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# Body Cooling Causes Normalization of Cardiac Protein Expression and Function in a Rat Heatstroke Model

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Cardiac dysfunction contributes to heatstroke genesis, which can be ameliorated by whole body cooling. A comparative analysis using two-dimensional in-gel electrophoresis of cardiac protein patterns is performed in rat controls, untreated heatstroke rats, and whole body cooling-treated heatstroke rats. After the onset of heatstroke, animals display hypotension and altered cardiac protein profiles, which can be reversed by whole body cooling. Thus, the proteomic mechanisms exerted by body cooling during heatstroke are elucidated by the current results.

**Keywords:** Proteomics • Myocardium • Heatstroke • Body cooling • Hypotension

## Introduction

Heatstroke is a life-threatening illness characterized by multiple organ dysfunction (including cardiac dysfunction), hyperpyrexia, and central nervous system disorders.[1,2] Arterial hypotension may result from decreased cardiac output (due to reduction in both stroke volume and total peripheral resistance) during heatstroke.[2,3] Whole body cooling (WBC) is the current therapy of choice for heatstroke because no pharmacologic agent is available.[1,4] Although the true mechanism of protection exerted by WBC during heatstroke remains unclear, ample evidence has accumulated to indicate that cardiac dysfunction is an attractive target for the therapy of heatstroke.[2]

The aim of this study was to identify the cardiac proteins that were involved in heatstroke-induced cardiac dysfunction by using two-dimensional in-gel electrophoresis coupled with mass spectrometry. First, we compared the precipitate fractions of heart samples obtained from normothermic controls and untreated or WBC-treated heatstroke rats and detected spots which changed more than 2-fold in expression level.[5] Second, we performed Western blotting or ELISA to confirm the spots in normothermic control rats, and untreated or WBC-treated heatstroke rats in order to elucidate the possible proteomic mechanisms exerted by WBC during heatstroke.

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## Materials and Methods

**Experimental Animals.** Adult Sprague−Dawley rats (weight, 255 ± 11 g) were obtained from the Animal Resource Center of the National Science Council of the Republic of China (Taipei, Taiwan). The animals were housed 4 in each group at an ambient temperature (Ta) of 22 ± 1 °C with a 12 h light/dark cycle. Pellet rat chow and tap water were available *ad libitum*. All protocols were approved by the Animal Ethics Committees of the Chi Mei Medical Center (CMMC 95083002) in accordance with the Guide for the Care and Use of Laboratory Animal of the National Institutes of Health and the Guidelines of the Animal Welfare Act. The ethical principle of this study was further approved by the National Science Council of the Republic of China (NSC 95-2314-B-218−001). Adequate anesthesia was maintained by means of a single intraperitoneal dose of urethane (1.4 g/kg of body weight) to abolish the corneal reflex and the pain reflexes induced by tail pinching throughout the entire experimental period (duration, approximately 8 h). At the end of the experiments, the control rats and any rats that had survived the heatstroke were killed with an overdose of urethane.

**Surgery and Physiological Parameter Monitoring.** The right femoral artery was cannulated with polyethylene tubing (PE50) for blood pressure monitoring. Physioloical parameters monitoring included core temperature (Tco), mean arterial pressure (MAP), cardiac output (CO), total peripheral resistance (TPR), stroke volume (SV), and heart rate (HR).

**Induction of Heatstroke.** Before the initiation of heatstroke, the Tco of urethane-anesthetized rats was maintained at about 36 °C by means of a folded heating pad, except during heat stresses at a room temperature of 24 °C. Heatstroke was induced by increasing folded heating pad temperature to 43 °C by means of circulating hot water. The time point at which the MAP dropped to 25 mmHg from the peak level was

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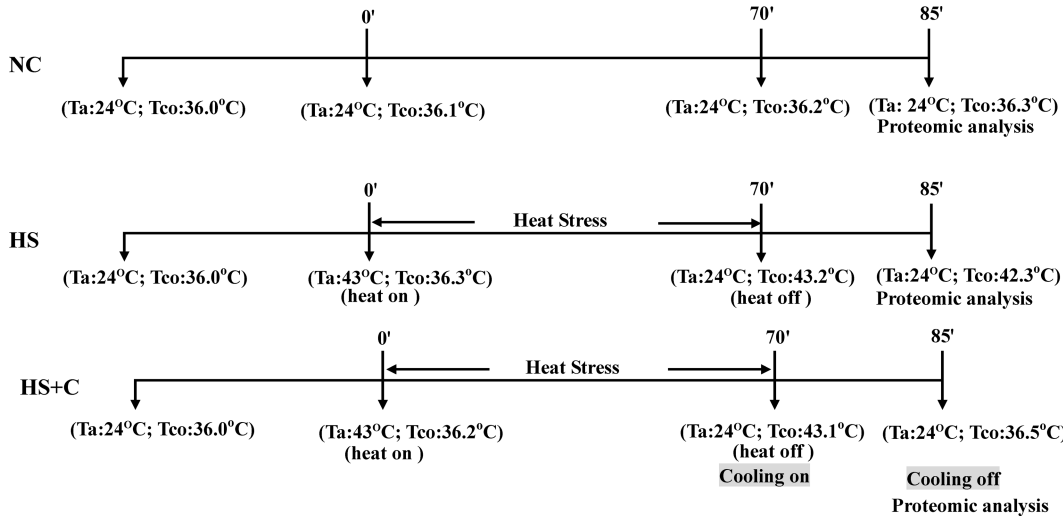


**Figure 1.** Experimental design. In normothermic controls (NC), their core temperatures (Tco) maintain a normal level of 36.0−36.3 °C at an ambient temperature (Ta) of 24 °C. In heatstroke (HS) rats with no treatment, upon heat stress (43 °C Ta for 70 min), their Tco increase to a new value of 43.2 °C. Then, the untreated HS rats are allowed to recover at room temperature (24 °C) and their Tco are decreased from 43.2 to 42.3 °C (at 85 min). In HS rats with body cooling, body cooling is conducted from 70 to 85 min for 15 min and their Tco values decrease from 43.1 to 36.5 °C. At 85 min, animals are killed for proteomic analysis.

considered as the onset of heatstroke.[6,7] At this time point, the average Tco was found to be 43.2 ± 0.2 °C. Immediately after the onset of heatstroke, the heating pad was removed and the animals were allowed to recover at room temperature (24 °C). Our pilot study showed that the latency for the onset of heatstroke (time interval between the start of heat stress and the onset of heatstroke) was found to be 70 ± 3 min for the heatstroke groups. Therefore, in the present study, the time point of 70 min denoted the onset of heatstroke, whereas the time point of 85 min denoted the 15th minute after the onset of heatstroke. The experimental design is summarized in Figure 1.

**Measurement of Cardiac Output.** The animal's trachea was intubated, and the animal was provided artificial respiration at 50 breaths/min, with tidal volume of 20 mL and inspiration-to-expiration ratio at 1:2. A 3S transonic flow probe (Transonic System, Taconic, NY) was implanted around the ascending aorta as described by Smith.[8] Briefly, the chest was opened at the third intercostal space to expose the heart. A small section (1 cm long) of the ascending aorta was freed from connective tissue. The flow probe was then implanted around the root of the ascending aorta. The chest incision was closed, and a negative intrathoracic pressure was restored. The values of total peripheral resistance (TPR) were obtained by dividing MAP by cardiac output (CO). The values of stroke volume (SV) were obtained by dividing CO by HR.

