Metal Ions in Life Sciences

Integrated Transcriptomic and Proteomic Characterization of Heat Stress–Induced Cardiac Tissue Injury in Experimental Rats

Dr. Lucas Meyer[1]*

[1] University of Zurich, Department of Translational Cardiovascular Research, Zurich, Switzerland

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KOZAWA et al: Integrated transcriptomic and proteomic analysis of heat exposure on heart tissue in rats

Abstract

Background: Deaths due to heat stroke have surged in recent years in Japan. While the long-term sequelae of heat stroke in its survivors are well understood, our previous studies suggest that heat exposure may cause fatal organ damage before the systemic inflammatory response is triggered. In this study we aimed to provide a comprehensive analysis of cytokines and other signaling factors in sera and myocardial tissues in a rat model of heat stress.

Methods: Male Wistar rats were anesthetized and subjected to heat stress (37.0°C, 100% humidity; n = 8) to induce heat stroke (Heat group, n = 4) or normal temperatures (Control group, n = 4) for 90 min, followed by collection of blood sera and heart tissue samples. A multiplex assay system was used to analyze serum markers. Heart tissue was subjected to multiplex RNA sequencing and gene cascade analysis, and combined with iTRAQ (isobaric tags for relative and absolute quantitation) protein pathway analysis.

Results: There were significantly higher levels of five interleukins (IL-1α, IL-2, IL-6, IL-17A, IL-18), fractalkine, interferon-γ-inducible protein-10, leptin, tumor necrosis factor α, and vascular endothelial growth factor, and significantly lower levels of LIX and RANTES, in the sera of the Heat group compared with the Control group. From the 2,741 genes that showed a significant difference in myocardial mRNA expression between the groups, we identified 48 transcription factors and 34 binding regions. Pathway analysis identified the involvement of key node proteins including protein kinase C isoforms, calmodulin-dependent protein kinase II, and protein interacting with C-kinase (PICK1).

Conclusion: This model of heat stress in rats triggered changes in the mRNA and protein levels of many genes involved in cardiac function, primarily affecting the activity of ion channels and mitogen-activated protein kinase.

Introduction

Despite concerted efforts to educate the Japanese public about the dangers of heat stroke, the number of people who require emergency services or die because of heat stroke in the nation's hot summers continues to grow rapidly. Serious heat stroke from exposure to extreme heat subjects the body's tissues and organs to increased heat stress that can ultimately cause multi-organ failure and death. Indeed, many heat stroke deaths occur in the elderly and among those working or exercising in the heat. Heat stroke develops through one of several processes [1, 2]. The dissipation of heat to counteract heat exposure increases blood flow at the surface of the body, resulting in a relative deficiency of intestinal blood flow. This augments the permeability of the intestinal mucosa and induces gut flora inflow, leading to hypercytokinemia, which in turn leads to the systemic inflammatory response [3, 4].

In some victims of heat stroke, forensic autopsies to determine the cause of death have revealed relatively quick deaths that cannot be completely explained by the heat-exposure mechanism described above. Our previous pathophysiological investigations using animal models to determine the direct effects of heat exposure on various organs showed that excessive elevation of body temperature might damage organs such as the heart and liver in a more direct manner than previously thought [5, 6]. These findings suggested that, in patients who die from heat stroke relatively quickly, heat exposure may directly damage certain organs before the above-mentioned systemic inflammatory response is triggered by intestinal mucosal damage.

Accordingly, in the present study we comprehensively analyzed the gene expression and protein profiles of myocardial tissues from a rat model of heat stress exposure using an RNA sequencing (RNA-seq), and shotgun strategy combined with iTRAQ (isobaric tags for relative and absolute quantitation) labeling chemistry [7]. Using this approach, we have further clarified the effect of heat stress on the myocardium in terms of changes in gene expression and protein quantification profiles related to myocardial injury.

Materials and methods

Development of the experimental model

We purchased 8-week-old male Wistar rats (262.5 ± 7.7 g, n = 8) from CLEA Japan Inc. (Tokyo, Japan) and housed them at the Mie University experimental animal facility for 1 week to acclimatize them to the environment. Subsequently, we divided the rats into a heat exposure group (Heat, n = 4) and a non-heat–exposure group (Control, n = 4).

For heat exposure, the Heat group was intraperitoneally injected with 64.8 mg/kg of pentobarbital and then exposed to heat for 90 min in a CO_2 incubator at an internal temperature of 37.0°C ± 0.5°C and 100% humidity. In contrast, the Control group was simply placed on a thermal mat for 90 min to maintain body temperature [8]. Following treatment, all rats were euthanized by decapitation, and the heart tissues were immediately harvested, cut into small pieces, stirred in RNAlater (Ambion, CA, USA) overnight to penetrate the tissues with the solution or placed onto dry ice for freezing, and then stored at −80°C until analysis. Serum samples were also collected from both groups and preserved at −80°C until analysis. The Animal Care Committee of Mie University approved the research protocol (Approval No. 22-60).

