Does the Rewarmed Heart Restore the Myocardial Proteome to That of the Pre-Cooled State? – A Proteomic Analysis of Surgical Samples –

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Background: Hypothermia is utilized in cardiac and aortic surgery to protect organs from ischemic reperfusion injury. Although the cooled body is invariably rewarmed after the procedure, it is still unknown whether the rewarmed body regains its former biological state. This study determined the modulatory effects of hypothermia on the human myocardial proteome and whether subsequent rewarming restores the proteome to the state prior to cooling.

Methods and Results: A quantitative proteomic analysis was performed using isobaric tags for relative and absolute quantification labeling tandem mass spectrometry. Right atrial samples were taken 3 times (pre, during and post cooling) during deep hypothermic cardiopulmonary bypass (CPB) from 8 patients with aortic arch aneurysms and 3 corresponding time points during normothermic CPB from 8 patients with ascending aortic or valsalva aneurysms. In total, 697 proteins were identified, with 222 proteins having high protein confidence. Bioinformatic analyses revealed significant downregulation of 19 proteins associated with energy production at hypothermic cardioplegic arrest. On rewarmed beating, 10 proteins remained downregulated, including those regulating cardiac contraction and adaptor proteins, although levels of the aforementioned 19 downregulated proteins returned to their initial values. Additional echocardiographic evaluation demonstrated that hypothermia preserved the variables of diastolic function to a greater extent than normothermic surgery.

Conclusions: Rewarming restores the human myocardial proteome to the pre-cooled state, except for proteins regulating cardiac contraction and adaptor proteins. (Circ J 2015; 79: 2648–2658)

Key Words: Hypothermia; Myocardium; Proteomics
surgery. Therefore, we can make comparisons among groups, and before and after treatment, and thereby differentiate the effects of hypothermia (cooling) and rewarming using this technology.

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The study objective was to clarify the effects of both deep hypothermia (22°C) and rewarming on the myocardial proteome during cardiopulmonary arrest and reperfusion by comparison with normothermic counterparts. By quantitatively analyzing and comparing 3 sets of iTRAQ labeling samples (ie, before cooling, during cooling, and after rewarming), we sought to verify whether the changes in the proteome profile induced by hypothermia could be fully corrected by rewarming.

**Methods**

**Patient Selection**

This prospective cohort study received approval from the Ethics Committee of Shimane University Faculty of Medicine and was carried out at Shimane University Hospital. We included 16 patients undergoing thoracic aortic repair in this study: 8 patients underwent aortic arch repair using deep hypothermic cardiopulmonary bypass (CPB) combined with coronary artery bypass grafting (CABG) (proteomic analysis; n=8) and 8 patients underwent aortic arch repair (AVR) with normothermic CPB (n=8). All participants gave written informed consent. To evaluate myocardial injury induced by hypothermia, we performed an echocardiographic examination preoperatively, and before and after rewarming, and thereby differentiated the effects of hypothermia (cooling) and rewarming using this technology. The same cardiopulmonary solution (mimiplegia, non-diluted cardioplegia including glutamate and aspartate as “substrates”) was administered at the same temperature as the circulating blood in both groups, namely warm solution in the normothermic CPB group and low temperature solution in the hypothermic CPB group, low temperature solution in the hypothermic CPB group. Therefore, we can make comparisons among groups: anesthesia was induced and maintained with fentanyl and propofol; catecholamines, vasodilators and antiarrhythmic drugs were administered similarly in both groups of patients. The CPB circuit was the same for both groups, consisting of a hollow-fiber polymer-coated membrane oxygenator (Quadrox-I, HM070000, Maquet, Germany), centrifugal pump (Rotaflow, BO-RF32, Maquet), arterial filter (40 μm Pall arterial line filter, AL6, Pall, Port Washington, NY, USA) and heparin-coated CPB circuit (MERA or JMS, Tokyo, Japan). The same cardiopulmonary solution (mimiplegia, non-diluted cardioplegia including glutamate and aspartate as “substrates”) was administered at the same temperature as the circulating blood in both groups, namely warm solution in the normothermic group, low temperature solution in the hypothermic CPB group.