**Heart Sample Preparation.** All hearts were harvested by 0.9% saline, washed, pulverized under liquid $N_2$, and lysed in lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 1 mM EDTA, 1 mM PMSF, 0.5% cocktail, and 100 mM dithiothreitol). Then, the samples were pulverized in sample grinding kit (Amersham-Pharmacia Biotech), and the sample solutions were centrifuged at 12 000*g* for 15 min. The supernatant was collected and centrifuged again at 75 000*g* in ultracentrifuge (Optima, Beckman). After centrifugation, the supernatant was cleaned with 2-D cleanup kit (Amersham-Pharmacia Biotech) and the protein pellet was dissolved in rehydration buffer and stored at −80 °C. The protein concentration was determined by DC protein assay kit (Bio-Rad).

**Isoelectric Focusing (IEF).** The Immobilized pH gradient (IPG) gel strips (GE Healthcare Bioscience) (18 cm, pH 4−7) were rehydrated for 16 h with 280 $\mu$L of rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 2% dithiothreitol, 0.5% IPG buffer and trace amount of bromophenol blue) containing 100 $\mu$g of protein. The proteins were then focused at 200, 1000, and 8000 V with a total of 32 000 Vh using IPGphor electrophoresis unit (Amersham-Pharmacia Biotech).

**SDS-PAGE.** After isoelectric focusing, the gel strips were equilibrated in equilibration buffer (6 M urea, 30% glycerol, 2% SDS) containing 2% dithiothreitol for 15 min and in equilibration buffer containing 5% iodoacetamide for a further 30 min. The gel was loaded onto the top of acrylamide gel (12.5%) and sealed with 0.5% agarose. The proteins were separated at 210 V per gel until bromophenol blue reached the bottom of the gel.

**Silver Staining.** The resulting gels were then visualized by silver stain. Briefly, the gel was fixed in fixation solution (ethanol/water/acetic acid, 4/5/1, v/v) after electrophoresis and treated with sensitizing solutions (0.5 M sodium acetate, 0.5% sodium thiosulphate) for 30 min. After sensitization, the gels were washed and incubated in 0.25% silver nitrate solutions for 20 min and then developed by incubating with the developing solution (2.5% sodium carbonate and 0.015% formaldehyde). All used two-dimensional electrophoresis protocol as described elsewhere.[5]

**Image Analysis.** To look for proteins showing disparity in expression, the proteome maps of heart tissues obtained from both normothermic controls and heatstroke rats were first analyzed by Image Master 2D Elite (Pharmacia) and by PDQuest 7.0 (Bio-Rad) softwares. A total of 9 pairs of well-focused gels from heart tissues of two groups of rat were compared. Differentially expressed spots were detected by computer analysis. The intensity of spots was calculated and normalized as percentage of total amount of all spots in the gel and analyzed by Student's *t* test. In all cases, a *P*-value less than 0.05 was considered as significantly different.

**In-Gel Digestion.** The in-gel digestion and mass spectrometric analysis were carried out as described previously[9] with

some modifications. The spots excised from the gel were incubated in a solution containing 15 mM of potassium ferricynate and 50 mM sodium thiosulfate until the brownish color disappeared. The destained gel piece was washed with 25 mM ammonium bicarbonate for 10 min and then with 25 mM ammonium bicarbonate/50% acetonitrile for 10 min. After drying in SpeedVac (ThermoSavant), the gel was incubated with 50 $\mu$L of 2% (v/v) 2-mercaptoethanol in the dark for 20 min and an equal volume of 10% (v/v) vinylpyridine in 25 mM ammonium bicarbonate/50% acetonitrile was added and incubated for a further 20 min. Then the gel was washed three times with 25 mM ammonium bicarbonate and dehydrated in 25 mM ammonium bicarbonate/50% acetonitrile. The gel was dried and treated with 50 ng of modified trypsin (Promega) in 100 $\mu$L of 25 mM ammonium bicarbonate at 37 °C overnight. The supernatant was collected after digestion and the gel was extracted with 200 $\mu$L of 0.1% formic acid. The extracts were combined and dried in SpeedVac and resuspended in 0.1% formic acid immediately for mass spectrometric analysis or stored at −20 °C until use.

**Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS).** Electrospray ionization (ESI) tandem mass spectrometry was performed using a ThermoFinnigan LCQ Deca ion trap mass spectrometer interfaced with an Agilent 1100 HPLC system. The digested proteins were separated in Agilent ZORBAX 300SB-C18 column (50 × 0.3 mm, 3 $\mu$m particle diameter, 300 Å pore size) using a mobile phase of solution A (0.1% formic acid in water) and solution B (0.1% formic acid in acetonitrile). The proteins were eluted at a flow rate of 1 $\mu$L/min with an acetonitrile gradient consisting of 5−16% solution B in 5 min, 16−20% solution B in 40 min, and 20−65% solution B in 40 min. The proteins eluted from the HPLC were introduced online to the ESI source and the spectra were acquired as successive sets of three scan modes (MS, Zoom and MS/MS scans) as described previously.[9] The acquired collision induced dissociation spectra were interpreted with TurboSequest software (ThermoFinnigan) that matches tandem mass spectra against nonredundant protein database. The matched proteins were selected based on four criteria: (i) cross correlation score (Xcore) = 2.0, 2.5, and 4.0 for proteins with charges +1, +2, and +3, respectively; (ii) delta cross correlation score (dCn) > 0.15; (iii) ranking of the primary score (Rsp) > 5; and (iv) primary score (Sp) < 200.

**Protein Extraction and Western Blot Analysis.** The heart tissue was ground under liquid nitrogen using a mortar and pestle, then mixed with RIPA lysis buffer [50 mM Tris-HCl pH 7.5, 0.1 M NaCl pH 7.5, 0.25% Na-deoxycholate, 1% IGEPAL-630, proteinase inhibitor cocktail tablet (Roche), phosphatase inhibitors with 1 mM $Na_3VO_4$, 1 mM NaF] and spun down at 12 000*g* for 5 min. The supernatant was collected and stored at −70 °C. Proteins were quantified with the DC protein assay kit (Bio-Rad) and 2× sample buffer (1 M Tris-HCl pH 6.8, 10% SDS, 0.01% $\beta$-ME, 0.01% Bromophenol blue, 20% Glycerol) and then separated in 10∼12% gradient sodiumdodecyl sulfate-polyacrylamide gel electrophoresis at 150 V for 3.5∼4 h. Electrophoresed proteins were transferred to PVDF membranes (Bio-Rad semidry transfer system) at 1.2 mA/cm$^2$ for 1.5 h. The PVDF membranes were incubated at room temperature for 1 h in blocking buffer (PBST, 5% (v/v) defat milk). Primary antibodies of anti-αB crystallin (stressgen), anti-HSP27 (upstate), anti-HSP20 (upstate), anti-MTCO1 (abcam) were diluted in PBST buffer (137 mM NaCl, 2.7 mM KCl, 8 mM $Na_2HPO_4$, 2 mM $KH_2PO_4$ pH 7.4, 0.1% (v/v) Tween 20). The membranes were incubated at room temperature for 1 h. The immunoblots were washed four times in PBST buffer for 15 min, immersed in the secondary antibody solution containing anti-rabbit (anti-HSP27, anti-HSP20) and anti-mouse (anti-αB crystallin, MT-CO1) antibodies for 1 h, and then diluted 15 000-fold in PBST buffer. The membranes were then washed in PBST buffer for 15 min four times and detected in ECL (PerkinElmer).

