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Quantitative Proteomics for Cardiac Biomarker Discovery Using Isoproterenol-Treated Nonhuman Primates

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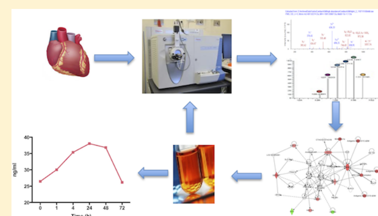
S Supporting Information

ABSTRACT: To identify new cardiac biomarkers, a quantitative proteomic analysis has been performed on serum and heart tissue proteins from three species of nonhuman primates following isoproterenol (ISO) treatment. Three serum proteins—serum amyloid A (SAA), α-1-acid glycoprotein (A1AG), and apolipoprotein A-1 (Apo A1)—were consistently identified as changed and remained altered 72 h post dose in all three species post ISO treatment, indicating the potential of including these proteins in preclinical or clinical evaluation of drug-induced cardiac injury. Furthermore, proteomic analysis of heart tissue proteins following ISO treatment demonstrated detrimental effects on calcium signaling and energy generation in cardiac myocytes. It is worth noting that cardiac troponins were not identified in serum but were identified as altered in heart tissue lysate along with other cardiac-specific proteins. This strategy for cardiac biomarker discovery by proteomic screening of heart tissue proteins, followed by verification in serum samples using immunoassays or targeted mass spectrometry, could be applied in future biomarker studies.



KEYWORDS: *tandem mass tag, proteomics, cardiotoxicity, serum biomarkers, isoproterenol, Cynomolgus macaque, Rhesus macaque, African green monkey*

INTRODUCTION

Cardiotoxicity and associated drug failures in clinical development or even withdrawals from the market contribute significantly to the rising cost of drug discovery and development in the pharmaceutical industry.[1] It has been reported that 28% of drug withdrawals in the USA are associated with negative cardiovascular events, and because of amortized drug failures, the cost of developing a new drug has been estimated to be as high as $5 billion.[2,3] Traditionally used biomarkers of cardiovascular diseases (CVD), however, have been proven unreliable in predicting cadiotoxicity in a number of settings. For example, although highly active antiretroviral therapy (HAART) has reduced AIDS-related deaths, CVD and CVD-related deaths have increased in the HIV-infected population by 1.5−2 times in comparison with HIV-negative individuals.[4−6] Yet, there is a lack of safety biomarkers addressing both drug-induced and immune-associated CVD in therapeutics for HIV. One of the strategies to address this problem is to use improved safety biomarkers to predict drug cardiotoxicity before advancing to human clinical trials. In particular, biomarkers that can reliably predict drug-induced cardiac injury in animals will decrease costs by excluding nonviable drug candidates and identifying those that have a greater probability of success in clinical trials.

Serum biomarkers provide a noninvasive measure for diagnosing cardiac injury. Numerous articles have reported the use of myocardium-specific proteins as biomarkers for congestive heart failure, myocardial infarction and other heart diseases. These cardiac biomarkers include the cardiac troponins I and T, cardiac natriuretic peptides, creatine kinase isoenzyme MB (CK-MB), and lactate dehydrogenase (LDH).[7−9] Among the biomarkers, cardiac troponins are perhaps the gold standard for cardiac injury, considering their sensitivity and specificity; however, the troponins have a half-life of ∼2 h, which makes them difficult to use for routine evaluation in toxicology studies.[8] One major problem associated with these current biomarkers is that they typically show significant changes only in the first 12−24 h postdose, returning to normal levels after 24 h.[10,11] Therefore, there is an urgent need for reliable cardiac biomarkers that can predict drug cardiotoxicity over longer periods of time and be incorporated in routine toxicology studies.

Acute myocardial infarction, the most common cause for CVD-related death, was found showing increased risk among HIV-positive people.[5] In our study, isoproterenol (ISO), a beta-adrenergic agonist used to treat bradycardia or heart block, has been used as a model compound to induce myocardial infarct-like lesions. Various studies have been conducted to evaluate myocyte damage following exposure to this drug in rat and monkey species.[12−15] We previously reported a panel of nine serum proteins showing significant change at 48 and 72 h after ISO treatment in African green monkeys (AFG); these proteins can potentially be used for predicting long-term cardiac

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injury.[16] To corroborate our studies, we conducted similar protein profiling of serum proteins at different times after ISO injection in Cynomolgus macaques (CM) and Rhesus macaques (RM). The serum proteins showing long-term alterations were compared among all three nonhuman primate (NHP) species. ELISA and Western-blot assays were conducted for verification of selected proteins. Moreover, proteins from four different heart compartments (left ventricle, right ventricle, left atrium, right atrium) were systematically analyzed to elucidate the cellular mechanism of the drug-induced cardiac injury. Energy imbalance, altered calcium flux, formation of reactive oxygen species, and structural degradation have been implicated in the literature as mechanisms of ISO-induced cardiotoxicity in mouse and rat species using immunoassays and histopathological and immunohistochemical studies;[17,18] however, few studies have been conducted on the protein profile of injured heart tissue.

We conducted 2D LC−MS/MS quantitative proteomics analysis on serum proteins and heart tissue proteins from CM and RM. A filter-aided sample preparation (FASP) strategy was used for protein extraction and digestion for individual animals; tandem mass tag (TMT) was used for isotopic labeling of the digested peptides from different animals for quantitation purposes.[19,20] The yield and reproducibility of this protocol has been evaluated for technical and biological replicates in previous studies, and the results showed it is highly reproducible and reliable for protein identification and quantitation.[16] In this study, we applied a similar approach to analyze serum proteins from two other NHP models to continue the cardiac biomarker discovery and expanded the analysis to include tissue proteins from four different compartments to understand the cellular mechanism of drug-induced heart injury. To our knowledge, this is the first systematic quantitative proteomic analysis on four different heart compartments from NHP models.