**CK-MB Measurement and Echocardiographic Examination**

Postoperative CK-MB levels were measured to evaluate myocardial injury. Transthoracic echocardiography was performed before and 2 weeks after surgery by the same cardiologist specialized in echocardiography. To determine diastolic function, spectral Doppler tracing of diastolic transmitral inflow velocity was obtained using pulsed Doppler imaging, and peak velocities were measured in early (E) and late diastole (A). The tissue Doppler imaging technique was used to record mitral annular velocity. Early diastolic mitral annular velocity (E') was measured by placing the sample volume at both the septal and lateral sides of the mitral annulus; the E/E' ratio was calculated to estimate left ventricular (LV) filling pressure and stiffness.

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**Table 1. Baseline Characteristics, Surgical Procedures and Intraoperative Variables**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Normothermic CPB; n=17 (proteomic analysis; n=8)</th>
<th>Hypothermic CPB; n=76 (proteomic analysis; n=8)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>60.7±15.2 (64.9±8.7)</td>
<td>72.3±12.5 (68.6±6.4)</td>
<td>0.001 (0.349)</td>
</tr>
<tr>
<td>Sex (male)</td>
<td>9 (3)</td>
<td>41 (3)</td>
<td>1.000 (1.000)</td>
</tr>
<tr>
<td>Hypertension</td>
<td>4 (4)</td>
<td>36 (6)</td>
<td>0.104 (0.608)</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>1 (1)</td>
<td>13 (0)</td>
<td>0.453 (1.000)</td>
</tr>
<tr>
<td>Dyslipidemia</td>
<td>6 (4)</td>
<td>11 (2)</td>
<td>0.076 (0.608)</td>
</tr>
<tr>
<td>Ischemic heart diseases</td>
<td>2 (1)</td>
<td>16 (2)</td>
<td>0.510 (1.000)</td>
</tr>
<tr>
<td>Ascending aorta replacement</td>
<td>7 (5)</td>
<td>32 (1)</td>
<td>1.000 (0.119)</td>
</tr>
<tr>
<td>Total arch replacement</td>
<td>0</td>
<td>32 (1)</td>
<td>0.0004 (1.000)</td>
</tr>
<tr>
<td>Hemi-arch replacement</td>
<td>0</td>
<td>9 (3)</td>
<td>0.203 (1.200)</td>
</tr>
<tr>
<td>Bentall operation</td>
<td>10 (3)</td>
<td>6 (2)</td>
<td>&lt;0.0001 (1.000)</td>
</tr>
<tr>
<td>Aortic valve replacement</td>
<td>5 (3)</td>
<td>1 (0)</td>
<td>0.0006 (0.200)</td>
</tr>
<tr>
<td>Coronary artery bypass grafting</td>
<td>2 (1)</td>
<td>4 (0)</td>
<td>0.301 (1.000)</td>
</tr>
<tr>
<td>Minimal core temperature (°C)</td>
<td>34.4±0.9 (34.9±1.1)</td>
<td>22.9±2.5 (22.0±1.1)</td>
<td>&lt;0.0001 (&lt;0.0001)</td>
</tr>
<tr>
<td>Aortic cross-clamping time (min)</td>
<td>128.0±45.8 (111.6±32.1)</td>
<td>151.8±62.3 (120.0±64.1)</td>
<td>0.083 (0.7452)</td>
</tr>
<tr>
<td>CPB time (min)</td>
<td>179.5±60.7 (150.0±37.6)</td>
<td>244.0±79.8 (217.1±66.4)</td>
<td>0.002 (0.0261)</td>
</tr>
</tbody>
</table>

Data are number of patients or given as mean±SD in patients undergoing either normothermic CPB or hypothermic CPB. Number, mean±SD and P value in each line indicate those patients analyzed retrospectively, and number in parenthesis are the proteomic study cohort group. CPB, cardiopulmonary bypass.
Tissue Sampling

The 3 right atrial wall tissue samples were excised as follows: T1 (before cooling, 5 min after pump initiation), T2 (during normothermic cardioplegic arrest, 107.5±52.1 min after pump initiation in normothermic CPB or during deep hypothermic cardioplegic arrest or ventricular fibrillation (VF), 67.9±38.6 min after pump initiation in DHCA+SCP), T3 (just before termination of CPB, 150.0±37.6 min after pump initiation in normothermic CPB or 217.1±66.4 min after pump initiation in DHCA+SCP) (Table 1).