**Adenine Phosphoribosyltransferase (APRT) Assay Procedure.** Cell homogenates for enzyme assay were prepared from rat heart tissue. Homogenization buffer containing 150 mM KCl, 20 mM Tris, 1 mM EDTA, 1 mM dithiothreitol (pH 7.0) plus 0.1% Triton X-100 (0.1 mL) was added in three portions to maximize cell recovery. Heart tissue homogenates were diluted with incubation buffer before assay. The incubation buffer consisted of 50 mM imidazole and 10 mM $MgCl_2$ (pH 7.0). The reaction was initiated by the addition of 20 $\mu$L of diluted cell extract to an equal volume of substrate solution, incubated at 37 °C for 10 min and terminated by the addition of 20 $\mu$L of 1.3 M $HClO_4$. The substrate solution contained 8 mM PRPP, 200 $\mu$M methylene adenosine 5′-diphosphate (AOPCP), 200 $\mu$M [8-$^{14}$C]adenine with a specific activity of 12.5 $\mu$Ci/$\mu$mol, all dissolved in incubation buffer. For blanks, perchloric acid was added to the substrate solution prior to the addition of cell extract. Samples were neutralized with 7 $\mu$L of 3 M $K_3PO_4$ and centrifuged at 13 000*g* for 3 min. The resultant supernatant was analyzed for conversion of substrate to products by collection of radioactive peaks separated by reverse-phase HPLC. The reaction was linear with respect to the amount of cellular protein and the incubation time for all the different heart tissues.

## Results and Discussion

**Heatstroke Causes Cardiac Dysfunction.** When the animals were exposed to a high ambient temperature, hypotension or circulatory shock may result from translocation of blood from the vital organs, including the heart and brain, to the skin in order to dissipate excessive heat.[10−12] Although only 32−35% of heatstroke patients are hypotensive when presented to the hospital,[13,14] hypotension does consistently occur during heatstroke in animal studies.[15] Hypotension leads to both splanchnic and cerebral ischemia which results in systemic inflammation, hypercoagulable state, multiorgan dysfunction and death during heatstroke. It turns out that maintaining an appropriate level of arterial pressure is an attractive target for therapy in heatstroke.

The present results further demonstrate that in the rat, heatstroke induces hypotension by reducing stroke volume, heart beat, and peripheral vascular resistance. As shown in Table 1, the values of core temperature (Tco) are significantly higher at 70−85 min after initiation of heat stress compared with those of normothermic controls. In contrast, the values of mean arterial pressure (MAP), heart rate (HR), cardiac output (CO), and total peripheral vascular resistance (TPR) are all significantly lower than those of the normothermic controls at 70−85 min after initiation of heat stress.

**Heatstroke Induces Alterations of Cardiac Proteins.** In an attempt to identify the cardiac proteins possibly involved in the heatstroke-induced cardiac dysfunction, heart samples are obtained at 85 min after the initiation of heat stress in untreated heatstroke rats or the equivalent time in normothermic controls.

Figure 2 illustrates the representative proteome maps of heart samples after separation by electrophoresis and visualization by silver staining. The proteins are well-separated in the

**Table 1.** Effect of Heat Exposure (43°C for 70 min) on Core Temperature (Tco), Mean Arterial Pressure (MAP), Cardiac Output (CO), Stroke Volume (SV), and Total Peripheral Vascular Resistance (TPR) in Normothermic Controls (NC), Heatstroke Rats (HS), and Heatstroke Rats Treated with Whole Body Cooling (HS+C)[a]

| groups/time course | Tco (°C) | MAP (mmHg) | HR (heat/min) | CO (mL/min) | SV (mL/heat) | TPR (mmHg/min mL$^{-1}$) |
|---|---|---|---|---|---|---|
| | | | **NC** | | | |
| 0 min | 36.1 ± 0.4 | 111 ± 2 | 370 ± 14 | 44 ± 4 | 0.12 ± 0.03 | 2.3 ± 0.2 |
| 70 min | 36.2 ± 0.3 | 112 ± 3 | 358 ± 16 | 48 ± 3 | 0.14 ± 0.04 | 2.2 ± 0.3 |
| 85 min | 36.3 ± 0.3 | 114 ± 3 | 364 ± 15 | 46 ± 4 | 0.13 ± 0.02 | 2.1 ± 0.2 |
| | | | **HS** | | | |
| 0 min | 36.3 ± 0.3 | 112 ± 4 | 372 ± 15 | 48 ± 5 | 0.13 ± 0.02 | 2.2 ± 0.2 |
| 70 min | 43.2 ± 0.5* | 88 ± 3* | 251 ± 14* | 36 ± 3* | 0.06 ± 0.01* | 1.0 ± 0.1* |
| 85 min | 42.3 ± 0.3* | 42 ± 4* | 83 ± 6* | 5 ± 1* | 0.03 ± 0.01* | 0.6 ± 0.1* |
| | | | **HS+C** | | | |
| 0 min | 36.2 ± 0.2 | 109 ± 3 | 377 ± 16 | 46 ± 4 | 0.13 ± 0.02 | 2.3 ± 0.2 |
| 70 min | 43.1 ± 0.4* | 89 ± 4* | 253 ± 15* | 38 ± 3* | 0.08 ± 0.01* | 1.0 ± 0.1* |
| 85 min | 36.5 ± 0.3† | 67 ± 3† | 346 ± 14† | 42 ± 3† | 0.12 ± 0.01† | 2.0 ± 0.2† |

[a] Values are means ± SEM of 8 rats per group. HS groups, exposed to 43°C, had heat exposure withdrawn at 70 min and then were allowed to recover at 24°C. In NC group, the animal were kept at 24°C throughout the whole course of experiments. In HS+C group, whole body cooling was adopted at time "70 min" for 15 min. *$P$ < 0.05 compared with corresponding control values in NC group; †$P$ < 0.05 compared with HS group (ANOVA followed by Duncan's test).

