Brief Report

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Quantitative proteomic study identified cathepsin B associated with doxorubicin-induced damage in H9c2 cardiomyocytes

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Summary **The study was performed to analyze the proteomic profiling of doxorubicin-treated H9c2 cardiomyocytes in order to identify novel protein biomarkers associated with doxorubicin-induced cardiomyopathy. The protein profiling of H9c2 cells in response to doxorubicin at an apoptosis-induced concentration of 0.5 μM were compared using iTRAQ analysis. Western-blot analysis was used to confirm differentially expressed proteins identified in the proteomic study. A total of 22 differently expressed proteins were identified in doxorubicin-treated H9c2 cells including 15 up-regulated and 7 down-regulated proteins. Gene Ontology (GO) analysis revealed that 10 altered proteins were enriched in the process of apoptosis. We further validated the expression of cathepsin B and its possible regulator nuclear factor kappa B (NF-κB) in H9c2 cells were increased during doxorubicin treatment using Western-blots. Differentially expressed proteins might provide clues to clarify novel mechanisms underlying doxorubicin-induced cardiomyopathy. Our results also suggest that increased cathepsin B expression might be associated with NF-κB up-regulation, and the exact mechanisms need to be clarified.**

Keywords: Doxorubicin, cardiomyopathy, proteomics, cathepsin B, nuclear factor kappa B

1. Introduction

Doxorubicin is one of the most effective chemo-therapeutic agents, and is indicated for many cancers including breast cancers, lung cancers, and ovary cancers. However, the optimal clinical use of this agent is limited by its major adverse effect of cardiomyopathy which leads to congestive heart failure, which would cause a mortality of approximately 50% (*1,2*). Extensive research has suggested several possible mechanisms for its cardiomyopathy including apoptosis, oxidative stress, inactivated cardiomyocyte specific genes, and altered molecular signaling (for example, MAPKs) (*3*). However, unfortunately, no effective treatment for established doxorubicin cardiomyopathy is available. Therefore, there is a great need to identify novel targets to protect the heart from doxorubicin damage.

Recent developments in proteomic technology provide an opportunity for the discovery of novel molecular pathways and biomarkers of drug-induced toxicity. Especially, in a recent proteomic study using the common two-dimensional electrophoresis (2-DE) and mass spectrometry technologies, Kumar *et al.* (*4*) identified several oxidative stress response-related proteins differently regulated in rat cardiomyocytes and heart tissues exposed to doxorubicin. Compared with the traditional proteomic methods such as 2-DE

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gel analysis followed by MS/MS, a stable-isotope labeled strategy iTRAQ-coupled 2-D LC-MS/MS approach takes advantage of higher detection sensitivity and quantitative accuracy, and holds the promise of effectively detecting a cellular protein profile in response to drugs (*5*).

Therefore, in this present study, we performed a differential proteomic analysis on doxorubicin-treated H9c2 cardiomyocytes using iTRAQ technology in order to identify novel biomarkers or potential targets for treatment of doxorubicin-induced cardiomyopathy.

2. Materials and Methods

2.1. *Cell culture and treatment*

Cardiomyocyte cell line H9c2 derived from embryonic rat heart by selective serial passages was obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China) with the original source as American Type Culture Collection (ATCC) (Manassas, VA, USA). H9c2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco, Carlsbad, CA, USA) with 10% fetal calf serum (Gibco) at 37°C with 5% CO_2. H9c2 cells were treated with doxorubicin (Sigma-Aldrich, St. Louis, MO, USA) at a clinically relevant concentration of 0.5 μM as described previously (*6*).

2.2. *Flow cytometric analysis of 0.5 μM of doxorubicin on apoptosis of H9c2 cells*

H9c2 cells without or with doxorubicin treatment at different time courses were collected and washed in PBS. After washing and staining with annexin V-FITC/propidium iodide (PI), the apoptosis rate of H9c2 cells were detected using flow cytometry as directed by an Annexin V-FITC/PI Kit (Nanjing Kaiji Biological Inc., Nanjing, China).