■ EXPERIMENTAL PROCEDURES

Animal Information

A total of six non-naïve Cynomolgus macaques (*Macaca fascicularis*, Mauritius origin, 7−10 years of age, naïve to isoproterenol) and six non-naïve Rhesus macaques (*Macaca mulatta*, unknown origin, 10−13 years of age, naïve to isoproterenol) were assigned to the study. For each species, two male and two female non-naïve animals were administered isoproterenol hydrochloride (ISO, Sigma-Aldrich, St. Louis, MO) as a single subcutaneous (s.c.) dose of 4 mg/kg (concentration 2 mg/mL) on day 1. An additional one male and one female of each species were administered saline and served as vehicle control animals. Blood was collected predose, and at 1, 4, 24, 48, and 72 h postdose from each animal, processed to serum, and flash-frozen with liquid nitrogen. On day 4, animals were sedated with ketamine (Buttler Schein, CA) and euthanized with an overdose of sodium pentobarbital (Buttler Schein, CA). Slices ∼4 mm^3 thick from the left and right ventricular free walls and the left and right atria of each animal were placed into screw-cap vials and flash-frozen with liquid nitrogen. Serum and tissues were stored at −80 °C until analysis.

Proteomics Sample Preparation

Heart tissue samples were thawed on ice, weighed, and washed with phosphate buffered saline (PBS). For tissue protein extraction, ∼100 mg of heart tissue was minced into 2 mm pieces in 1 mL of lysis buffer (50 mM Tris, pH 7.4, 0.5% SDS, EDTA-free protease inhibitor cocktail). About 0.2 mL of glass beads (0.5 mm, Sigma-Aldrich, St. Louis, MO) was added to each sample. Subsequently, samples were homogenized five times for 1 min each by a Bullet-Blender (Next Advance, Averill Park, NY) at 4 °C and then left on ice for 1 h. Debris was removed by centrifugation at 12 000*g* for 15 min at 4 °C, and supernatants were stored at −80 °C for proteomics sample preparation. Total protein of the samples was measured using a Nanodrop 2000 (Thermo Scientific, San Jose, CA).

Both serum samples and tissue protein lysates were purified and digested using the FASP protocol developed by Mann's group.[19] Briefly, 200 μL of diluted (1:20 dilution ratio) serum or tissue lysate was transferred into a 1.5 mL Microcon YM-10 centrifugal unit (Millipore, Billerica, MA). Protein reduction, alkylation, and tryptic digestion were conducted in the centrifugal unit. After overnight tryptic digestion at 37 °C, the peptides were eluted twice with 150 μL of 50 mM ammonium bicarbonate. The total protein or peptide concentration in each step was measured using a Nanodrop 2000. The eluted peptides were dried by vacuum centrifugation and then resuspended in 50 mM tetraethylammonium bromide for TMT labeling. For each sample, 50 μg of digested peptides was incubated with an amine-reactive 6-plex TMT tag (Thermo Scientific, San Jose, CA) for 2 h at room temperature (RT). Reactions were quenched by adding 8 μL 5% hydroxylamine and incubated at RT for 15 min. The labeled peptides were then combined, and excess TMT tags were removed by a 3 × 8 mm strong cation exchange (SCX) trap column, then desalted by a 3 × 8 mm C18 reverse phase (RP) trap column (Bruker-Michrom, CA). Purified labeled peptides were dried by vacuum centrifugation and then resuspended in 0.1% formic acid (FA) for multidimensional protein identification technology (MudPIT) analysis.

MudPIT Conditions

Each TMT labeled sample was separated using a nano-LC system (Agilent 1200, Palo Alto, CA). Briefly, 15 μg peptide mixtures were separated first by an SCX column (self-packed, 100 μm × 100 mm, 5 μm, 300 Å polysulfethyl). Fractions were eluted stepwise with a nine-step ammonium formate concentration gradient (5, 15, 25, 50, 75, 100, 200, 500, and 1500 mM). Each salt step eluent was further separated on a capillary C_{18} RP column (75 μm × 150 mm, 3 μm particle size) with a 146 min gradient from 2 to 97% acetonitrile in 0.1% FA at a constant flow rate of 300 nL/min.

The peptides eluted from the C_{18} column were directly analyzed with an LTQ Orbitrap XL (Thermo Fisher Scientific, San Jose, CA) equipped with a nanoelectrospray ionization source. Full MS spectra were acquired in positive mode over a 350−1800 m/z range, followed by four CID (collision induced dissociation) and four HCD (higher-energy collisional dissociation) events on the four most intense ions selected from the full MS spectrum using a dynamic exclusion time of 30 s. Four CID scans (maximum inject time 100 ms, minimum signal threshold 500 counts, collision energy 35%, activation time 30 ms, isolation width 1.0 m/z) were used for peptide identification, and four corresponding HCD scans (maximum inject time 300 ms, minimum signal threshold 500 counts, collision energy 45%, activation time 30 ms, isolation width 1.0 m/z) were used for quantitation.

Database Search

Acquired tandem mass spectra were searched against the National Center for Biotechnology Information Macaca protein database. A decoy database containing the reverse sequence of all the proteins was appended to estimate false discovery rate (FDR).[21] The search was performed using the SEQUEST algorithm incorporated in Proteome Discoverer 1.3.0.339 (Thermo Finnigan, San Jose, CA), and a cutoff of 5% FDR was applied. The precursor mass accuracy was limited to 13 ppm, and fragment ion mass tolerance was set at 1.1 Da. Fully tryptic enzyme specificity and up to two missed cleavages were allowed. Fixed modifications included carbamidomethylation on cysteines, and variable modifications included oxidation on methionines and TMT adduction to peptide N-termini, lysines, and tyrosines. The FDR, calculated through a decoy database, was set as 0.05 using Percolator scoring with posterior error probability validation. For protein identification, only the first best-matching peptide was taken into consideration. Peptide quantitation was also performed in Proteome Discoverer 1.3.0.339 in a same workflow. A TMT 6-plex quantitation method was used for HCD-based quantitation. Mass tolerance was set at 150 ppm for reporter TMT tags. The intensity of each peptide was normalized to protein median intensity before calculating the ratio of different tags from the same peptides. Peptide quantitation data from both unique and nonunique peptides were averaged to determine the protein relative abundance at different time points versus predose.[22] A Student's t-test was applied on the proteins showing average abundance change >1.5-fold upon ISO treatment, and a cutoff of p-value < 0.05 was applied. Pathway and network analysis on changed heart tissue proteins was performed using Ingenuity Pathway Analysis software (Redwood City, CA).