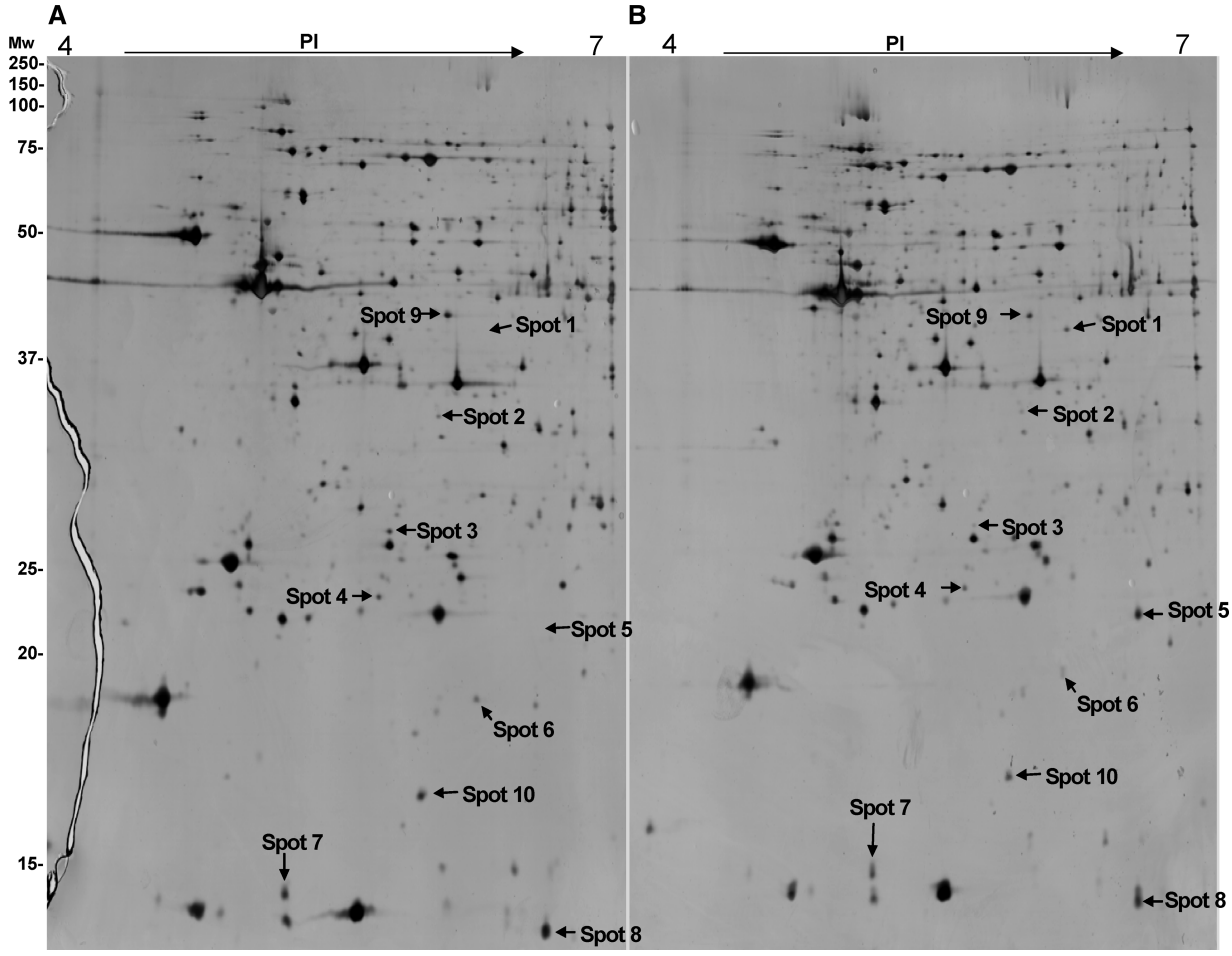


**Figure 2.** 2-DE pattern of a normothermic control rat (NC) (A) and a heatstroke rat (HS) (B) heart samples. Image and statistical analysis revealed that the differential protein expression between A and B and 10 deregulated spots were observed after comparison of 6 pairs of well-separated profiles using PDQUEST software.

18 cm gel with PH range 4−7. Each gel resolved up to ~780 protein spots. Image and statistical analysis reveals that the differential protein expression between normothermic control and heatstroke heart samples and 10 deregulated spots are observed after comparison of 9 pairs of well-separated profiles using PDQUEST software. The differentially expressed protein spots are scored when the difference is greater than 2-fold in magnitude. Of the deregulated spots (indicated by arrows in Figure 2) that are consistently found in our studies, eight are down-regulated (spots 2, 3, 4, 6, 7, 8, 9, 10) and two are up-regulated (spots 1 and 5). The selected areas in the gel containing each differentially expressed spot are amplified in

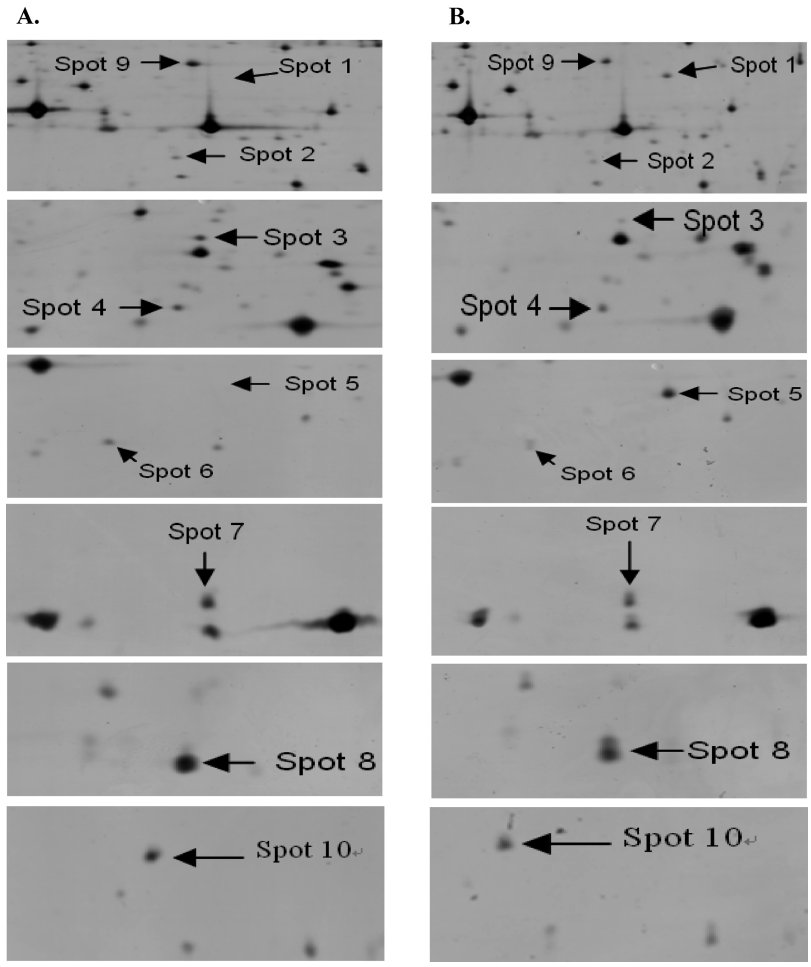


**Figure 3.** Differential protein expressing during heatstroke. Selected area of the spots showing intensity difference between normothermic control (NC) (A) and heatstroke (HS) (B) rat heart samples were amplified and indicated by arrows.

Figure 3. The variation between gels using the sample and between animals in each group is statistically insignificant. For example, the numbers of protein spots (Mean ± SD) are 789 ± 85 ($n$ = 9) and 784 ± 84 ($n$ = 9) for normothermic controls and heatstroke animals, respectively. In addition, the match rate is found to be 96−100%.

After mapping the proteome, the proteins with altered expression are identified (Table 2). For this purpose, spots are excised, digested with trypsin, and analyzed by tandem mass spectrometry as described in Materials and Methods. The results of mass spectrometric analyses are summarized in Table 3. The down-regulated spots (spot 2−4, 6−10) in heatstroke heart samples are identified as annexin III, HSP27, APRTase, HSP20, MTC01, NDUFA5, NDUFA10, and MYHC-alpha. The up-regulated spots (spots 1 and 5) were identified as NDUFA10 and alpha B crystallin. Apparently, spot 1 shares with spot 9 the same protein NDUFA10. Although a more quantitative or specific approach (such as DIGE or immunohistochemistry) was not used, we performed Western blotting technique to confirm the data in the current study.

**Whole Body Cooling Causes Attenuation of Heatstroke-Induced Hypotension.** Whole body cooling is the current therapy of choice for heatstroke because no pharmacologic agent is available.[1,4] Our previous results have shown that whole body cooling improves survival during heatstroke by attenuating circulatory shock and cerebral ischemia.[16] The present findings further show that whole body cooling causes amelioration of hypotension by reducing stroke volume, heart beat, and total peripheral resistance in the rat during heatstroke (as shown in Table 1).

