion: We first identified ERp29 and prohibitin as novel markers for DOX toxicity, and ERp29 might be a candidate target to develop novel therapeutic strategies to alleviate adverse effects of doxorubicin-based chemotherapies.

Keywords: Apoptosis, cardiac toxicity, doxorubicin, ERp29, proteomics

Doxorubicin (DOX) has been used widely to treat multiple malignancies such as lung, breast, ovary and uterine cancers. However, DOX chemotherapy is prone to induce a cumulative and delayed cardiomyopathy, which greatly limits its utility in clinical practice [1]. The proposed mechanism of DOX cardiotoxicity includes oxidative stress, mitochondrial dysfunction and cardiomyocyte apoptosis [2-4]. However, the exact molecular mechanism of DOX-induced cardiotoxicity, especially the novel targets for intervention, still needs further investigation.

Proteomics provides a useful analytical tool for the generation of global protein expression profiles of the DOX-affected cardiomyocytes. Recently, Kumar et al. [5], analyzed the protein profiling of primarily isolated adult rat cardiomyocytes and heart tissues exposed to low levels of DOX, and identified a panel of proteins related to oxidative stress response differently regulated after DOX treatment. Therefore, these findings suggest that proteomic techniques might be helpful for identifying novel drug-associated biomarkers, and might also provide potential targets for alleviating the cardiotoxicity in patients treated by DOX.

In this study, we first performed a differential proteomic analysis of H9c2 cells, a widely used model of DOX toxicity, in response to apoptosis-induced concentrations of DOX. And then further study indicated that ERp29, an identified potential target by proteomics, might attenuate toxicity induced by DOX in H9c2 cardiomyocytes.

Materials and methods

Cell culture and treatment

The H9c2 cardiomyocyte cell line used in this study was derived from embryonic rat heart by selective serial passage and obtained from American

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Type Culture Collection (ATCC). H9c2 cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal calf serum at 37°C with 5% CO_2. H9c2 cells apoptosis were induced by adding 0.5 μM of DOX to the medium for 48 hours, and then the cells were harvested for relevant assays. This concentration is a clinically relevant, at which cells exhibit apoptosis, but not exhibit significant cellular damage [6].

Proteomics analysis

Sample preparation and two-dimensional electrophoresis (2-DE) separation were performed as described. Briefly, H9c2 cells treated with DOX at 0h and 48h were lysed in a buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 40 mM DTT, 0.5% IPG buffer. 300 μg of extracted proteins mixed with rehydration buffer were applied to 24 cm non-linear IPG strips (pH 3-10 NL), and isoelectric focusing was carried out on the Ettan IPGphor Isoelectric Focusing System (GE Healthcare). After equilibration, the proteins were further separated by 12% SDS-PAGE gels using an Ettan DALT twelve system (GE Healthcare). After Commassie blue (R350) staining, the images of scanned gels were analyzed using the ImageMaster 2D Platinum software (GE Healthcare). Differently expressed spots were selected for trypsin digestion and tandem mass spectrometry analysis (Applied Biosystems 4700 Proteomics Analyzer MALDI-TOF/TOF mass spectrometer). Database searching of MS data was performed using Mascot software.

Western-blot

H9c2 cells were treated by 0.5 μM DOX for 0, 12, 48, 72 hours, and then cell lysates were extracted with RIPA Buffer; 30 μg of the isolated protein samples was separated by 12% SDS-polyacrylamide gel and transferred to nitrocellulose membrane. The membranes were immunoblotted with anti-ERp29 (1:1000, Santa Cruz) and anti-prohibitin antibody (1:1000, Santa Cruz), followed by the addition of horseradish peroxidase-conjugated secondary antibody. After washing, the membranes were detected using an ECL chemiluminescence reagent (Milipore). β-actin was used as a loading control.

ERp29 transfection and doxorubicin toxicity assay

Cells transfected with pcDNA/ERp29 or pcDNA were generated as previously described [7]. Transfection was performed in the same culture medium without fetal bovine using Lipofectamine according to the manufacturer's manual. After treatment with doxorubicin for 0, 12, 48, 72 hours, cell viability was determined by MTT assay. Viability was assessed at 570 nm absorbance; MTT reduction results were expressed as the percentages of control group.

Statistics

All the experiments on cultured cells were performed at least in triplicate. In 2-DE analysis, only the spots showing at least a 2-fold change between DOX-treated and control groups in intensity gels were defined as differently expressed spots. MTT reduction data are presented as mean□standard deviation (SD). Student's t tests were used to analyze the significance of differences. $p < 0.05$ was considered as significant.