iTRAQ Labeling and Strong Cation Exchange (SCX) Chromatography

Samples were prepared according to the manual published by AB Sciex (Foster, CA, USA) and as described previously. In brief, equal amounts of the T1, T2, and T3 samples from each patient were denatured and reduced, the cysteines alkylated, and finally digested with trypsin (AB Sciex). Each digest was labeled with a different iTRAQ tag using the iTRAQ reagent 4-plex kit (AB Sciex). iTRAQ label 114 was used for the T1 sample, and iTRAQ labels, 116 or 117 were selected for the T2 and T3 samples; the 3 samples from each patient were then combined. The combined samples were fractionated into 6 fractions by SCX chromatography according to the manufacturer’s instructions (AB Sciex) and each fraction was desalted, also according to the manufacturer’s instructions (Waters, Milford, MA, USA).

NanoLC and MALDI-TOF/TOF MS/MS Analysis

One fraction from the SCX chromatography was fractionated to 171 spots using a DIna nanoLC system (KYA Tech, Tokyo, Japan) and collected onto an Opti-TOF LC/MALDI 384 target plate (AB Sciex) according to the manufacturer’s instructions and as described previously. Spotted peptide samples were analyzed by a 5800 MALDI-TOF/TOF MS/MS Analyzer with TOF/TOF Series software (version 4.0, AB Sciex). MS/MS data were analyzed using ProteinPilot™ software (version 3.0) and the Paragon™ protein database (AB Sciex). Quantitative changes of proteins at T2 or T3 were calculated using iTRAQ ratios T2:T1 or T3:T1, respectively.

Western Blot Analysis

Western blot analyses were performed as described previously. In brief, myocardial samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblotted using rabbit polyclonal IDH3A antibody (Proteintech, Chicago, IL, USA), mouse monoclonal gelsolin (GSN) antibody (Sigma, St. Louis, MO, USA) and anti-rabbit IR dye 680-conjugated IgG (LI-COR, Lincoln, NE, USA). Protein bands were visualized using an Odyssey (LI-COR) infrared imaging system and their intensities measured for densitometric analyses of IDHA3 and GSN. Data from duplicate or triplicate experiments were normalized as the ratio T2:T1 or T3:T1 and analyzed statistically.

iTRAQ Data Analysis and Bioinformatic Analysis

Proteins identified as showing expression changes were examined for conformity to the following conditions: (1) false discovery rate (FDR) <5% (FDR was estimated by “decoy database searching” using ProteinPilot Software); (2) protein confidence >99% (“unused ProtScore” >2). Unused ProtScore is defined as -log (1-% confidence/100). Proteins satisfying these criteria were regarded as having statistical significance.

The KEGG was used to assess whether these significant proteins could be associated with well-defined canonical or signaling pathways. PANTHER software (version 9.0, http://www.pantherdb.org) was used to test for statistical overrepresentation of gene ontology (GO, http://www.geneontology.org) terms as described in detail elsewhere. If the number of identified genes in a GO term was significantly larger than...
Proteomic Profiling of Hypothermic Cardiac Changes

Among the 222 identified proteins, 203 were also matched to the annotation number (k number) and subsequently searched using the “Search & Color Pathway” tool in the “KEGG Mapper”. (A) The 66 identified proteins were matched to a metabolic pathway (ko 01100) and highlighted by black in the pathway map, indicating that identified proteins were intensively located on glycolysis (marked by “G”), TCA cycle (marked by “T”) and oxidative phosphorylation (marked by “O”) in the map. (B) TCA cycle (“T,” ko 00020) map is illustrated and 13 matched proteins/associated enzyme commission numbers (EC) are highlighted in pink. In these, 6 proteins were downregulated with statistical significance (indicated by red arrows) during deep hypothermia (T2) in DHCA patients. These were MDH2 (EC: 1.1.1.37), IDH3 (EC: 1.1.1.41), SDHA (EC: 1.3.5.1), DLD (EC: 1.8.1.4), DLAT (EC: 2.3.1.12) and ACO (EC: 4.2.1.3). (C) Oxidative phosphorylation (“O”, ko 00190) map is illustrated and 27 matched proteins/associated EC numbers are shown in pink. In these, 6 proteins were downregulated with statistical significances (indicated by red arrows) during deep hypothermia (T2) in DHCA patients. These proteins were SDHA, CYC1, UQCRC1, ATP5C1, COX5B, and NDUFV2.