2.3. *Protein digestion and labeling with iTRAQ reagents*

Protein extraction at 48 h was performed as described previously (*7*). Briefly, H9c2 cells treated with and without doxorubicin for 48 h were dissolved in lysis buffer (7 M urea, 2 M thiourea, 65 mM DTT, 0.1 mM PMSF) and protein concentrations were determined using the 2D Quant Kit (GE Healthcare, Uppsala, Sweden). Protein trypsin digestion and iTRAQ labeling were performed in accordance with the manuscript's protocol (Applied Biosystems, Carlsbad, CA, USA). After reduction and alkylation, 100 μg protein of each sample were digested with trypsin and labeled with iTRAQ reagents as follows: H9c2 cells treated with doxorubicin, iTRAQ reagent 113; and H9c2 cells treated without doxorubicin, iTRAQ reagent 115. The labeled digests were then combined for peptide separation.

2.4. *Trypsin peptides fraction and mass spectrometry*

The mixed peptides were fractionated using strong cation exchange (SCX) chromatography into 10 fractions. The peptides in these fractions were further separated using a C18 column on a Tempo LC nano-flow system, and spotted on stainless steel target plates using a MALDI spotting system (Applied Biosystems). Mass spectrometer 4800 MALDI TOF/TOF Analyzer (Applied Biosystems) was used to perform tandem mass spectrometry acquisition in the positive ion mode. Only the most intense ion signals with S/N above 40 and excluding common trypsin autolysis peaks were selected as precursors for MS/MS acquisition. The MS/MS data were searched using the MASCOT Database search engine (Matrix Science, London, UK) embedded into GPS-Explorer Software (Applied Biosystems) against the Swiss-Prot database with the following search parameters: peptide tol: 0.3 Da, MSMS tol: 0.6 Da, variable modification: oxidation (M), fixed modification: carboxymethyl (C). Proteins were identified with at least a 95% confidence level, and relative quantification of proteins was based on the ratio of peak areas of m/z 113 and 115 from the MS/MS spectra.

2.5. *Gene ontology (GO) analysis*

GO analysis was performed with the GSEABase package from the R statistical platform (*http://www.r-project.org*).

2.6. *Western-blot*

The iTRAQ analysis has been performed only once in this study, to confirm the expression variation of cathepsin B in H9c2 cells during doxorubicin treatment, and we further performed a Western-blot experiment in another independent series of samples, which is not the same as those used in the proteomic study. Whole cell extracts and nuclear protein extracts were prepared using a protein extraction kit (Beyotime Bio-tech, Haimen, China) according to the manufacturer's protocol. Thirty μg of the extracted protein samples were separated using 12% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Primary antibody incubation was carried out overnight at 4°C using mouse monoclonal antibodies against cathepsin B (1: 1000, Abcam, Cambridge, UK) and nuclear factor kappa B (NF-κB) p50 (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Membranes were then probed with the corresponding IgG-HRP. Protein bands were visualized using enhanced chemiluminescence reagents (Milipore, Billerica, MA, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control.

3. Results and Discussion

As seen in Figure 1, our data indicates 0.5 μM doxorubicin exerted a considerable apoptosis-inducing effect on H9c2 cardiomyocytes in a time-dependent manner. We then applied an iTRAQ-based proteomic strategy to assess differential expression of H9c2 cells affected by doxorubicin at 48 h on the proteomic level. Proteins giving tryptic peptides with a 113/115 ratio ≥ 2 were classified as up-regulated and a ratio ≤ 0.5 as down-regulated. We confidently identified a total of 212 non-redundant proteins in the iTRAQ analysis. Twenty-two differentially expressed unique proteins were revealed including 15 up-regulated and 7 down-regulated proteins. The protein and gene names, database ID, molecular weight, isoelectric point (pI), and fold changes of identified proteins are listed in Tables 1 and 2. GO analysis on apoptosis related biological process revealed that 3 up-regulated genes (*Anxa1, Cryab, Lmna*) and 6 down-regulated genes (*Hspa5, Phb, Hspb1, Hspd1, Vdac1, Pdia3*) were enriched in the process of apoptosis (Figure 2).

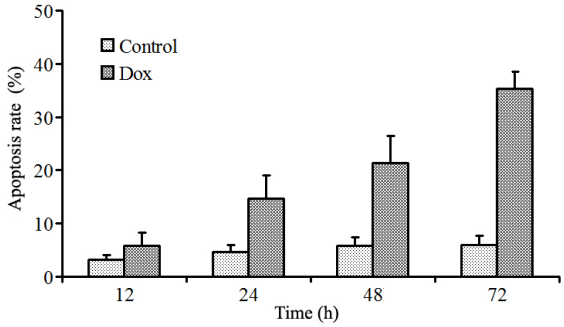

Figure 1. Flow cytometric analysis of 0.5 μM doxorubicin on apoptosis of H9c2 cells.