**Whole Body Cooling Causes Normalization of Cardiac Protein Expression during Heatstroke.** Small molecular weight HSPs, including HSP27, HSP20, and alpha B crystallin, possess cytoprotection and the intracellular assembly, folding, and translocation of oligometric proteins and represent a rapid response to altered redox states.[17,18] To validate spots 3, 6, and 5 of Figure 3 as HSP27, HSP20, and alpha B crystallin, we performed Western blotting with anti-alpha B crystallin, anti-HSP20, and anti-HSP20 antibodies, respectively (Figures 4−6). The Western blotting shows that the relative expressions of both HSP27 and HSP20 are decreased while the relative expression of alpha B crystallin is increased during heatstroke. The heatstroke-induced decreased cardiac expressions of both HSP27 and HSP20 are significantly reversed by whole body cooling. On the other hand, the increased cardiac expression of alpha B crystallin during heatstroke is further enhanced by whole body cooling. These findings suggest that the decreased cardiac HSP20 and HSP27 may play a compensatory role in the pathophysiology of heatstroke-induced cardiac dysfunction. However, alpha B crystallin may play a cardioprotective role against the progression of cardiac dysfunction during heatstroke. In fact, overproduction of HSP20,[19] HSP27,[20−22] and alpha B crystallin[23−25] by transfection into rodent and Chinese hamster cell lines is found to be correlated with survival from

**Table 2.** Protein Identification of Cardiac Tissue Protein Markers in Heat Stroke Heart Tissues by LC-MS/MS Based on the Swiss-Prot Database

| spot no. | protein identity | Swiss-Prot acc. no. | % coverage[a] | experimental p*I*/molecular mass (kDa) | theoretical p*I*/molecular mass[b] (kDa) | spectra mached | identitfied sequences (Xcorr, dCn, RSP)[c] |
|---|---|---|---|---|---|---|---|
| 1 | NADH dehydrogenase 1 alpha subcomplex 10-like protein (NAUFA 10) | Q561S0 | 23 | 6.3/41 | 7.14/40.5 | 16 | YGLLASILGDK (3.61, 0.41, 1) |
| | | | | | | | VITVDGNICSGK (1.08, 0.03, 38) |
| | | | | | | | DIAEQLGMK (3.16,0.39, 1) |
| | | | | | | | LQSWLYASR (2.55, 0.26, 1) |
| | | | | | | | VTSAYLQDIEDAYKK (4.25, 0.52, 1) |
| | | | | | | | VVEDIEYLNYNK (5.34, 0.53, 1) |
| | | | | | | | IYDSFR (1.49, 0.19, 124) |
| | | | | | | | YAPGYNADVGDK (2.23, 0.46, 1) |
| 5 | Alpha-Crystallin B chain | P23928 | 39 | 6.5/21 | 6.76/20 | 12 | RPFFPFHSPSR (2.51, 0.35, 73) |
| | | | | | | | DRFSVNLDVK (1.83, 0.09, 6) |
| | | | | | | | HFSPEELK (2.95, 0.21, 4) |
| | | | | | | | VLGDVIEVHGK (3.6, 0.41, 1) |
| | | | | | | | QDEHGFISR (1.89, 0.05, 34) |
| | | | | | | | TIPITR (1.57, 0.09, 6) |
| | | | | | | | EEKPAVTAAPK (1.39, 0.24, 1) |
| 2 | Annexin III | P14669 | 24 | 6.1/36 | 5.96/36 | 9 | QYQEAYEQALK (2.95, 0.44, 2) |
| | | | | | | | EISQAYYTAYK (2.65, 0.36, 1) |
| | | | | | | | DDISSETSGDFRK (2.97, 0.58, 1) |
| | | | | | | | DESLKVDEHLAK (2.01, 0.09, 1) |
| | | | | | | | DAQTLYDAGEKK (2.27, 0.28, 1) |
| | | | | | | | NTPAFLAGR (1.70, 0.31, 3) |
| | | | | | | | GAGTDEFTLNR (3.46, 0.35, 1) |
| 3 | Heat-shock protein beta-1 (HSP 27) | P42930 | 34 | 5.9/26 | 6.12/22.8 | 7 | SPSWEPFR (2.05, 0.08, 13) |
| | | | | | | | DWYPAHSR (1.67, 0.18, 4) |
| | | | | | | | LFDQAFGVPR (3.31, 0.31, 4) |
| | | | | | | | QLSSGVSEIR (1.80, 0.07, 42) |
| | | | | | | | VSLDVNHFAPEELTVK (2.26, 0.26, 1) |
| | | | | | | | AVTQSAEITIPVTFEAR (3.44, 0.41, 1) |
| 4 | Adenine phosphoribosyltransferase (APRTase) | P36972 | 21 | 5.8/23 | 6.17/19.5 | 4 | DISPLLKDPDSFR (3.96, 0.50, |
| | | | | | | | 1)IDYIAGLDSR (2.29, 0.23, 1) |
| | | | | | | | AELEIQKDALEPGQK (2.06, 0.12, 4) |
| 6 | Alpha B Crystallin-related protein (HSP 20) | P97541 | 35 | 6.2/19 | 6.05/17.5 | 10 | VPVQPSWLR (2.64, 0.32, 1) |
| | | | | | | | RASAPLPGFSTPGR (3.92, 0.43, 1) |
| | | | | | | | HFSPEEISVK (2.69, 0.31, 1) |
| | | | | | | | VVGDHVEVHAR (1.34, 0.06, 36) |
| | | | | | | | HEERPDEHGFIAR (3.08, 0.32, 1) |
| 7 | Cytochrome c oxidase polypeptide Vb, mitochondrial precursor (MTCO1) | P12075 | 32 | 5.1/14 | 7.68/13.9 | 6 | EIMIAAQR (2.55, 0.04, 1) |
| | | | | | | | GLDPYNMLPPK (2.20, 0.27, 5) |
| | | | | | | | EDPNLVPSVSNK (1.63, 0.36, 1) |
| | | | | | | | LVPYQMVH (1.63, 0.24, 2) |
| 8 | NADH:ubiquinone oxidoreductase (NAUFA 5) | Q63362 | 55 | 6.5/13 | 7.07/13.2 | 11 | TTGLVGLAVCDTPHER (3.06, 0.49, 2) |
| | | | | | | | LTILYTK (1.91, 0.13, 14)) |
| | | | | | | | ILDLLK (2.11, 0.06, 1) |
| | | | | | | | KYTEQITSEKLELVK (4.58, 0.54, 1) |
| | | | | | | | KLENLLQGGEVEEVILQAEK (4.55, 0.50, 1) |
| 9 | NADH dehydrogenase 1 alpha subcomplex 10-like protein (NAUFA 10) | Q561S0 | 30 | 6.1/41 | 7.14/40.5 | 16 | YGLLASILGDK (3.79, 0.49, 1) |
| | | | | | | | VITVDGNICSGK (2.35, 0.38, 1) |
| | | | | | | | DIAEQLGMK (3.18, 0.30, 1) |
| | | | | | | | HYPEAGIQYSSSTTGDGR (3.74, 0.38, 1) |
| | | | | | | | LQSWLYASR (2.49, 0.37, 1) |
| | | | | | | | VTSAYLQDIEDAYKK (2.21, 0.16, 1) |
| | | | | | | | VVEDIEYLNYNK (5.02, 0.51, 1) |
| | | | | | | | IYDSFR (1.46, 0.01, 2) |
| | | | | | | | KYAPGYNADVGDK (2.84, 0.38, 1) |
| 10 | MYH6_RAT Myosin heavy chain, cardiac muscle alpha isoform (MyHC-alpha) | P02563 | 0.3 | 5.9/17 | 5.59/223 | 5 | TECFVPDDKEEYVK (2.37, 0.59, 1) |
| | | | | | | | EDQVMQQNPPKFDK (1.86, 0.21, 1) |
| | | | | | | | SEAPPHIFSISDNAYQYMLTDR (3.51, 0.65, 1) |
| | | | | | | | ENQSILITGESGAGK (4.87, 0.52, 1) |