DHCA, deep hypothermic cardiopulmonary bypass combined with circulatory arrest.
Figure 3. Scatterplot of proteins identified with high protein confidence demonstrating log-transformed iTRAQ ratio and P values. Fold changes >1.2 or 0.833 were defined as significant up- or downregulation, shown by the 2 vertical lines. P<0.05 was defined as statistically significant and shown by the horizontal line. Proteins located in the upper left or right fields were statistically down- or upregulated. (A) Scatterplot of identified proteins at midway (T2) in normothermic cardiopulmonary bypass (CPB) patients; 10 proteins in the left upper corner were downregulated with statistical significance. (B) Scatterplot of identified proteins at termination of CPB (T3) in normothermic CPB patients. There were 6 downregulated proteins and 1 upregulated protein with statistical significance. (C) Scatterplot of identified proteins during cooling (T2) in DHCA patients. There were 50 downregulated proteins with statistical significance, showing an enlarged view on the right. Numbers indicate proteins shown in Table S3. (D) Scatterplot of identified proteins after rewarming (T3) in DHCA patients. There were 10 downregulated proteins with statistical significances. DHCA, deep hypothermic CPB combined with circulatory arrest.
that in the whole genome classified by the same GO term (ie, the number of observed genes in a GO term was significantly larger than the number of expected genes in the same GO term by the binomial test), the GO term was described here as ‘overrepresented’ with statistical significance after Bonferroni correction for multiple testing. The annotations of identified proteins were obtained from the Uniprot database (http://www.uniprot.org/).

Statistical Analysis
Continuous variables are expressed as mean±standard deviation; they were tested for statistically significant differences between patient groups using Student’s t-test or Welch’s t-test. Categorical variables were compared using Fisher’s exact test. Echocardiographic variables were tested for statistically significant differences between preoperative and postoperative values using paired t-tests. For analysis of iTRAQ ratios (ie, T2:T1, T3:T1 ratios) for each significantly identified protein, P values were calculated by 1-sample t-test of averaged protein ratio against 1 to assess the validity of the protein expression change. To assess the western blot analysis data, statistical comparisons were performed by 2-way analysis of variance (general linear model) with repeated measures followed by a post-hoc Bonferroni test to identify individual differences. P<0.05 was defined as statistically significant.

Results
Proteomic Profiling by PANTHER and KEGG Software
Mass spectrometry identified 697 proteins satisfying the criteria (FDR <5%) (Table S1), of which 222 fulfilled the criteria of protein confidence >99% (“unused ProtScore” >2) in at least 5 of 8 samples in either group. These proteins were classified according to the GO biological process using PANTHER software. The “metabolic process” was the most prominent GO category (Figure 1). Of the 222 proteins, 203 were matched to the genome annotation (k number) in the KEGG and searched using the “Search&Color Pathway” tool in the “KEGG Mapper.” Among 178 pathways detected, metabolic pathway (ko 01100) included the largest number of proteins (66 proteins) (Figure 2A); glycolysis/gluconeogenesis (ko 00010) included 14 proteins; citrate cycle (tricarboxylic acid (TCA) cycle, ko 00020) had 13 proteins (Figure 2B); oxidative phosphorylation (ko 00190) had 27 proteins (Figure 2C).

Table 2. GO Categories (Biological Process) Showing Statistical Overrepresentation for Myocardial Proteins Taken Under Conditions of Both Hypothermic Cardioplegic Arrest and Rewarmed Beating