Of the differentially expressed proteins, cathepsin B (with two matched peptides: GENHCGIESEIVAGIPR and SGVYKHEAGDVMGGHAIR, peptide coverage of 11%), which had not been associated with doxorubicin-induced cardiac toxicity identified in previous studies, was selected to be further confirmed by Western-blot analysis. As seen in Figure 3, the expression of cathepsin B in H9c2 cells was increased during doxorubicin treatment and peaked at 48 h, which was in accordance with the proteomic results. Furthermore, expression of possible cathepsin B regulator NF-κB p50 protein in the nucleus increased after doxorubicin induction, which is consistent with the variation of cathepsin B.

In the current study, we quantitatively compared the global proteomic profiling of rat cardiomyocyte H9c2 cells with and without doxorubicin addition at a clinical relevant concentration, which exhibited an apoptosis-inducing effect, but not significant cellular damage in H9c2 cells (*6,8*). Our proteomic analysis revealed a series of proteins varied in cardiomyocytes after doxorubicin treatment for 48 h, in which 10 differentially regulated proteins were enriched in the biological process of apoptosis, which have been

Table 1. List of the up-regulated proteins identified in the iTRAQ experiments

No.	Accession number	Protein name	Gene	Molecular weight (Da)	pI	Ratio
1	P23928	α-Crystallin B chain	*Cryab*	20,088	6.76	2.3
2	P07150	Annexin A1	*Anxa1*	38,829	6.97	3.1
3	P55260	Annexin A4	*Anxa4*	35,848	5.30	2.1
4	P04639	Apolipoprotein A-I	*Apoa1*	30,062	5.52	2.6
5	P15999	ATP synthase subunit α, mitochondrial	*Atp5a1*	59,753	9.22	4.4
6	P00787	Cathepsin B	*Ctsb*	37,469	5.36	3.6
7	P48675	Desmin	*Des*	53,456	5.21	2.2
8	P04764	α-Enolase	*Eno1*	47,127	6.16	3.1
9	O35814	Heat shock cognate 71 kDa protein	*Stip1*	70,871	5.37	2.0
10	P48679	Lamin A/C	*Lmna*	72,418	6.41	2.7
11	Q9ER34	Aconitate hydratase, mitochondrial	*Aco2*	85,433	7.87	3.7
12	Q68FX0	Isocitrate dehydrogenase subunit β, mitochondrial	*Idh3B*	42,353	8.89	3.5
13	O35814	Stress-induced-phosphoprotein 1	*Stip1*	62,570	6.40	6.2
14	P48721	Stress-70 protein, mitochondrial	*Hspa9*	73,857	5.97	2.3
15	A9YUA5	Cardiac troponin T2	*Tnnt2*	34,452	5.19	3.1

Table 2. List of the down-regulated proteins identified in the iTRAQ experiments

No.	Accession number	Protein name	Gene	Molecular weight (Da)	pI	Ratio
1	P63039	60 kDa heat shock protein, mitochondrial	*Hspd1*	60,955	5.91	0.32
2	P06761	78 kDa glucose-regulated protein	*Hspa5*	72,347	5.07	0.21
3	P60711	Actin, cytoplasmic 1	*Actb*	41,737	5.29	0.14
4	P11598	Protein disulfide-isomerase A3	*Pdia3*	56,623	5.88	0.19
5	P42930	Heat shock protein β-1	*Hspb1*	22,893	6.12	0.41
6	P67779	Prohibitin	*Phb*	29,820	5.57	0.31
7	Q9Z2L0	Voltage-dependent anion-selective channel protein 1	*Vdac1*	30,756	8.62	0.11

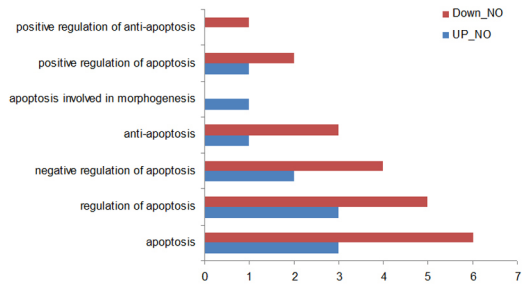

Figure 2. GO annotation on the apoptosis biological process on up-regulated proteins (UP) and down-regulated proteins (Down) identified in the proteomic experiments.