[a] Total percentage of sequence coverage of the matched peptides. [b] Theoretical p*I* and mass obtained from the Swiss-Prot database. [c] Xcorr = cross-correlation, dCn = delta cross correlation score, RSp = ranking of preliminary score.

heatstroke. In addition, ischemia injury in cardiac myocytes can be protected by overproduction of both HSP27 and alpha B crystallin.[26] In vitro studies further show that overexproduction of HSP20 protects against necrotic and apoptotic death of cardiomyocytes.[27] Taking these observations together, we propose that WBC might cause attenuation of cardiac dysfunc-

**Table 3.** Protein and Gene Name Identification, Spot Quantity Ratio/Regulation and Description of Cardiac Protein Markers in Heat Stroke Heart Tissues

| spot no. | protein identity (gene name) | quantity ratio (HC/NC)/ regulation[a] | description |
|---|---|---|---|
| 1 | NADH dehydrogenase 1 alpha subcomplex 10-like protein, NAUFA 10 (Ndufa 10) | 2.5/Up | This protein is a component of the hydrophobic protein fraction and has NADH dehydrogenase activity and oxidoreductase activity. It transfers electrons from NADH to the respiratory chain. |
| 5 | Alpha-crystallin B chain (Cryab) | 5.0/Up | Hsp27 and αB-crystallin share considerable sequence and structural similarity, associate in vivo, and are induced by oxidative stress. Both small heat shock proteins function as molecular chaperones in protein biosynthesis to facilitate protein folding and translocation. Protective effects of the small heat shock proteins have been described against several noxious stresses like hyperthermia, hypertonic stress, and various cytotoxic agents, including cytoskeletal disruptors, and ischemia-mediated injury in cardiac myocytes. |
| 2 | Annexin III (Anxa3) | 0.2/Down | Inhibitor of phospholipase $A_2$, also possesses anticoagulant properties. Interacting selectively with phospholipids, a class of lipids containing phosphoric acid as a mono- or diester, in the presence of calcium. Annexins have been implicated in various cellular functions such as exocytosis, formation or modulation of ion channels, and membrane attachment of cytoskeletal elements. |
| 3 | Heat-shock protein beta-1, HSP 27 (Hspb1) | 0.05/Down | Small HSP, including HSP20, HSP25, HSP27, αB-crystallin, and myotonic dystrophy kinase binding protein, are a group of proteins expressed in muscle tissues and share sequence homology of approximately 80−100 amino acids at the C terminus, known as the α crystallin domain. It was reported that overexpression of HSP27 by transfection into rodent and Chinese hamster cell lines directly correlated with survival from hyperthermia. Overexpression of αB-crystallin has a similar effect. It was also reported that overexpression of both HSP27 and αB-crystallin protected against ischemic injury in cardiac myocytes. |
| 4 | Adenine phosphoribosyltransferase, APRTase (Aprt) | 0.5/Down | APRTase is an enzyme involved in the purine nucleotide salvage pathway. It functions as a catalyst in the reaction between adenine and phosphoribosyl pyrophosphate (PRPP) to form AMP. |
| 6 | Alpha B crystallin-related protein, HSP 20 (Hspb6) | 0.4/Down | B crystallin mRNA and protein have been found to occur in high levels in heart lung, skeletal muscle, and kidney. In heart, B crystallin is bound to myocardial cytoskeletal proteins, suggesting its role in cytoskeletal stabilization. HSP20 is a newly discovered small HSP that was copurified with αB-crystallin and HSP27 from skeletal muscle by affinity chromatography. Previous reports demonstrated that HSP20 redistributed from the cytosol to insoluble fractions and dissociated from the aggregated form to the small form when rat diaphragm was exposed to heat stress in vitro. Stable overexpression of HSP20 in Chinese hamster ovary cells results in enhanced survival after heat shock, which is similar to the results for αB-crystallin. The cardioprotective effect of HSP20 in vitro might be mediated mainly by inhibiting cardiomyocyte necrotic and apoptotic cell death, possibly via the PKC/mito $K^+_{ATP}$ pathway. Hsp20 has been shown to regulate vasodilation21 and suppress platelet aggregation. |
| 7 | Cytochrome c oxidase polypeptide Vb, mitochondrial precursor, MTCO1, (Cox5b) | 0.5/Down | This protein is one of the nuclear-coded polypeptide chains of cytochrome c oxidase, the terminal oxidase in mitochondrial electron transport. |
| 8 | NADH:ubiquinone oxidoreductase, NAUFA5 (Ndufa5) | 0.4/Down | The NADH:ubiquinone oxidoreductase (Complex I), provides the input to the respiratory chain from the NAD-linked dehydrogenases of the citric acid cycle. The complex couples the oxidation of NADH and the reduction of ubiquinone, to the generation of a proton gradient which is then used for ATP synthesis. |
| 9 | NADH dehydrogenase 1 alpha subcomplex 10-like protein, NAUFA 10 (Ndufa10) | 0.6/Down | Transfer of electrons from NADH to the respiratory chain. The immediate electron acceptor for the enzyme is believed to be ubiquinone. This protein is a component of the hydrophobic protein fraction and has NADH dehydrogenase activity and oxidoreductase activity. |
| 10 | MYH6_RAT Myosin heavy chain, cardiac muscle alpha isoform, MyHC-alpha (Myh6) | 0.3/Down | Two isoforms of MyHC (and) are expressed in mammalian heart. Myosin consisting of MyHC has a higher ATPase activity than myosin composed of MyHC, and in the rodent heart, contractile velocity correlates with the relative amount of each MyHC. |

[a] The spot quantity ratio is defined as up- and downregulation of expressed protein depending on protein spot intensity.

tion during heatstroke by enhancing cardiac expression of HSP20, HSP27, and alpha B crystallin simultaneously. Although a possible protective role of alpha B crystallin has been described in vitro, to demonstrate a protective role in vivo is a much more complex matter.

Evidence has accumulated to indicate that mitochondrial dysfunction leads to impaired energy production, increased intracellular calcium, generation of free radicals, activation of the mitochondrial permeability transition, and secondary excitotoxicity.[28] More specifically, damage to the mitochondrial respiratory chain has been suggested to be an important factor in the pathogenesis of a variety of neurodegenerative disorders.[29] Cytochrome C oxidase catalyzes the last step in the electron-transport chain (ETC) and results in the transfer of electron from cytochrome C to molecular oxygen.[30] Inhibition of cytochrome C oxidase activity also leads to increased production of mitochondrial reactive oxygen species via indirect actions on complexes I and III of the ETC.[31] MTCO 1 is the largest of the five mitochondrial ETC complexes with an approximate molecular weight of 98 kDa. There is strong evidence that mitochondrial MTCO 1 inhibition can be induced in part through accumulated damage caused by oxidative stress.[32−34] MTCO 1 deficiency represents one of the most severe and frequent disorders in the mitochondrial energy metabolism associated with human diseases.[35] Adenine phosphoribosyl transferase (APRTase) is also responsible for the