<table>
<thead>
<tr>
<th>GO term</th>
<th>Description</th>
<th>Homo sapiens (n=21,804)*</th>
<th>No. of expected genes</th>
<th>No. of observed genes (no. of mapped genes=57)**</th>
<th>P value</th>
<th>Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>At hypothermic arrest</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0006091</td>
<td>Generation of precursor metabolite and energy</td>
<td>280</td>
<td>0.72</td>
<td>12</td>
<td>1.16E-09</td>
<td>CYB5R3 UQCR1 SDHA AC02 DLD COX5B IDH3A NDUFV2 MDH2 NNT CYC1 ETF A</td>
</tr>
<tr>
<td>0022904</td>
<td>Respiratory electron transport chain</td>
<td>232</td>
<td>0.6</td>
<td>8</td>
<td>2.59E-05</td>
<td>CYB5R3 UQCR1 SDHA DLD COX5B NDUFV2 CYC1 ETF A</td>
</tr>
<tr>
<td>0006631</td>
<td>Fatty acid metabolic process</td>
<td>188</td>
<td>0.48</td>
<td>6</td>
<td>1.61E-03</td>
<td>ANXA1 CYB5R3 ANXA5 ETF A ECHS1 DECR1</td>
</tr>
<tr>
<td>0006119</td>
<td>Oxidative Phosphorylation</td>
<td>57</td>
<td>0.15</td>
<td>4</td>
<td>2.69E-03</td>
<td>SDHA COX5B NDUFV2 NNT</td>
</tr>
<tr>
<td>0006099</td>
<td>Tricarboxylic acid cycles</td>
<td>23</td>
<td>0.06</td>
<td>3</td>
<td>5.46E-03</td>
<td>AC02 IDH3A MDH2</td>
</tr>
<tr>
<td>0008152</td>
<td>Metabolic process</td>
<td>8,613</td>
<td>22.12</td>
<td>37</td>
<td>9.20E-03</td>
<td>MYOM3 CYB5R3 UQCR1 APOA1 RPS27A HK1 PHB PRDX2 AC02 SDHA CKB HSPA8 DECR1 HSD1 MYOM1 ARL6IP5 IDH3A DLD ATP5C1 SERPIN1 S100A1 ANXA1 MDH2 HSPA1A ETF A DLAT HSP90B1 CKM ALDH2 COX5B MYH9 SORBS2 SQD1 NNT NDUFV2 TUFM ANXA5</td>
</tr>
<tr>
<td>0006635</td>
<td>Fatty acid β-oxidation</td>
<td>35</td>
<td>0.09</td>
<td>3</td>
<td>1.88E-02</td>
<td>ETF A ECHS1 DECR1</td>
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<tr>
<td>0005975</td>
<td>Carbohydrate metabolic process</td>
<td>650</td>
<td>1.67</td>
<td>8</td>
<td>4.32E-02</td>
<td>MDH2 SORBS2 HK1 DLAT ECHS1 SDHA AC02 IDH3A</td>
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<tr>
<td>At rewarmed beating</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>0032989</td>
<td>Cellular component morphogenesis</td>
<td>646</td>
<td>0.3</td>
<td>4</td>
<td>2.45E-02</td>
<td>MYH9 SORBS2 TMOD1 GSN</td>
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<tr>
<td>0009653</td>
<td>Anatomical structure morphogenesis</td>
<td>691</td>
<td>0.32</td>
<td>4</td>
<td>3.18E-02</td>
<td>MYH9 SORBS2 TMOD1 GSN</td>
</tr>
</tbody>
</table>

Of 222 proteins fulfilling the criteria, proteins that changed (<0.83-fold or >1.2-fold) significantly were analyzed by PANTHER software (version 9.0). The test for statistical overrepresentation (see Methods for more detail) of each GO (see Methods for more detail) term (biological process) identified no proteins group (GO) in normothermic patients, but 8 GO categories in hypothermic patients. *Total number of genes in the whole genome (Homo sapiens, n=21,804) classified by the GO term; **number of genes that would be detected in the input list for a particular GO category on the basis of the reference list (Homo sapiens); †number of genes from the input list (n=57) classified by the GO term. GO, gene ontology.
Proteomic Changes During DHCA and Normothermic CPB

To demonstrate both the fold changes and P values of proteins having statistical significance, fold changes (T2/T1, T3/T1) were combined with their P values and demonstrated by log-transformed iTRAQ ratio and P values, with the 2 vertical lines showing a fold change=1.2 and =0.833, respectively, and the horizontal line showing P<0.05 (Figure 3). At a glance, a scatterplot of proteins had a V-shaped distribution in the normothermic CPB patients, indicating there was an even distribution among up- and downregulated proteins (Figures 3A,B; Table S2). Immunoglobulin and acute phase protein expressions were significantly decreased midway (T2) of CPB (Figure 3A); heat shock protein 1 (HSPE1) and acute phase proteins, including α-1-acid glycoprotein 1 (ORM1), α-1-antitripsin (SERPINA1), and transthyretin (TTR), were significantly decreased on termination (T3) of CPB (Figure 3B; Table S2). Conversely, protein scatterplots were located in the left half field only, indicating that most proteins were uniformly downregulated in DHCA patients, with 50 downregulated proteins having statistical significance (Figure 3C: Table S3). These downregulated proteins are also indicated by the red arrow on the enzyme commission (EC) number or gene name in Figure 2B,C. After rewarming, the number of significantly downregulated proteins decreased and the number of upregulated proteins increased (shown in the left upper corner and right lower corner in Figure 3D). Cardiac samples were taken at deep hypothermic VF before aortic clamping in 3 patients in order to examine the effects of simple deep hypothermia; the remaining 5 patients had cardiac samples taken at deep hypothermic cardioplegic arrest. However, there were no significant differences in proteome between the 2 groups. In DHCA patients, levels of downregulated proteins mostly returned to the pre-cooling values after rewarming and declamping (T3). However, 10 proteins remained downregulated at T3 (Figure 3D; Table S3). These included myosin light chain 3 (MYL3), tropomodulin-1 (TMOD1), GSN and adaptor proteins regulating many signaling pathways.