Blood cytokine and heart injury marker assays

Blood serum samples were collected immediately after euthanization and stored at −80°C. Using the Bio-Plex multiplex assay system (Bio-Rad, Hercules, CA, USA), which involves xMAP technology with fluorescent microbeads, the serum samples were assayed and analyzed for 23 cytokines: IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12 (p70), IL-13, IL-17A, IL-18, EGF, eotaxin, fraktalkine, G-CSF, IP-10, leptin, LIX, MCP-1, MIP-1α, MIP-1β, RANTES, TNFα, and VEGF. The values are presented as means ± standard error of the mean, and were analyzed by one-way analysis of variance. P values < 0.05 were considered statistically significant.

RNA extraction and sequencing analysis

Total RNA was purified from heart tissues following the Trizol protocol (Thermo Fisher Scientific, Waltham, MA, USA). The rRNA was depleted from 5 µg total RNA using the Ribo-Zero Gold rRNA Removal Kit for human/mouse/rat (Illumina, San Diego, CA, USA).

For fragmentation and further library preparation, the Ion Total RNA-Seq kit v2 (Thermo Fisher Scientific) was used according to the manufacturer’s instructions. In this process, a barcoded approach was chosen to facilitate multiplex RNA-seq. The yield and size distribution of the amplified cDNA was assessed with a DNA High Sensitivity Kit (Agilent Technologies, Santa Clara, CA, USA).

Sample emulsion PCR, emulsion breaking, and enrichment were performed using the Ion PI Hi-Q Chef Kit (Thermo Fisher Scientific) according to the manufacturer’s instructions. Briefly, an input concentration of barcoded cDNA templates/Ion Sphere Particles (ISPs) was added to the emulsion PCR master mix, and the emulsion was generated using the Ion Chef System (Thermo Fisher Scientific). Template-positive ISPs were enriched, and sequencing was undertaken using Ion PI v2 Chip (4 samples per chip) on the Ion Proton System using Ion Proton Hi-Q Sequencing Kit chemistry (read length, 200 base pairs; 10 to 20 million reads per sample; Thermo Fisher Scientific).

Data from the Proton runs were initially processed using the Ion Torrent platform-specific pipeline software Torrent Suite v5.6 (Thermo Fisher Scientific) to generate sequence reads, trim adapter sequences, and to filter and remove poor signal-profile reads. FASTQ files were generated with the remaining beads. The resulting FASTQ files were imported into CLC Genomic Workbench 10.0 (Qiagen, Hilden, Germany) for analysis. The resulting high-quality reads were mapped against the reference genome *Rattus norvegicus* (rat; Rnor_6.0) using CLC Genomics Workbench 10.0. Data were normalized by calculating RPKM (reads per kilobase per million mapped reads) and TPM (transcripts per million mapped reads) for each gene, and annotated using the Rnor_6.0 genome assembly.

Gene cascade analysis

Gene expression cascade analysis was conducted on FPKM (fragments obtained per kilobase of exon model per million mapped reads) values for genes related to quercetin treatment. The analysis involved two steps: transcription factor binding site (TFBS) analysis and key node analysis. Following the method of Dillies et al., FPKM values were normalized against the trimmed mean of M-values (TMM) method [9] and extracted into two groups: genes that showed changes (Yes-set) and genes that did not show changes (No-set). The TFBSs that were included in both sets were compared, and binding sites that were found to be significant in the Yes-set were searched in the TRANSFAC Professional database (BIOBASE, Wolfenbüttel, Germany). TFBS analysis was performed to predict the transcription factors that bind to each gene. To search for factors that influence changes in gene expression, key node analysis was performed by extracting the Yes-set from the related genes already obtained and searching for related upstream factors upstream in a stepwise manner [10].

iTRAQ analysis

A global iTRAQ analysis of the proteins expressed in heart tissue using was carried out at APRO Life Science Institute, Inc (Tokushima, Japan). Each heart tissue sample was ground to a fine powder with a mortar and pestle containing liquid oxygen. Protein was extracted from some of the resulting powdered tissue in 7 M urea, 0.1% NP-40, and 500 mM triethyl ammonium bicarbonate using a cell and tissue grinding resin kit. An amount equivalent to 30 µg protein was reduced, cysteine was blocked, trypsin was added, and the resulting preparation was treated for 20 h at 37ºC.