ELISA and Western Blot Assays

Human C-reactive protein and pancreatic α-amylase ELISA kits were purchased from Abcam (Cambridge, MA). Monkey serum amyloid A, and α-1-acid glycoprotein ELISA kits were purchased from Kamiya Biomedical (Seattle, WA). All ELISA assays were conducted according to the manufacturers' protocols. Each serum sample was tested in duplicate, and the average value was calculated and plotted graphically. For Western blot assays, cardiac troponin I antibody and β-2-microglobulin antibody were purchased from Abcam (Cambridge, MA). Cytochrome c1 (CYC1) antibody was purchased from Proteintech Group Inc. (Chicago, IL). β-Actin antibody purchased from Abcam was used as a loading control. Briefly, 50 μg of proteins was run on 4−20% precast polyacrylamide gel (Bio-Rad, Hercules, CA) and transferred to nitrocellulose membranes. Membranes were blocked with Odyssey Blocking Buffer (LI-COR, Lincoln, NE), followed by incubation with primary antibodies at 1:1000 dilutions. Membranes were washed three times with 1X PBS containing 0.05% Tween20 (v/v), incubated with IRDye secondary antibodies (LI-COR, Lincoln, NE) for 1 h, and washed again to remove unbound antibody. Odyssey CLx (LI-COR, Lincoln, NE) was used to detect bound antibody complexes.

RESULTS

Cardiotoxicity Evaluation

Monkeys treated with ISO demonstrated a variety of adverse cardiac effects. The cardiac effect of ISO in AFG has been previously reported.[16] Heart rate for CM, as measured by electrocardiograms (ECG), decreased within 1 h and in general remained below control and prestudy values for 72 h. At 24 h postdose, heart rate was 34.7% and 22% lower than the control group for males and females, respectively (Figure 1). Cardiac abnormalities observed in CM during the first 24 h postdose included hypotension; occasional ventricular premature complexes; ventricular bigeminy; atrial premature complexes, with or without aberrant conduction; and ST segment elevation. A majority of the treatment-related findings were noted within 15−60 min or 45−65 min. Heart rates for RM were also measured by ECG. These increased within 1 h post-treatment and remained elevated for up to 24 h, then dropped and remained below the control and prestudy values for the remainder of the study (Figure S1, Supporting Information).

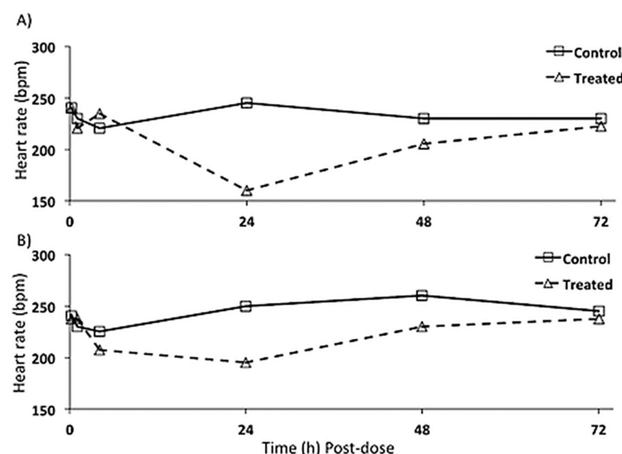

Figure 1. Heart rates of (A) male and (B) female Cynomolgus macaques following a single s.c. administration of 4 mg/kg ISO. Heart rates were measured by ECG at time 0 (immediately prior to dose administration), 15 min, 1, 4, 24, 48, and 72 h postdose.

Serum Protein Identification and Quantitation

A total of 375 proteins were identified and quantified in CM serum samples. The 0 h time point was used as control, and the ratios of the intensity of the reporter ions associated with other time points (1, 4, 24, 48, 72 h) versus the reporter ion of 0 h time point were acquired as relative peptide abundance. Table 1 shows that after ISO injection, seven proteins were significantly up-regulated, and three proteins were down-regulated in CM serum (Table S2, Supporting Information). These effects lasted through 72 h, except pancreatic α-amylase isoform 2, which still maintained a high level of concentration at 48 h post dose but dropped to baseline at 72 h. Data on protein relative abundance was acquired via the ratio of intensity of reporter ions from HCD spectra (Figure S3, Supporting Information). All the CID spectra of selected proteins were manually inspected (Figure S4, Supporting Information) to ensure accurate identification.