Bioinformatic Analysis by PANTHER Software

Proteins decreased or increased (<0.833-fold or >1.2-fold) with statistical significance (P<0.05) found in at least 5 of 8 samples, which were located in the upper left or right field in Figures 3A–D, were identified to obtain both an adequate sample size and statistical power, and were subsequently analyzed by PANTHER software. The statistical overrepresentation test identified no protein group (GO) overrepresented statistically in normothermic CPB patients; however, there were 8 statistically overrepresented GO categories (biological processes) mainly related to energy production in DHCA patients, in which 19 significantly downregulated proteins were directly related to energy production. These downregulated proteins mostly returned to the pre-cooling levels after rewarming and declamping (T3); thus there were only 2 GO groups relating to cardiac structure morphogenesis statistically overrepresented at T3 in DHCA patients (Table 2).
Table 3. Echocardiographic Variables Before and After Surgery

<table>
<thead>
<tr>
<th></th>
<th>Normothermic (n=17†)</th>
<th>Hypothermic (n=29)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before surgery</td>
<td>After surgery</td>
</tr>
<tr>
<td>LVMi (g/cm)</td>
<td>149.9±43.9</td>
<td>133.7±47.1</td>
</tr>
<tr>
<td>LVEDVI (ml/m²)</td>
<td>79.1±36.0</td>
<td>58.0±15.6**</td>
</tr>
<tr>
<td>LVMi/LVEDVI (g/ml)</td>
<td>2.2±0.7</td>
<td>2.6±0.7</td>
</tr>
<tr>
<td>LVEF (%)</td>
<td>60.5±8.0</td>
<td>56.8±13.4</td>
</tr>
<tr>
<td>E (cm/s)</td>
<td>53.6±14.1</td>
<td>67.3±14.6**</td>
</tr>
<tr>
<td>A (cm/s)</td>
<td>70.1±21.3</td>
<td>70.7±21.7</td>
</tr>
<tr>
<td>E/A</td>
<td>1.00±0.29*</td>
<td>0.76±0.22</td>
</tr>
<tr>
<td>DcT (ms)</td>
<td>235.2±75.7</td>
<td>191.1±39.3</td>
</tr>
<tr>
<td>E'/E</td>
<td>5.2±2.6</td>
<td>4.9±2.0</td>
</tr>
<tr>
<td>E/E’</td>
<td>12.4±6.2</td>
<td>15.6±7.1*</td>
</tr>
</tbody>
</table>

Data are given as mean ± SD. †Patients evaluated by echocardiography pre- and postoperatively. Paired t-tests were carried out between “before surgery” and “after surgery” in each group, *P<0.05; **P<0.01; ***P<0.001. DcT, deceleration time; LVEDVI, left ventricular end-diastolic volume index; LVEDVI, left ventricular end-diastolic volume index; LVEF, left ventricular ejection fraction; LVMi, left ventricular mass index.

Western Blot Analysis

Among the protein levels that decreased after cooling in DHCA patients, IDH3A, a protein that returned to its pre-cooling level (protein no. 46 in Table S3), and GSN, a protein that did not recover its level (no. 26 protein in Table S3), were both validated by western blotting. The IDH3A level decreased after cooling with statistical significance (P=0.013, between T1 and T2) and increased after rewarming to some extent in DHCA patients, although there were no significant changes in normothermic CPB (Figure 4A). On the other hand, GSN levels decreased during both cooling (T2) and rewarming (T3) similarly to the iTRAQ ratio, but without reaching statistical significance, because of large variations in the DHCA patients (Figure 4B); however, these levels decreased at T2 and increased to the initial level at T3 in normothermic CPB patients.