Results

To identify the changes in protein profiling induced by DOX, H9c2 cells were exposed to DOX at a clinically relevant concentration, then the extracted proteins were subjected to 2-DE. **Figure 1** shows representative 2-DE images of H9c2 cells exposed to DOX at 0h and 48 hours. Gel analysis revealed 10 reproducible protein spots with more than 2-fold change in density during DOX treatment. As showed in **Table 1**, the expression of 6 proteins (Gamma-enolase; Annexin A5; Heterogeneous nuclear ribonucleoprotein H; Proteasome subunit beta type-3; Stress induced phosphoprotein 1; T-complex protein 1 subunit alpha) were significantly increased, whereas 3 proteins (Endoplasmic reticulum protein ERp29; 60 kDa Heat shock protein; Prohibitin) were significantly down-regulated in DOX-treated H9c2 cells.

To further confirm the results of proteomics, we carried out western blot analysis of ERp29 and prohibitin. As seen in **Figure 2**, we found that DOX decreased the expression of prohibitin and ERp29 in H9c2 cells during DOX induction, which in accordance with the results of 2-DE.

To determine whether ERp29 could attenuate cardiomyocytes toxicity induced by DOX, ERp29 was over-expressed in H9c2 cells by transfection. MTT reduction was measured after ERp29 were over-expressed in H9c2 cells; the results showed that over-expression of ERp29 partially recovered the MTT reduction (**Figure 3**).

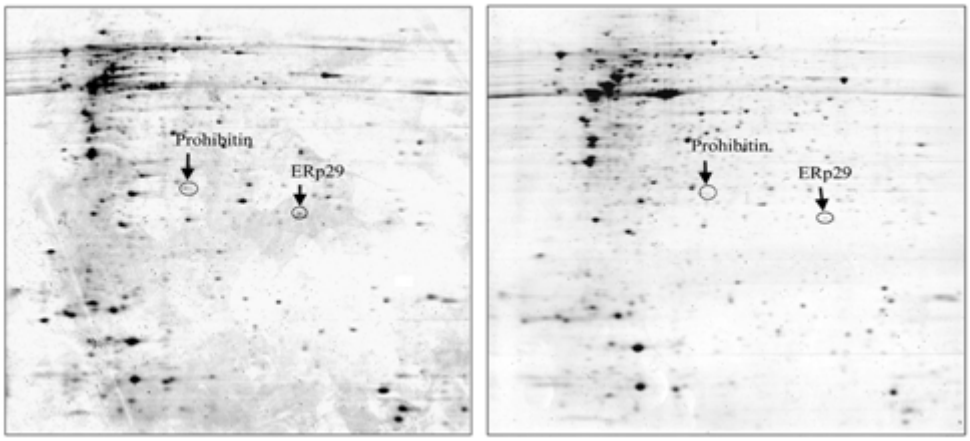

Figure 1. Representative 2-DE maps and differentially regulated proteins in H9c2 cells after treated with 0.5 μM doxorubicin for 48 hours compared with control.

Table 1. Differently expressed proteins in H9c2 cells identified by 2-DE and mass spectrometry.

Protein name	UniProt accession	MW (Da)	pI	Gene Oncology annotation	Mascot Score	Sequence Coverage (%)
Up-regulated proteins						
Gamma-enolase	P07323	47510	5.03	(GO:0042493) Response to drug	80	34
Annexin A5	P14668	35744	4.91	(GO:0043065) Positive regulation of apoptosis	78	20
Heterogeneous nuclear ribonucleoprotein H	Q8VHV7	49188	5.70	(GO:0006397) mRNA processing (GO:0051603) Proteolysis	76	20
Proteasome subunit beta type-3	P40112	23235	6.15	involved in cellular protein catabolic process	116	44
Stress-induced-phosphoprotein 1	O35814	62570	6.40	(GO:0051087) (HSP70) binding (GO:0051082) Unfolded protein	70	20
T-complex protein 1 subunit alpha	P28480	60359	5.86	binding; (GO:0005524) ATP binding	125	28
Down-regulated proteins						
Endoplasmic reticulum protein ERp29	P52555	28574	6.23	(GO:0006950) Response to stress; (GO:0005515) Protein binding	68	33
60 kDa Heat shock protein	P63039	60955	5.91	(GO:0006916) Anti-apoptosis (GO:0006950) Response to stress;	116	25
Prohibitin	P67779	29820	5.57	(GO:0006950) Negative regulation of apoptosis	170	63

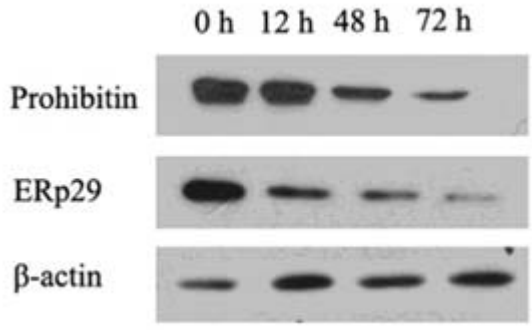

Figure 2. Western blot analysis confirmed the level of prohibitin and ERp29 expression decreased after treated with doxorubicin.