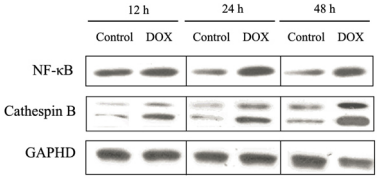

Figure 3. Western blot analysis detected the level of cathepsin B and nuclear NF-κB p50 expression in H9c2 cells during doxorubicin (DOX) treatment.

proved to be closely associated with doxorubicin-induced cardiac toxicity in previous studies. Among these aberrantly regulated proteins, several proteins such as cardiac troponin-T, prohibitin, and HSP60 have already been described to be involved in the generation of doxorubicin-induced cardiomyopathy (*6,9-11*).

We selected cathepsin B as a novel candidate doxorubicin-associated protein for the further validation and function study for the following reasons. First, this protein in H9c2 cells increased significantly in the proteomic study. Secondly, the role of cathepsin B in the pathogenesis of doxorubicin-induced cardiomyopathy has not been reported. However, the evidence in cancer cells indicates that cathepsin B is a crucial component in doxorubicin-induced apoptosis. For example, Bien *et al*. (*12*) found that cathepsin B could mediate doxorubicin-induced cell death by the mechanisms of loss of activating caspase 3, cleavaging PARP and inhibiting cdk1 *et al*. in tumor cells. Our Western-blot analysis indicated that doxorubicin induced a time-dependent up-regulation of cathepsin B protein increase in H9c2 cells, which is in accordance with those of our proteomic analysis. This finding suggests cathepsin B is a key regulatory component in doxorubicin-induced cardiomyopathy, which might be similar to its modulatory role in doxorubicin-induced tumor cell death. Bien *et al*. (*12*) also found that doxorubicin induces cathepsin B expression and activity *via* NF-κB in tumor cells, and Li *et al*. (*13*) found that expression of NF-κB p50 protein in the nucleus and NF-κB binding activity increased significantly in cardiomyopathy induced by adriamycin. In this study, we also demonstrate that doxorubicin induced cathepsin B expression was accompanied by up-regualtion of NF-κB. Recently, Qiao *et al*. (*14*) also found a co-occurrence of NF-κB activation and cathepsin B up-regulation in delayed anesthetic preconditioning protection against myocardial infarction. Together with the data of the previous studies, our findings suggest that up-regulation of cathepsin B by doxorubicin in cardiomycytes might be associated closely with NF-κB, and the exact mechanisms still deserve further investigation.

Cathepsin B is a prominent lysosomal protease, and plays an important role in the apoptosis process and myocardial damage (*15*). Tsuchida *et al*. (*16*) demonstrated that cathepsin B was involved in degradation of myofibrillar proteins in myocardial infarction. Ge *et al*. (*17*) also found elevated cathepsin B expression might modulate myocardial apoptosis in patients with dilated cardiomyopathy. Therefore, we proposed that cathepsin B could be increased by doxorubicin, and act as a potent apoptosis inducer during the following myocardial toxicity. However, the exact mechanisms of cathepsin B in doxorubicin induced cardiomyopathy remain not fully elucidated, and deserve further investigation.

Considering its role in doxorubicin-induced tumor cell death, it has been proposed that increasing cathepsin B expression could be a novel strategy to modify susceptibility towards doxorubicin (*18*). But our results indicated that up-regulated cathepsin B would also aggravate cardiomyopathy. Therefore, when cathepsin B is considered as an attractive target for sensitizing chemotherapy in cancer, its potential cardiac toxicity must be evaluated carefully first.

In conclusion, in this present study, using iTRAQ-based proteomic methods, we identify a panel of differentially expression proteins related to doxorubicin treatment in cardiomyocytes, which might provide clues to clarify novel mechanisms underlying doxorubicin-induced cardiomyopathy. Among these proteins, cathepsin B expression was further confirmed by Western-blots, and our results also suggest that increased cathepsin B expression might be associated with the up-regualtion of NF-κB in doxorubicin-induced cardiomyopathy.

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