Of the 10 proteins showing significant alteration in CM serum, 5 were also detected as significantly changed in AFG serum after ISO injection. They are α-1-acid glycoprotein 1-like isoform 2 (A1AG), C-reactive protein isoform 2 (CRP), fructose-bisphosphate aldolase A isoform 1 (FBAA), serum amyloid A protein-like isoform 1 (SAA) and apolipoprotein A-1 isoform 1 (Apo A1). To further assess the potential of these proteins to serve as biomarkers for evaluating heart injury, we tested the serum samples from four RMs after ISO injection. Serum samples were taken at 4 and 72 h post dose, and the

Table 1. Proteins Demonstrating Significant Change (>2-Fold) in Cynomolgus Macaque Serum after Isoproterenol Treatment[a]

accession	description	0 h	1 h	4 h	24 h	48 h	72 h
109110480	α-1-acid glycoprotein 1-like isoform 2	1.00	1.00	1.09	1.98	1.97	1.53
297279399	pancreatic α-amylase isoform 2	1.00	1.24	4.69	7.22	2.12	1.17
297298533	α-1-antichymotrypsin isoform 2	1.00	1.18	1.23	2.98	2.98	2.37
109017524	c-reactive protein isoform 2	1.00	1.16	2.20	16.09	5.28	2.61
109128138	fructose-bisphosphate aldolase A isoform 1	1.00	1.09	1.38	2.89	3.16	2.67
109107123	serum amyloid A protein-like isoform 1	1.00	1.25	1.28	8.95	5.00	2.48
297296595	tropomyosin α-4 chain	1.00	1.82	2.44	4.12	4.82	2.71
109108768	apolipoprotein A-I isoform 1	1.00	0.97	0.95	0.76	0.60	0.54
109086845	carbonic anhydrase 1 isoform 1	1.00	1.13	0.86	0.38	0.33	0.40
109042272	fetuin-B isoform 1	1.00	0.86	0.79	0.64	0.40	0.48

[a]Values shown represent protein relative abundance over 0 h values averaged from four ISO-treated monkeys (males and females combined).

protein levels were compared with the sample from the same monkey taken predose. Three proteins—A1AG, SAA and Apo A1—were detected as significantly changed after ISO injection in all three species (Figure 2). The level of SAA reached the peak at 24 h post dose and still maintained a high level (5-fold increase) at 48 h in CM. In AFG, SAA showed its highest level at 48 h post dose, which was about a 7-fold increase compared with predose. Because the 24 and 48 h time points were not analyzed in RM, we cannot tell at which point SAA showed maximum concentration; however, at 72 h post dose, SAA still showed as much as a 10-fold increase. The other two proteins, A1AG and Apo A1, also showed consistent increases or decreases in all three species after ISO injection.

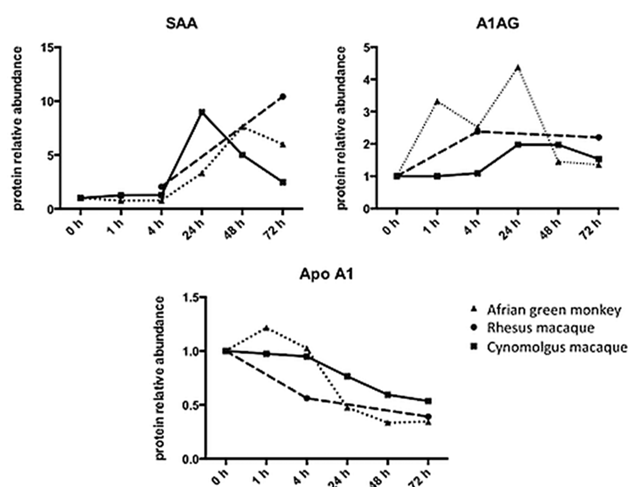

Figure 2. Proteins showed significant change after ISO injection in serum in all three species (African green monkey, Rhesus macaque, and Cynomolgus macaque). The protein relative abundances over 0 h (predose) were determined by LC−MS/MS.

ELISA Validation of Serum Proteins

Four serum proteins—CRP, SAA, A1AG, and pancreatic α-amylase (PA)—were selected for ELISA validation in CM (Figure 3 and Table S5, Supporting Information). SAA and A1AG were confirmed to be significantly changed after ISO injection in all three species and were also validated in RM sera by ELISA (Table S6, Supporting Information). CRP was on the list of changed proteins in both AFG and CM studies. CRP and SAA have been previously reported as acute-phase proteins, and their levels are often correlated.[23] Previous studies showed that elevated levels of CRP and SAA can be correlated with higher

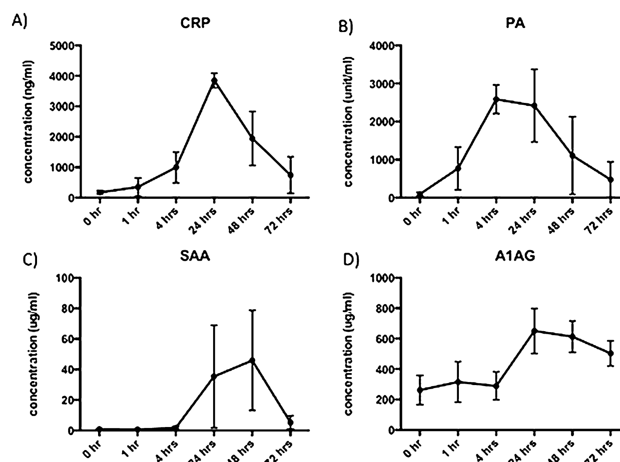

Figure 3. ELISA validation of (A) C-reactive protein; (B) pancreatic α-amylase; (C) serum amyloid A; (D) α-1-acid glycoprotein in serum from four ISO-treated Cynomolgus macaques.

mortality rates in patients with acute coronary syndromes, even in the patients with negative rapid cardiac troponin T (cTnT) assay.[24,25] Quantitative CRP and SAA can thus provide additional prognostic information for predicting cardiac necrosis beyond the information provided by cTnT alone. Serum samples from four CM following ISO treatment were used for ELISA validation. Serum concentration of CRP increased from 173 ± 64 ng/mL at 0 h to the maximum concentration of 3846 ± 240 ng/mL at 24 h post dose. The concentration of SAA was also elevated significantly after ISO injection and reached its peak concentration at 48 h post dose (45 ± 32 μg/mL, which is about a 50-fold increase compared with the concentration at 0 h of 0.9 ± 0.09 μg/mL).