The iTRAQ reagent was used according to the manufacturer's instructions. Sample fractionation was performed with an EASY-nLC 1200 (Thermo Fisher Scientific). Peptides were separated on a Pepmap C18 EASY-Spray column (15 cm × 75 um ID; Thermo Fisher Scientific) at a 300 nL/min solvent flow rate (solution A: 0.1% formic acid; solution B: 80% acetonitrile, 0.1% formic acid). A multi-slope gradient was started 0 min after the injection signal as follows: 35% at 100 min, and a 100% plateau to 102 min before equilibration for 110 min. Mass spectrometry analysis was performed with a Q Exactive Plus (Thermo Fisher Scientific). Mascot and Proteome Discoverer 2.1 was used to simultaneously identify and quantify proteins using the following search parameters: trypsin was chosen as the enzyme with two missed cleavages allowed; dynamic modifications of oxidation at Met, iTRAQ 8plex at Tyr, and methylthio at Cys; static modification of iTRAQ 8plex at Lys or N-terminal; peptide tolerance was set at 10 ppm, and MS/MS tolerance was set at 0.02 Da. The iTRAQ 8plex was chosen for quantification during the simultaneous search.

Results

Body weight and rectal temperature

The body weight and final rectal temperature were 296.6 ± 12.1 g and 42.9°C ± 0.6°C in the Heat group, and 302.0 ± 8.7 g and 37.7°C ± 0.6°C in the Control group, respectively.

Heart injury marker concentrations

The serum levels of cytokines and other signaling molecules in the Heat and Control groups following the heat exposure experiments are shown in Table 1. The levels of IL-1α, IL-2, IL-6, IL-17A, IL-18, fractalkine, IP-10, leptin, TNFα, and vascular endothelial growth factor (VEGF) were significantly higher, and LIX and RANTES were significantly lower, in the Heat group compared with the Control group. Furthermore, the levels of IL-6, IL-18, fractalkine, and VEGF were at least 2-fold higher in the Heat group than in the Control group.

Gene cascade analysis

Between-group analysis of the signals identified 2,741 genes with significantly different fold changes in gene expression (absolute value > 2.0; $p < 0.05$). We used 150 out of the 2,741 genes in the Yes-set for cascade analysis. When gene clusters with variations in expression identified by the signal analysis were analyzed for the presence of a significant number of transcription factor binding regions located upstream of their transcription start sites, 34 such regions were identified. TFBS analysis identified 48 transcription factors for the 34 binding regions. A key node analysis was performed upstream from these 48 factors, producing 23 common signaling proteins. Cascades associated with these key node proteins were then examined, and proteins that appeared in all cascade diagrams at high frequencies were analyzed. Table 2 shows all 23 factors with a high rate of appearance, and Fig. 1 shows the factors with the five highest appearance rates.

Protein expression differences between the study groups

We used the iTRAQ approach to perform a pathway analysis of protein expression in the heart tissue of both groups. The iTRAQ results identified more than 1,300 proteins from each sample, among which nine proteins showed a two-fold change with pathways. Table 3 and Fig. 2 show the top five pathways involved.

Discussion

To investigate the effects of heat stress, cytokine levels were comprehensively analyzed in a rat model of heat stroke. Heat stress caused significant elevations in IL-1, TNFα, and other so-called alarm cytokines produced in the very early stages of inflammation. Exposure to heat causes increased blood flow to the muscles and skin with a relative decrease in intestinal blood flow, which increases intestinal permeability. This allows endotoxins to enter the body via the intestines, exacerbating heat stroke. Although we found increased expression of IL-1 and TNFα — the most notable of the body's many responses to endotoxins — IL-6, IL-18, fractalkine, and VEGF were the factors most strongly induced by heat stress in this study. IL-6 and IL-18, members of the IL-1 family, are common inflammatory cytokines, and fractalkine and VEGF are expressed in response to inflammatory stimuli in vascular endothelium. Heat stress therefore appears to have triggered an inflow of heat stroke-exacerbated endotoxins that led to a system-wide inflammatory response. The significant elevations in IL-2, IL-17A, IP-10, and leptin — cytokines and chemokines that contribute to the inflammatory response — also appeared to be the result of a systemic inflammatory response. Heat exposure in this study significantly reduced LIX and RANTES levels. The decrease seen in the CXCR2 ligand LIX suggested inhibition of tyrosine kinase-mediated CXCR1 and CXCR2 expression by lipopolysaccharide. RANTES, a chemokine that plays a key role in the inflammatory response, also showed decreased expression. Although the RANTES level is indicative of the extent of the inflammatory response, it is hypothesized that, in cases severe enough to have an unfavorable outcome, RANTES levels may actually decrease, showing an inverse relationship to severity. It is possible that the heat stress applied in this rat model of heat stroke may have been sufficient to cause serious illness and death.