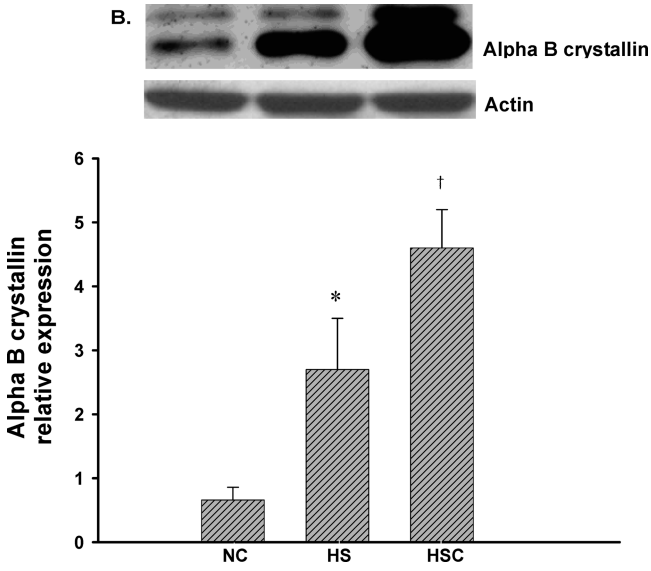


**Figure 4.** Increased total alpha B crystallin levels during heatstroke could be enhanced by whole body cooling. (Upper panel) Western blot analysis of alpha B crystallin in heart extracts from a normothermic control (NC), a heatstroke rat (HS), and a heatstroke rat treated with whole body cooling (HS+C). (Lower panel) Band intensities were quantitated by densitometry (Quantity one, Bio-Rad). *$P < 0.05$, compared with NC group; †$P < 0.05$, compared with HS group (Student's $t$ test). The data are represented as means ± SEM of 5 rats for each group.

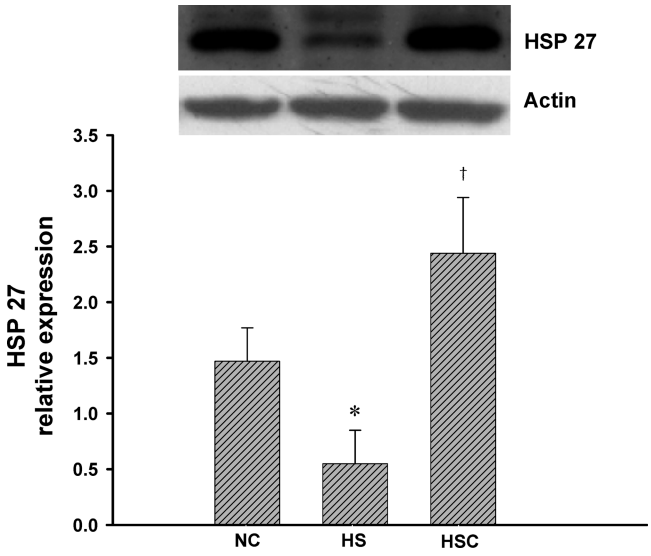


**Figure 5.** Decreased HS P27 expression during heatstroke could be reversed by whole body cooling. (Upper panel) Western blot analysis of HSP27 in heart extracts from a normothermic control (NC), a heatstroke rat (HS), and a heatstroke rat treated with whole body cooling (HS+S). (Lower panel) Band intensities were quantitated by densitometry (Quantity one, Bio-Rad). *$P < 0.05$, compared with NC group; †$P < 0.05$, compared with HS group (Student's $t$ test). The data are represented as means ± SEM of 5 rats for each group. Ta, ambient temperature; Tco, core temperature.

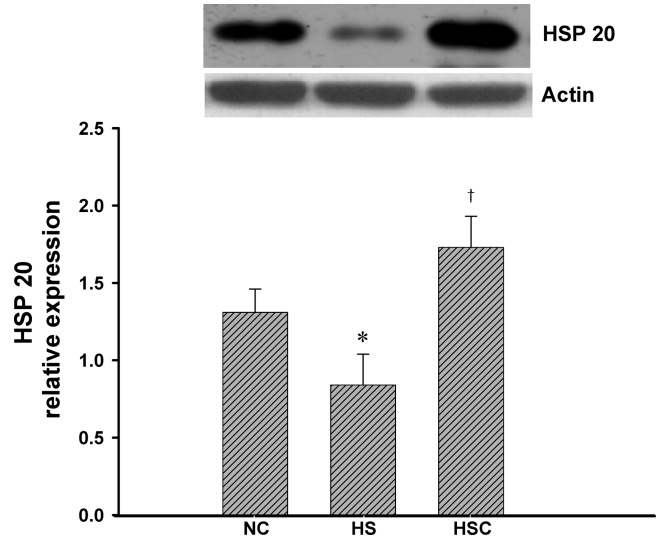


**Figure 6.** Decreased HS P20 expression during heatstroke could be reversed by whole body cooling. (Upper panel) Western blot analysis of HSP20 in heart extracts from a normothermic control (NC), a heatstroke rat (HS), and a heatstroke rat treated with whole body cooling (HS+S). (Lower panel) Band intensities were quantitated by densitometry (Quantity one, Bio-Rad). *$P < 0.05$, compared with NC group; †$P < 0.05$, compared with HS group (Student's $t$ test). The data are represented as means ± SEM of 5 rats for each group.

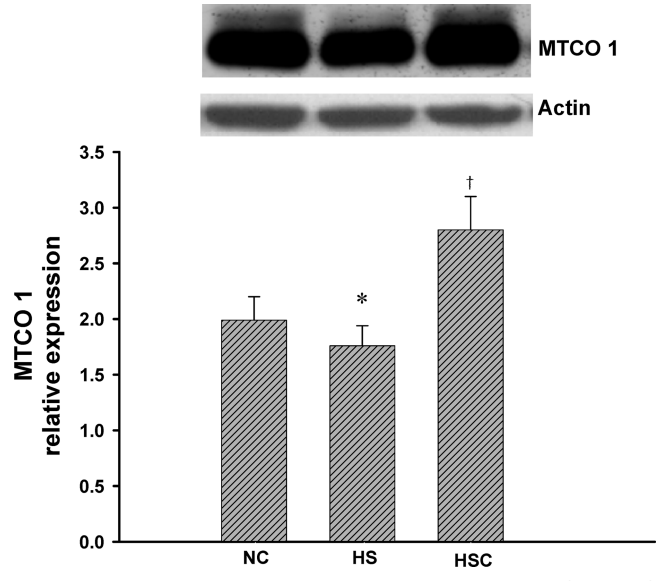


**Figure 7.** Decreased Cytochrome c oxidase subunit 1 (MTCO1) expression during heatstroke could be reversed by whole body cooling. (Upper panel) Western blot analysis of MTCO1 in heart extracts from a normothermic control (NC), a heatstroke rat (HS), and a heatstroke rat treated with whole body cooling (HS+S). Tissues were harvested at 85 min after the start of heat exposure. (Lower panel) Band intensities were quantitated by densitometry (Quantity one, Bio-Rad). *$P < 0.05$, compared with NC group; †$P < 0.05$, compared with HS group (Student's $t$ test).

reversible production of adenosine monophosphate and pyrophosphate from adenine and 5-phospho-α-D-ribosyl-1-pyrophsphate.[36] Adenosine is rapidly metabolized in the mitochondrial space to the following metabolites: Adenosine → Inosine → Hypoxanthine → Xanthine → Uric acid. Interstitial concentration of the adenosine metabolites increased during myocardial ischemia.[37] Indeed, as shown in both Figures 7 and 8, the myocardial expression of both MTCO 1 and APRTase are decreased during heatstroke, which could be reversed by WBC. In the present findings, WBC may cause attenuation of cardiac dysfunction during heatstroke by reversing the decreased expression of both MTCO 1 and APRTase in rat myocardium.