The serum concentration of A1AG, also an acute-phase protein, has been reported to increase in response to tissue injury, inflammation, infection, and cancer.[26,27] The ELISA results showed that A1AG increased from 262 ± 95 μg/mL at 0 h to 650 ± 147 μg/mL at 24 h post dose and stayed as high as 502 ± 81 μg/mL at 72 h post dose. The elevation of PA in serum after ISO treatment was also validated by ELISA. The concentration of PA increased significantly and stayed at the peak concentration (2500 U/mL) from 4 to 24 h post dose, which is ~25-fold higher than predose. PA can regulate the blood glucose level, and it has been used as a drug target for treating diabetes and obesity.[28] There are, as yet, no studies showing a direct correlation between PA elevation and cardiac injury, making this the first report of increased PA level in serum associated with heart injury. All the validated proteins

Table 2. Proteins Changed >1.5-Fold in Cynomolgus Macaque Left Ventricle 3 Days after Isoproterenol Treatment

accession	gene	description	Σ coverage	Σ no. unique peptides	fold change	*p* value
297279864	*SV2A*	synaptic vesicle glycoprotein 2A-like	1.34	1	−1.76	0.019
297275492	*CNDP2*	cytosolic nonspecific dipeptidase isoform 3	21.65	6	−1.76	0.010
109124398	*HSPB6*	heat shock protein beta-6-like isoform 2	21.25	3	−1.72	0.001
297278451	*WDR65*	WD repeat-containing protein 65-like	0.79	1	−1.60	0.001
109094852	*DDT*	d-dopachrome decarboxylase isoform 1	32.20	3	−1.58	0.001
284925118	*ACOT13*	acyl-coenzyme A thioesterase 13	25.00	3	−1.56	0.008
109087718	*CYC1*	cytochrome *c*1, heme protein, mitochondrial-like isoform 2	25.23	4	−1.51	0.015
109112231	*HSPA5*	78 kDa glucose-regulated protein isoform 2	29.66	15	1.52	0.048
297290660	*RPL10A*	60S ribosomal protein L10a-like	18.43	3	1.64	0.046
109071710	*EEF1A1*	elongation factor 1-α 1 isoform 9	33.98	6	1.65	0.046
109123592	*CALR*	calreticulin isoform 2	21.10	6	1.71	0.002
274324259	*LGALS1*	galectin-1	5.93	1	1.82	0.020
297287337	*IQCG*	IQ domain-containing protein G-like	3.39	1	1.83	0.005
109096389	*LOC1003597 63*	40S ribosomal protein S25-like	8.94	1	1.85	0.047
297273563	*GRIN2C*	glutamate [NMDA] receptor subunit epsilon-3-like	2.83	1	2.02	0.001
297279918	*SETDB1*	histone-lysine *N*-methyltransferase SETDB1	2.17	1	2.19	0.026
114051850	*B2M*	beta-2-microglobulin precursor	24.37	1	2.33	<0.001
297263747	*LRRC43*	leucine-rich repeat-containing protein 43-like	4.20	1	2.59	0.024
109084629	*TRIP11*	thyroid receptor-interacting protein 11	0.57	1	3.01	0.036

did not show any changes in the control animals, except for CRP, which showed elevation in one control animal but still lower than the protein level in the treated animals.

Protein Identification and Quantitation in Heart Tissue

To understand the cellular mechanism of drug-induced cardiac injury, heart tissue proteins from each CM sacrificed at 72 h post dose were analyzed to determine tissue component-specific protein changes. Each heart tissue was divided into four compartments: left ventricle, right ventricle, left atrium, and right atrium. Tissue proteins from each compartment were extracted, processed, and isotopically labeled. Six TMT tags were used to label six different macaques (four ISO-treated and two controls). Each sample was conducted in two technical replicates. A total of 1320 and 1335 proteins were identified and quantified with high confidence (FDR < 0.05) in the left ventricle and right ventricle compartments, respectively. The protein expression profiles in monkeys nos. 3, 4, 5, and 6 (ISO-treated) were compared with the vehicle-treated monkeys nos. 1 and 2. A Student's *t*-test was applied to screen the proteins showing an average abundance change >1.5-fold upon ISO treatment. Previous studies on ISO-induced cardiac toxicity in a rat model reported severe hypertrophy and myocardial necrosis in the left ventricle.[17] In our study, moderate myofiber degeneration was observed in the left ventricle of ISO-treated CM by histopathology (data not shown). The results of left ventricle protein profiling showed that the expression level of 12 proteins increased and 7 proteins decreased more than 1.5-fold in ISO-treated animals compared with the control with a *p*-value < 0.05 (Table 2 and Table S7, Supporting Information). Network analysis of the changed proteins in the left ventricle showed that their functions are associated with cardiac necrosis, cardiovascular system development and function, and cell death (Figure 4). Compared with the left ventricle, protein profiling showed fewer and less severe changes in the right ventricle, suggesting that ISO might have less deleterious effects on the right ventricle (Table S8, Supporting Information).

Interestingly, we observed the most ISO treatment-induced protein expression level changes in the right atrium compartment. Because of the limited amount of right atrium tissue sample from monkey no. 2, the right atrium protein profiling was performed only for monkeys 1, 3, 4, 5, and 6. Of the total 1140 identified proteins in the right atrium, 19 proteins were shown to be down-regulated, and 2 proteins were up-regulated in ISO-treated monkeys compared with the controls using a *p*-value <0.05 (Table 3 and Table S9, Supporting Information). Levels of atrial natriuretic factor (ANF), a polypeptide hormone secreted by the heart muscle cells in response to atrial stretch, were significantly increased in both left and right atria upon ISO injection. An enhanced plasma concentration of ANF has been reported in the literature as associated with cardiac dysfunction and has been suggested for diagnosis and risk stratification in heart failure.[7] We observed less and moderate protein changes in the left atrium than in the right atrium after ISO injection (Table S10, Supporting Information).