Myocardial Injury and Echocardiographic Findings

Additional retrospective analysis revealed that there were 2 hospital deaths, from septic shock and rupture of abdominal aortic aneurysm after thoracic aortic repair, in the deep hypothermic CPB group (n=76), but there were no hospital deaths in the normothermic group (n=17). There were 3 major complications, comprising 2 strokes and 1 graft infection, in the deep hypothermic group, and there was 1 case of mediastinitis in the normothermic group. There was no incidence of severe low output syndrome necessitating IABP or any assisted circulation in either group. The postoperative peak CK-MB value was lower in the deep hypothermic group than in the normothermic group (34.2±22.2 ng/ml, n=70 vs. 44.7±33.3 ng/ml, n=16, P=0.246), although aortic cross-clamping time was longer in the deep hypothermic group (Table 1, P=0.083). Echocardiographic evaluation did not demonstrate significant changes in ejection fraction pre- and post-surgery in either group, although left ventricular end-diastolic volume index (LVEDVI) significantly decreased after surgery in both groups (Table 3). E/A similarly increased post-surgery in both groups with statistical significance. E/E’ slightly decreased postoperatively in the deep hypothermic group (P=0.239, Figure 5A), but this ratio significantly increased postoperatively in the normothermic group (P=0.017, Figure 5B).

Discussion

Downregulation of Metabolic Proteins by Deep Hypothermia

Bioinformatic analyses demonstrated that deep hypothermic cardioplegic arrest/VF produced downregulation of metabolic proteins (Table 2). However, no such downregulation of proteins was observed in normothermic CPB counterparts that were similar to the DHCA group regarding cardioplegic solution, ischemic time, but not the temperature and T3 sampling time. These are unexpectedly new findings that have not been previously reported, although several studies have demonstrated reduced oxygen consumption or relatively constant ATP levels in cardiac tissue during hypothermic cardioplegic arrest compared with normothermic cardioplegic arrest.34,25 Chitwood et al have demonstrated in the potassium-arrested heart model that asystole at 37°C decreased MVO2 from 5.18±0.55 to 1.85±0.20 ml O2/min/100 g of left ventricle (35% of normal value); asystole at 22°C further decreased MVO2 to 0.54±0.05 ml O2/min/100 g of left ventricle (10% of normal value).4 In the present proteomic analysis, levels of proteins belonging to the TCA cycle and ETC did not decrease in the normothermic arrested heart, implying that enzymatic protein levels might be too high relative to the decreased oxygen consumption (35% of normal); however, enzymatic activity would decrease accordingly. In the deep hypothermic (22°C) arrested heart, the levels of proteins were downregulated but remained much higher than the levels of myocardial oxygen consumption (10% of normal). Aragones et al recently demonstrated that restricting movement of glycolytic intermediates into the TCA cycle could induce hypoxia tolerance by inactivating the pyruvate dehydrogenase complex (PDC).38 In our study, we revealed that expression of the dihydrolipoyl-sine-residue acetyltransferase component of the PDC (DLAT) was decreased at hypothermic arrest and returned to initial values at rewarmed beating (carbohydrate metabolic process in Table 2; no. 19 protein in Table S3), together with decreased expression of many other proteins in the TCA cycle and ETC. It can be speculated that hypothermia could induce ischemia tolerance by globally reducing the expression of proteins related to oxygen consumption, which could constitute a distinct approach from that targeting a specific protein for inhibition.

Our recent animal proteomic study demonstrated that two-thirds of myocardial proteins in the left ventricle were down-
regulated after surface cooling (23°C, unpublished data). Among these proteins, ATP5C1 was downregulated, and also decreased in the right atrial tissue from DHCA patients. Analyses of protein abundance in the left ventricle are clinically valuable; however, it is quite difficult to take LV tissue samples safely during surgery. Therefore we analyzed right atrial tissue as a surrogate myocardial sample, as has been previously done in other studies. However, recent proteomic and transcriptomic analyses showed that >90% of proteins belong to both ventricles and atria, while only 6.7% of proteins were atria-specific and 9.1% were ventricle-specific. In the ventricles, protein expression preferentially relates to muscular contraction and energy production. In contrast, fibrosis and apoptotic pathways are concentrated in atrial myocardium, indicating the higher susceptibility of atrial myocardium to apoptosis. Although the present study analyzed changes in protein expression during a short CPB time, key regulatory enzymes may have t1/2 values as low as 0.5 to 2 h.