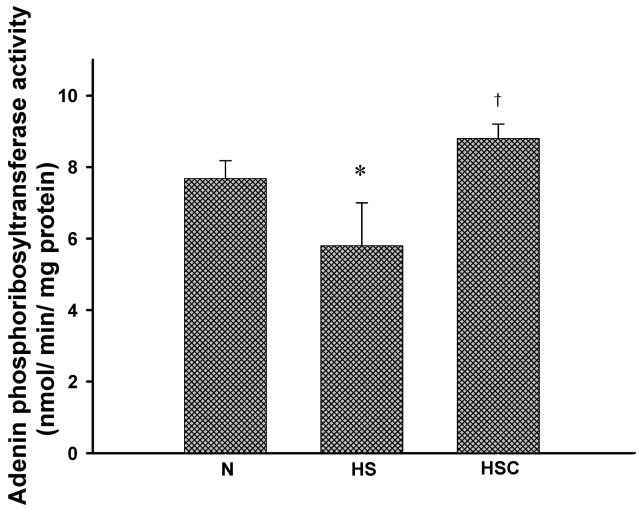


**Figure 8.** Decreased xanthine levels during heatstroke could be revered by whole body cooling. ELISA analysis of xanthine in heart extracts from a normothermic control (NC), a heatstroke rat (HS), and a heatstroke rat treated with whole body cooling (HS+S). $^{*}P < 0.05$, compared with NC group; $^{\dagger}P < 0.05$, compared with HS group (Student's *t* test). The data are represented as means ± SEM of 5 rats for each group.

A novel phopho-specific, fluorescent dye has been introduced to identify phosphoproteins. Using this method, Schulenburg and colleagues[38,39] observe staining of two subunits of MTCO 1 with molecular weight of 42 and 18 kDa, respectively. In these experiments, the protein migrating at 42 kDa is assigned by mass spectrometry as NDUFA10. This observation indicates that protein phosphorylation is the basis for this heterogeneity. It is worth noting that NDUFA10 has been referred to as "mammalian specific" subunit of MTCO 1.[40] In the current experiment, the myocardial expression of NDUFA10 is up- and downregulated during heatstroke (as shown in Table 3). Future studies are required to examine the consequence of NDUFA10 phosphorylation in heatstroke animal treated or untreated with WBC.

Annexin III is also called "lipocortin 3" or "placental anticoagulant protein 3".[41] Annexin III has been shown to have anticoagulant and anti-phospholipase $A_2$ properties in vitro[42] and $Ca^{2+}$-dependent aggregation of isolated specific granules from human neutrophils.[43] The hypercoagulable state (evidenced by increased levels of activated partial thromhoplastin time, prothronbin time, and D-dimers, as well as decreased levels of platelet counts and protein C) that occurred during heat stroke is significantly suppressed by brain cooling.[7] In the present study, as shown in Table 3, cardiac annexin III expression is reduced during heat stroke. Again, future studies are required to assess the effect of whole body cooling on the reduced annexin III expression during heatstroke.

Genes encoding $\beta$-myosin heavy chain have been associated with dilated cadiomyopathy.[44] Two cardiac myosin heavy chain isoforms have been identified in humans with the genes tandemly located on chromosome 14.[45] MYH6 encodes αMYHC and MYH7 encodes $\beta$MYHC.[45] Both αMYHC and $\beta$MYHC are present in different amounts in mammalian hearts.[46] In humans, an MYH6 mutation has been found in one case of elderly onset sporadic hypertrophic cardiomyopathy.[47] Mutation in MYH6 may cause a spectrum of phenotype ranging from dilated cardiomyopathy to hypertrophic cardiomyopathy.[48] The present results demonstrate that decreased stroke volume is associated with decreased MYHC-alpha in rat myocardium during heatstroke.

**Cardiac Ischemia, Rather than Hyperthermia, Is the Main Cause of Heatstroke-Induced Cardiac Dysfunction.** As described in Figure 1, the Tco of the normothermic controls (NC) are maintained at 36.1−36.3 °C and heat stress increases the Tco from 36.0 to 43.2 °C. In addition, in HS+C animals, normal Tco are recovered (36.5 °C) after 15 min whole body cooling. It should be stated that cardiac ischemia (due to arterial hypotension), rather than hyperthermia, is the main cause of cardiac dysfunction. As mentioned before, heat stress induces hyperthermia, stimulates metabolism, and progressively reduces blood flow to the heart. Cellular ischemia and hypoxia in the heart can produce cellular injury and inflammation in sitsu. The current choice for treatment of heatstroke is whole body cooling.[1] However, heatstroke is often fatal following adequate body cooling.[49,50] Tissue damage continues to develop after cooling to normal body temperature in 25% of heatstroke patients.[51] It has also been reported that normal volunteers can passively endure a core temperature of about 42 °C with no or minimal tissue injury.[52,53] Evidence has accumulated to indicate that cardiac and cerebrovascular dysfunction, rather than hyperthermia, is an attractive target for therapy in heatstroke.[2,15] In the absence of body cooling, the heatstroke reactions can still be improved by the following measures, adopted immediately after the onset of heatstroke: (i) fluid replacement with 3% NaCl solution, 10% human albumin, or hydroxyl starch; (ii) intravenous delivery of anti-inflammatory drugs or free radical scavengers; (iii) hyperbaric oxygen therapy; or (iv) transplantation of human umbilical cord blood cells.[15] It is likely that these above-mentioned measures, like whole body cooling, may help to maintain normal proteomic mechanisms in hearts and to keep appropriate levels of blood pressure during heatstroke.

## Conclusions

In this study, we identify decreased protein expression spots as HSP27, APRTase, HSP20, MTCO1, annexin III, NDUFA5, NDUFA10 and MYHC-alpha. HSP27 and HSP20 are small molecular weight HSPs associated with cytoskeletal integration. APRTase, MTCO1, NDUFA5, and NDUFA10 are associated with mitochondria function, and MYHC-alpha is a muscle contractile protein. Annexin III is associated with anticoagulation. Therefore, failing hearts during heat stroke are characterized by reduced mitochondrial activity, and loss of the structural integrity of the cardiomyocytes. On the other hand, we identify 1 increased expression spot during heatstroke as alpha B crystallin, a cardiac protective protein against cardiac ischemia and damage. WBC, in addition to restoring the appropriate levels of HSP27, HSP20, APRTase and MTCO1, enhances preservation of alpha B crystallin and maintains appropriate cardiac function during heatstroke.

**Abbreviations:** 2D-DIGE, two-dimensional differential in-gel electrophoresis; WBC, whole body cooling; Ta, ambient temperature; Tco, core temperature; MAP, mean arterial pressure; CO, cardiac output; TPR, total peripheral resistance; SV, stroke volume; HR, heart rate; NAUFA 10, NADH dehydrogenase 1 alpha subcomplex 10-like protein; HSP27, heat shock protein beta-1; APRTase, Adenine phosphoribsyltransferase; HSP20, Alpha B crystallin-related protein; MTC01, Cytochrome C oxidase polypeptide Vb, mitochondrial precursor; NAUFA5, NADH: ubiquinone oxidoreductase; MyHC-alpha, MYH6 RAT Myosin heavy chain, cardiac muscle alpha isoform; NC, nor-

mothermic control; HS, a heatstroke rat; HS+S, a heatstroke rat treated with WBC.

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