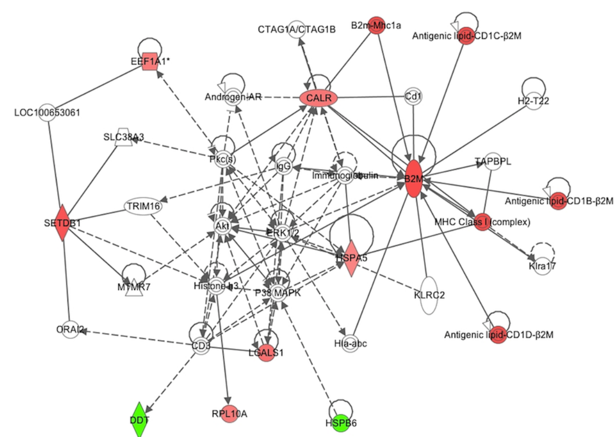

Figure 4. Network analysis of the changed proteins in Cynomolgus macaque left ventricles after ISO treatment: cardiac necrosis/cell death, cardiovascular system development and function, cell death, and survival. Proteins labeled red are up-regulated; proteins labeled green are down-regulated; proteins shown in gray are not identified in our data. The figure was generated using Ingenuity Pathway Analysis software.

Table 3. Proteins Changed >1.5-Fold in Cynomolgus Macaque Right Atrium 3 Days after Isoproterenol Treatment

accession	gene	description	Σ coverage	Σ no. of unique peptides	fold change	*p* value
108997197	*NPPA*	atrial natriuretic factor	5.88	1	4.89	0.025
108999269	*CLIC4*	chloride intracellular channel protein 4 isoform 3	5.14	1	2.25	0.012
109111058	*TPM2*	tropomyosin beta chain isoform 11	32.75	3	1.87	0.017
297265595	*HADHB*	a functional enzyme subunit beta, mitochondrial	36.67	16	−1.86	0.021
92110045	*UQCRC1*	cytochrome *b*−*c*1 complex subunit 1, mitochondrial	23.75	7	−1.88	0.014
109081387	*Tpm1*	tropomyosin α-4 chain isoform 17	65.49	15	−2.03	<0.001
297281135	*TNNT2*	troponin T, cardiac muscle-like isoform 6	23.89	5	−2.06	<0.001
109122723	*ATP5D*	ATP synthase subunit delta, mitochondrial	5.36	1	−2.15	0.005
297300460	*ATP5C1*	ATP synthase subunit gamma, mitochondrial-like isoform 1	18.61	4	−2.15	0.003
109093170	*PYGB*	glycogen phosphorylase, brain form isoform 1	28.80	13	−2.17	0.002
109066521	*ATP5B*	ATP synthase subunit beta, mitochondrial	59.17	3	−2.19	<0.001
109048544	*ATP5J2*	ATP synthase subunit f, mitochondrial-like isoform 2	27.27	2	−2.42	<0.001
109013523	*ATP5F1*	ATP synthase subunit b, mitochondrial isoform 1	6.25	2	−2.80	<0.001
297298685	*C14orf2*	6.8 kDa mitochondrial proteolipid	48.28	3	−2.81	<0.001
109126074	*TNNI3*	troponin I, cardiac muscle isoform 1	15.71	3	−3.00	<0.001
297285706	*CACNA2D2*	voltage-dependent calcium channel subunit α-2/δ-2-like	5.15	4	−3.42	<0.001
297297517	*MYH6*	myosin-6-like	50.13	87	−3.69	<0.001
109116524	*MYL4*	myosin light chain 4-like	49.74	10	−4.07	<0.001
94421002	*COX5A*	cytochrome c oxidase subunit 5A, mitochondrial precursor	11.33	2	−4.15	<0.001
109087718	*CYC1*	cytochrome *c*1, heme protein, mitochondrial-like isoform 2	4.92	1	−4.27	<0.001
297285630	*TNNC1*	troponin C, slow skeletal and cardiac muscles-like	7.21	1	−4.61	<0.001
109076348	*SLC25A4*	ADP/ATP translocase 1	21.81	3	−4.83	<0.001

Western-Blot Validation of Tissue Proteins

To verify the changed proteins identified in heart tissue upon ISO treatment, Western blot assays were conducted on three tissue proteins: cardiac troponin I (TNNI3), cytochrome c1 (CYC1), and β-2-microglubulin (B2M), from both the left ventricle and the right atrium compartments (Figure 5).

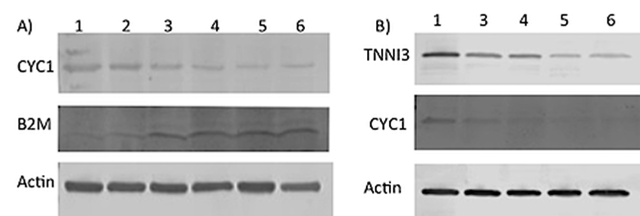

Figure 5. Western blot analysis of (A) CYC1 and B2M in left ventricle tissue lysate; (B) TNNI3 and CYC1 in right atrium tissue lysate. Actin was used as a loading control. Each lane was labeled with an animal number. Animals 1 and 2 were vehicle-treated; animals 3−6 were ISO-treated. Data show the CYC1 level decreased in both the left ventricle and right atrium of ISO-treated animal tissue lysate compared with vehicle-treated ones. The level of B2M elevated upon ISO administration in the left ventricle tissue lysate, whereas TNNI3 decreased after ISO treatment in the right atrium tissue lysate.