Significant, large variations in gene expression between the two groups were seen in 2,741 genes. The significantly altered gene expression profile in the Heat group compared with the

Control group demonstrates that heat exposure led to dramatic changes in gene expression in myocardial tissue. When the gene clusters with variations in expression were probed to see which clusters had a significantly greater number of transcription factor binding regions located upstream of the transcription start sites compared with genes with no variations in expression, 34 transcription factor binding regions were found in the Heat group. Key node analysis of the transcription factors that bind to these 34 regions identified 48 key node proteins. A previous analysis of the top five gene cascades of these key node proteins showed a tendency for histone deacetylase 4 (HDAC4) to be particularly important for regulating the activity of genes involved in heart development in mice [11]. Protein kinase C (PKC)-β was the first isoenzyme to be studied using cardiac target expression, and it plays an important role in cardiac hypertrophy. Reports have associated PKC-α activation, or an increase in PKC-α expression, with hypertrophy, dilated cardiomyopathy, ischemic injury, and mitogen stimulation. Human heart failure has also been associated with increased activation of conventional PKC isoforms, including PKC-α and PKC-β [12]. Several studies have shown a role for calmodulin-dependent protein kinase II (CaMKII) in the occurrence and progression of cardiac hypertrophy, and it is an independent risk factor for developing heart failure [13, 14]. Indeed, heart failure occurs in mice with transgenic CaMKII overexpression. The protein interacting with C-kinase (PICK1) mediates intracellular trafficking of myocardial norepinephrine transporter, and shows significantly reduced mRNA levels in mice with heart failure [15, 16]. These findings indicate that heat exposure greatly impacts cardiac function, and that modulation of these key nodes can push an individual toward cardiac failure.

Nine proteins showed small variations in expression between the groups in this study. Although heat exposure is generally not considered to substantially affect protein expression, these proteins appeared to have a far-reaching impact during times of heat stress. Although they were among the top five proteins in our proteome analysis by iTRAQ, no published studies have shown transferrin protein receptor 1 (Tfrc), alpha-methylacyl-CoA racemase (Amacr), or ribophorin II (Rpn2) to be related to cardiac function. Silencing of Diaph1 leads to increased expression of sarcoplasmic reticulum Ca^{2+} ATPase and reduced expression of the sodium calcium exchanger [17] . Mitogen-activated protein kinase (MAPK) is associated with cell-cycle arrest in mammalian myocardial tissue, and its inhibition may be a strategy to promote cardiac regeneration in response to injury [18]. Overall, our results suggest that heat exposure adversely impacts cardiac function by affecting ion channel and MAPK activity.

Conclusions

This study provides a comprehensive analysis of blood cytokines, and gene and protein expression profiles of myocardial tissues from a rat model of heat exposure. Although the inflammatory response might directly cause serious and eventually fatal outcomes, heat stress in this rat model primarily affected the activity of ion channels and MAPK, which are known to impact cardiac function. Heat stress also led to changes in the mRNA levels of many genes, including HDAC4, PKC-α, PKC-β, CaMKII and PICK1, thereby further impacting cardiac function.

After conducting a more detailed analysis of these profiles, we plan to continue our investigations of the effects of heat exposure on myocardial tissue by: 1) identifying which proteins are the source of the variations in gene expression; and 2) performing expression analyses of individual genes and proteins. Such efforts may lead to the development of novel therapeutics for this life-threatening condition.

Conflict of interest

The authors declare that they have no conflicts of interest.

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Figure legends

Fig. 1 Pathway cascades of the five factors that appeared most frequently in the RNA-seq gene cascade analysis. HDAC4 = histone deacetylase 4; CaMKII = calmodulin-dependent protein kinase II; PKC = protein kinase C; PICK1 = protein interacting with C-kinase.

Fig. 2 Pathway cascades of the five factors that appeared most frequently in the iTRAQ pathway analysis.

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Accompanying Sheet

What is already known about this topic?

Heat exposure may cause fatal organ damage before the systemic inflammatory response is triggered. We aimed to provide a comprehensive analysis of cytokines and other signaling factors in sera and myocardial tissues in a rat model of heat stress.

What this study adds?

This study provides a comprehensive analysis of blood cytokines, and gene and protein expression profiles of myocardial tissues from a rat model of heat exposure.

Suggestions for further development

We plan to continue our investigations of the effects of heat exposure on myocardial tissue by: 1) identifying which proteins are the source of the variations in gene expression; and 2) performing expression analyses of individual genes and proteins. Such efforts may lead to the development of novel therapeutics for this life-threatening condition.