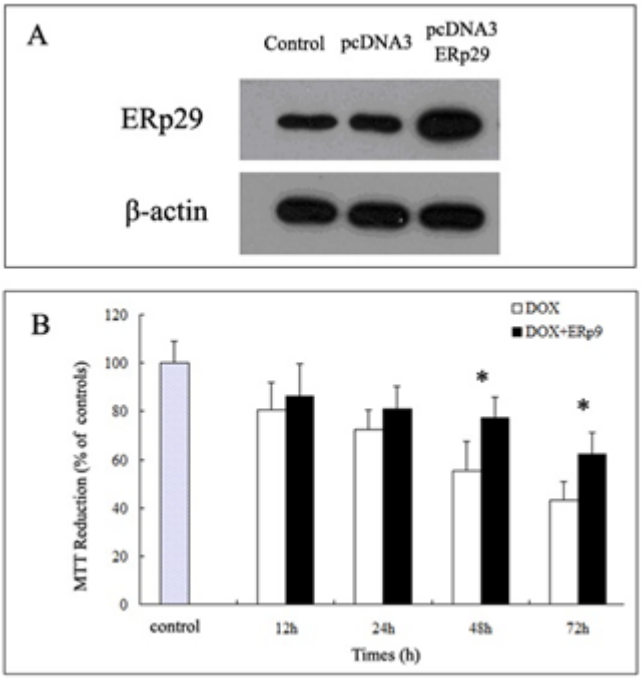

Figure 3. The effect of ERp29 overexpression on doxorubicin-induced toxicity in H9c2 cells measured by MTT reduction analysis at the indicated times. **A**: Western-blot analysis of ERp29 overexpression in H9c2 cells. **B**: Overexpression of ERp29 partially recovered the MTT reduction induced by doxorubicin in H9c2 cells. *$p < 0.05$.

Discussion

In this study, the global proteomic profiling of rat cardiomyocyte H9c2 cells before and after DOX treatment at a clinical relevant concentration were compared. The results showed a slightly different set of protein changes, including 6 up-regulated proteins and 3 down-regulated proteins. The identified proteins involved in various aspect of response to stress, oxidation-reduction process, and apoptosis, which is closely associated with DOX-induced cardiotoxicity. Part of above proteins has already been described in previous studies on DOX-induced cardiomyopathy. For example, stress induced phosphoprotein 1 was identified to be up-regulated in DOX-treated isolated rat cardiomyocytes, whereas proteasome subunit alpha type 3 has been found to be overexpressed in DOX-treated rat heart tissues in a previous proteomic study [5]. Shan et al. [8] reported that overexpression of Heat shock protein 60 (Hsp60) increased the expression of the anti-apoptotic Bcl-xl and Bcl-2, reduced the expression of the pro-apoptotic Bax and BAD, and also inhibited ubiquitination of Bcl-xl. Therefore, Hsp60 might exhibit a cardiac protective effect on doxorubicin-induced cardiomyopathy.

Meanwhile, our study also found several novel DOX-associated proteins such ERp29 and prohibitin, which might be valuable as candidate targets for treating cardiomyopathy. We further confirmed that the expression of ERp-29 and prohibitin decreased in H9c2 cells during DOX-treatment, which is in accordance with the results of our proteomic analysis.

The generation of hydrogen peroxide may play an important role in DOX cardiotoxicity. Recent studies indicated that prohibitin protect cell injury induced by hydrogen peroxide in cardiomyocytes [9]. Most recently, Muraguchi et al. [10] also demonstrated that prohibitin protects H9c2 cardiomyocyte against cell death under hypoxia stress status. Therefore, we proposed that up-regulating prohibitin expression in cardiomyocytes might be a novel strategy to alleviate DOX cardiotoxicity.

ERp29 is an endoplasmic reticulum (ER) lumenal protein involving in the folding/export of secretory proteins [11]. Accumulated evidence has implicated that the expression of ERp29 was increased and associated with resistance to oxidative stress, suggesting a potential protective role against stress [12, 13]. Zhang et al. [14] indicated that over-expression of ERp29 attenuates the doxorubicin cytotoxicity in breast cancer cells by up-regulating expression of Heat shock protein 27 (Hsp27) through

modulating eukaryotic translational initiation factor 2□ (eIF2□). Several studies have demonstrated that Hsp27 could reduce oxidative stress-induced apoptosis in cardiomyocytes, and over-expression of hsp 27 could attenuate doxorubicin-induced cardiotoxicity in vitro and in vivo [15, 16]. However, the role of ERp29 in DOX cardiotoxicity has not been investigated. In this present study, we found over-expression of ERp29 would also protect the H9c2 cells against cell toxicity induced by DOX. This finding, together with the data of the previous studies, suggests that ERp29 might play a protective role in preventing DOX-triggered cardiomyopathy.

In conclusion, in this study, we demonstrated the protein changes in DOX-induced apoptosis in H9c2 cells by proteomics approach. We first identified ERp29 and prohibitin as novel markers for DOX toxicity, and ERp29 exhibits a protective effect during DOX-triggered apoptosis, indicating a potential target to develop novel therapeutic strategies to alleviate adverse effects of doxorubicin-based chemotherapies.

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