Dissociation and release of troponin from the myofibril has been reported in the literature after myocardial infarction, which result in the observed elevation of troponin serum level.[29] In our proteomic analysis, cardiac troponin I and T and troponin C were all identified as decreased in the right atrium tissue lysate, indicating dissociation of troponin from the myofibril. Western blot verification showed that the level of TNNI3 significantly decreased in the right atrium tissue lysate of ISO-treated monkeys (animals 3, 4, 5, 6) compared with the control monkey (animal 1). CYC1 is a subunit of the cytochrome *b*−*c*1 complex in the electron transport chain. Alteration of CYC1 could cause the disruption of mitochondrial function and contribute to cell apoptosis.[30] The decrease in CYC1 identified by proteomic analysis was also verified by Western blot in both the left ventricle and right atrium tissue lysate. In addition, B2M was indicated as a biomarker to evaluate cardiovascular diseases, and an elevated B2M level in plasma was reported to be associated with cardiovascular mortality and cardiovascular events in patients at different stages of chronic kidney disease.[31] In our current study, an increase in B2M in the left ventricle was determined by LC−MS/MS and verified by Western assay, as well.

■ DISCUSSION

Cardiac toxicity is now the leading cause for drug discontinuation in clinical trials and even postmarket surveillance in the U.S.[32] With antiretroviral therapy, patients infected with HIV are living longer; however, studies showed increased rates of CVD and CVD-related mortality in this population. These cardiac events may be due to a combination of factors from antiretroviral therapy and the HIV virus.[5,6] Preclinical evaluations provide an invaluable opportunity to identify drug toxicity before administration of potential new antiretroviral therapeutics or other drugs to human subjects. Although cardiac troponins have gained much attention and interest for their potential to provide sensitive early warning of heart injury, significant uncertainty surrounding the interpretation, narrow kinetic range, and variability in troponin response level among species make routine application of troponin in preclinical trials controversial.[33,34] Thus, there is an urgent need for safety biomarkers that can be used in routine evaluation of short- and long-term cardiac injury in preclinical trials. We previously reported nine serum proteins that showed significant changes at 48 and 72 h after ISO injection in AFG monkeys.[16] To expand on this study, similar proteomic screening has been conducted on the serum proteins from two other NHP models, CM and RM. A panel of 10 proteins showed significant alteration at 48 and 72 h after ISO injection in CM. Among these, three proteins (SAA, A1AG, and Apo A1) were confirmed to be changed in all three species. Multiple

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proteins such as SAA, A1AG, and CRP are involved in inflammation, whereas several other proteins are associated with cardiac hypertrophy and necrosis, such as carbonic anhydrase, tropomyosin, and α-1-antichymotrypsin.[35−37] Our results suggest that these proteins have the potential to be used in preclinical evaluation of cardiac toxicity for both short- and long-term studies.

Understanding the cellular mechanism of drug-induced heart injury is essential for physicians to be aware of potential interactions with other drugs and dose limitation for prevention of adverse effects.[38] In our study, interrogation of proteins from four different heart compartments identified the left ventricle and right atrium as the compartments showing the greatest alterations after ISO administration. Pathway analysis of the altered proteins in heart tissue suggested two major pathways that are responsible for cardiotoxicity: calcium signaling and mitochondrial dysfunction.

Calcium Signaling and Troponin Complex Dissociation

Since Sydney Ringer showed over 100 years ago that the heart could not beat in the absence of Ca^{2+}, numerous studies have been conducted to understand the role of calcium in cardiac function and the progression of heart disease, such as cardiac hypertrophy and heart failure.[39,40] Calcium plays a crucial role in regulating contraction and intracellular signaling, which are vital for healthy heart function. In our study, two of the up-regulated proteins identified in left ventricle were involved in the calcium signaling pathway: calreticulin and glutamate [NMDA] receptor subunit ε-3-like (GRIN2C). Calreticulin is an endoplasmic reticulum luminal Ca^{2+} buffering chaperone. It is essential for cardiac development, and its gene is tightly regulated during cardiogenesis. Calreticulin is involved in regulation of intracellular Ca^{2+} homeostasis, and overexpression of calreticulin can disrupt Ca^{2+} storage and release.[41,42] Seidman's group reported increased expression levels of calreticulin in calsequestrin 2 mutant mice with ventricular tachycardia.[43] GRIN2C is a subunit of glutamate receptors, which are found in the central nervous system and also play a role in the calcium signaling pathway.[44] Although glutamate receptors are mostly implicated in neurological disorders such as Parkinson's and Huntington's diseases, these receptors have also been found to be overactivated or abnormal in diabetes and ischemia.[45,46] There are currently no reports of altered GRIN2C levels associated with any cardiac diseases in the literature.

At the end of the calcium signaling pathway, Ca^{2+} directly activates the myofilaments for heart muscle contraction by binding to the troponin complex.[37] When Ca^{2+} concentration declines, it dissociates from troponin, and muscle fiber relaxation occurs. The troponin complex has three units: troponin I, T, and C. Although troponin C has no cardiac specificity, both troponin I and T have cardiac isoforms. Thus, cardiac troponin I (cTnI) and troponin T (cTnT) are used as biomarkers for the detection of cardiac injury.[8] Interestingly, we observed decreased levels of TnC, cTnI, and cTnT in right atria upon ISO treatment. This could be due to the degradation of the structural pool of the troponin complex and release of troponin into the circulatory system,[47] which is consistent with the elevated blood levels of troponin observed in myocardial infarction and other cardiac injuries in clinical studies. Other myofilament proteins, such as myosin-6, myosin light chain 4, tropomyosin β-chain isoform 11, and tropomyosin α-4 chain isoform 17, are also detected as altered upon ISO treatment. The decrease in troponins and other interacting proteins suggests that myofilaments, the contractile elements of the heart, could be one of the drug targets.