Rewarming and the Myocardial Proteome
After reperfusion and rewarming, the myocardial proteome recovered to pre-cooling levels, especially in energy production (Figure 3D; Table S3). However, there were 10 proteins that had reduced expressions. Among these, MYL3 and...
Adaptor Proteins and IR Injury
Expression of 2 major adaptor proteins remain decreased after rewarming: sorbin and SH3 domain-containing protein 2 (SORBS2) did not change during deep hypothermia, but their expressions decreased after rewarming (protein no. 2 in Table S3); 14–3–3 protein zeta/delta (YWHAZ) decreased during deep hypothermia and its expression did not recover after rewarming (protein no. 40 in Table S3). To our knowledge, this is the first demonstration that adaptor proteins are downregulated by hypothermia and rewarming. SORBS2 can form a complex with tyrosine-protein kinase ABL1, which activates apoptotic signaling resulting from DNA injury, is abundantly expressed in the heart, and is located in the Z-disk of the cardiac sarcomere.39 When severe IR injury occurs, SORBS2 proteins are released from the heart into the bloodstream after reperfusion.40 However, we could not detect SORBS2 protein in plasma taken after reperfusion in our previous proteomic analysis.14 The 7 isoforms of 14-3-3 proteins have multiple roles in the apoptotic signaling network and work for signaling integration.41 14-3-3 proteins can contribute to cell survival by sequestering pro-apoptotic proteins, such as BAD, BAD and c-ABL.41 Apoptotic activation is facilitated by JNK-mediated phosphorylation of 14-3-3, triggering release of these apoptotic effectors.41 It is unclear how hypothermia-induced downregulation of 14-3-3 protein and SORBS2 could be implicated in regulating the abovementioned apoptotic process. A further study including protein phosphorylation is needed.

Temperature of Cardioplegia/CPB and Postoperative Cardiac Function
In terms of postoperative peak CK-MB measurement and echocardiographic systolic function, there was no significant difference in cardioprotective effect between warm and hypothermic cardioplegia/CPB. However, in the normothermic patients, peak CK-MB levels slightly increased over those in the hypothermic patients. Furthermore, E/E’ (most relevant predictor of LV filling pressure) significantly increased postoperatively, suggesting diastolic dysfunction caused by inadequate protection during normothermic surgery and subsequent fibrosis. Pathological examination of LV samples demonstrated such a chronic fibrosis with normal cardiac function 50 days after transplantation in a prolonged (>4–8h) myocardial preservation model.42 Although there were several differences, including age and surgical procedure, between the present groups except for temperature (Table 1), differences in surgical procedures could not have significantly impacted E/E’ because there was no change in E/E’ value pre and post AVR.31 Although this ratio is a clinically useful tool, its accuracy has some limitations.43 Whether hypothermic (cold) cardioplegia is superior to its warm counterpart is still undetermined.19,44 Because a uniform study design has not been followed in these studies, many different conditions other than temperature have been used, such as antegrade/retrograde or intermittent/continuous perfusion, crystalloid or blood cardioplegia.

Study Limitations
Because the amounts of the clinical samples of cardiac tissue were very limited, it was not possible to fully validate large proteomic data sets. In fact, western blotting was limited to examination of 2 important proteins. iTRAQ labeling and repeat samplings could be effective in serial measurements of proteomic changes during the course of surgery. However, many factors relating to surgical intervention, such as deep hypothermic circulatory arrest of the lower body, may have affected the cardiac proteome in this study. Clinical data were retrospectively analyzed; furthermore, there were several differences between groups. Therefore, the effect of hypothermia/rewarming on the cardiac proteome and function would best be validated by animal studies.

Conclusions
Deep hypothermia produced downregulation of cardiac proteins related to energy production and rewarming restored these proteins to pre-cooling levels, except for adaptor proteins and regulators of cardiac contraction. To our knowledge, these are novel findings that may contribute to a global understanding of the effects of hypothermia and rewarming.

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