Oxidative Phosphorylation and ATP Generation

Mitochondria generate most of the cell's supply of adenosine triphosphate (ATP). In addition to supplying cellular energy, mitochondria play an important role in many other tasks, such as cellular proliferation, cell death, regulation of the cellular redox state, and fatty acid oxidation. Mitochondrial mutation and dysfunction have been implicated in many diseases, including heart and neurological diseases, and may also play a role in the aging process.[48] Oxidative phosphorylation is the metabolic pathway in which the mitochondria can generate ATP by the oxidation of nutrients. In our study of CM heart tissue proteins, we identified several proteins involved in the oxidative phosphorylation pathway that are significantly down-regulated upon ISO treatment (Figure 6). Among these proteins, ATP5B, ATP5C1, ATP5D, ATP5F1, and ATP5J2 belong to complex V, which is the final enzyme in the oxidative phosphorylation pathway to generate ATP. UQCRC1 and CYC1, and COX5A are present in complex III and IV, respectively, and are responsible for transferring electrons to proteins and oxygen in the electron transfer chain. Down-regulation of these proteins could lower the rate of oxidative phosphorylation and impair energy production. Damage to mitochondrial function could also cause the release of cytochrome *c*, which is a key step leading to programmed cell death.[49] We observed decreased levels of CYC1, which is a subunit responsible of transferring electrons to cytochrome *c*, in the heart tissue. Previous studies on cardiac diseases reported damage to mitochondria by decreased energy production, accumulation of reactive oxygen species, and release of cytochrome *c* in ischemia, reperfusion, cardiomyopathy, and heart failure.[46] Thus, a drug-induced decrease in energy production and loss of mitochondrial function could very likely contribute to myocyte apoptosis and heart tissue injury.

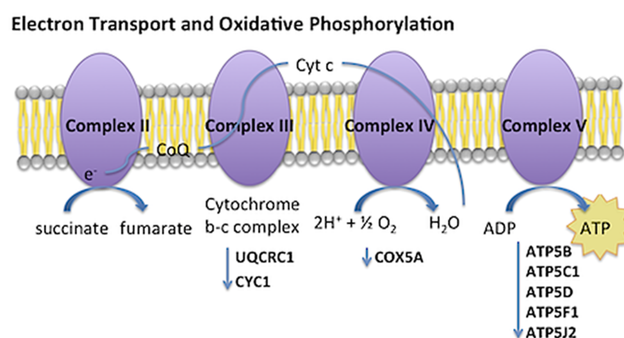

Figure 6. Proteins showing significant down-regulation upon ISO treatment are involved in the electron transport and oxidative phosphorylation pathway.

In summary, we applied a TMT labeling-based quantitative proteomics approach to three NHP species for safety biomarker discovery to evaluate drug cardiotoxicity. The results showed consistent alteration of three proteins—SAA, A1AG, and Apo A1—in all three species upon ISO injection, indicating the potential of including these proteins in preclinical evaluation of drug-induced cardiac injury. In addition, proteomic analysis of injured heart tissue proteins demonstrated detrimental effects on energy generation and mitochondrial function of cardiac

myocytes, which can provide insights for reducing cardiotoxicity in future drug discovery and development. It is worth noting that cardiac troponins were not identified in serum, but were identified as decreased in heart tissue lysate along with other cardiac-specific proteins. In serum samples, the top 10 most abundant proteins account for 90% of the total protein content, making it difficult to detect these medium- or low-abundance cardiac-specific proteins with nontargeted profiling mass spectrometry. Immunoaffinity enrichment and targeted mass spectrometry were used for detection of cardiac troponins in serum samples.[50] Our results suggest that proteomic screening of heart tissue proteins, followed by verification in serum samples using targeted mass spectrometry or other sensitive measures, could be a new strategy to discover improved cardiac biomarkers with high specificity in future studies.

■ ASSOCIATED CONTENT

Supporting Information

Figure S1. Heart rates of male and female Rhesus macaques following a single s.c. administration of 4 mg/kg ISO. Heart rates were measured by ECG at time 0 (immediately prior to dose administration), 15 min and 1, 4, 24, 48, and 72 h postdose. Table S2. Proteins demonstrating significant change (>2-fold) in Cynomolgus macaque serum after isoproterenol treatment. Figure S3. Representative MS/MS spectra of peptides (A) YEGGQEHFAHLLILR from protein α-1-acid glycoprotein and (B) RLYGSEAFATDFQDSAVAK from protein α-1-antichymotrypsin in serum sample. The left panel shows the CID spectra for protein identification; the right panel shows the corresponding HCD spectra for protein quantitation. Each peak from different TMT tags was labeled with corresponding time points (0, 1, 4, 24, 48, 72 h). Figure S4. CID spectra of proteins demonstrating significant change in Cynomolgus macaque serum after isoproterenol treatment. Table S5. ELISA validation of C-reactive protein (CRP), pancreatic α-amylase (PA), serum amyloid A (SAA) and α-1-acid glycoprotein (A1AG) in serum from four ISO-treated and two control Cynomolgus macaques. Table S6. ELISA validation of serum amyloid A (SAA) and α-1-acid glycoprotein (A1AG) in serum from four ISO-treated and two control Rhesus macaques. Table S7. Proteins changed >1.5-fold in Cynomolgus macaque left ventricle 3 days after isoproterenol treatment. Table S8. Proteins changed >1.5-fold in Cynomolgus macaque right ventricle 3 days after isoproterenol treatment. Table S9. Proteins changed >1.5-fold in Cynomolgus macaque left atrium 3 days after isoproterenol treatment. Table S10. Proteins changed >1.5-fold in Cynomolgus macaque right atrium 3 days after isoproterenol treatment. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS:

ISO,isoproterenol; CVD,cardiovascular disease; HAART,highly active antiretroviral therapy; AFG,African green monkey; CM,Cynomolgus macaque; RM,*Rhesus macaque*; TMT,tandem mass tag; FASP,filter-aided sample preparation; NHP,nonhuman primates; SCX,strong cation exchange; RP,reversed phase; CID,collision induced dissociation; HCD,higher-energy collisional dissociation; FDR,false discovery rate; ECG,electrocardiograms

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