Transgenic sickle cell trait mice do not exhibit abnormal thermoregulatory and stress responses to heat shock exposure

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ABSTRACT

There remains controversy over whether individuals with sickle cell trait (SCT) are vulnerable to health risks during physical activity in high temperatures. We examined thermoregulatory and stress-related responses to heat exposure in SCT and wild-type (WT) mice. No significant differences in core temperature (T_c) were observed between SCT and WT mice during heat exposure. There was no correlation between peak T_c during heat exposure and levels of hemoglobin S in SCT mice. Basal levels of circulating inflammatory and stress-related markers were not significantly different between SCT and WT mice. Although heat exposure caused significant increases in plasma interleukins 1 β and 6, and 8-isoprostane in SCT and WT mice, no differences were found between SCT and WT mice with similar thermal response profiles during heat exposure. SCT mice had significantly higher expression of heat shock protein 72 (Hsp72) in heart, liver and gastrocnemius muscle than WT mice under control and post-heat conditions. In conclusion, there is neither thermoregulatory dysfunction nor abnormal stress-related response in SCT mice exposed to moderate heat. The hemoglobin variant in mice is associated with altered tissue stress protein homeostasis.

Keywords:

sickle cell trait, heat stress, hsp72, oxidative stress, rodent

1. Introduction

Sickle cell trait (SCT), which affects about 1 in 12 African Americans, is often viewed as a benign condition. There is long-standing controversy over whether individuals with SCT are more vulnerable to injury during physical challenges than non-SCT persons [1-4]. Screening for SCT has long been used in professional sports and military recruits, and more recently in college sports as a precautionary measure, but its legitimacy remains contested among both clinicians and scientists [5-7]. Concerns have been raised over potentially unfair precautions or limitations on individuals with SCT that may stigmatize large numbers of people, both socially and economically. Research on SCT has been conducted based primarily on clinical case studies. To date, few studies have examined physiological and biochemical effects of exposure to high temperatures on SCT carriers.

Over the last two decades, several transgenic mouse models that express human hemoglobin S (HbS) have become available [8, 9]. These mice that heterozygously express human β S globin gene provide a unique opportunity to experimentally investigate the physiological characteristics associated with SCT. The objective of this study was to determine whether physiological homeostasis is altered locally and systemically in SCT mice under basal and acute heat stress conditions, compared to wild-type (WT) mice. We examined thermoregulatory activities and stress-related markers in tissues and plasma in SCT and WT mice in response to acute heat exposure.

2. Materials and methods

2.1. Animals

All procedures performed on animals were approved by the Uniformed Services University Institutional Animal Care and Use Committee. Mice (male, 8-10 weeks of age when experiments began) were purchased from The Jackson Laboratory (Bar Harbor, ME). The SCT mice were obtained by breeding males exclusively expressing human α - and β^S -transgene globins with hemizygous females expressing mouse β globin gene. They were backcrossed to C57BL/6J one generation at The Jackson Laboratories and had a background of >75% C57BL/6 genomes. The most widely used inbred strain C57BL/6J, which is listed as the “approximate” control for the SCT strain by the supplier, was used as WT controls in this study. No abnormalities or organ injuries were detected in the SCT mice used in this study by the current supplier. This study included four groups of mice: control SCT (n = 9), heat-exposed SCT (n = 16), control WT (n = 15) and heat-exposed WT (n = 22). The mice of the control groups were not exposed to heat. All mice were maintained in conventional animal facilities (~22 °C) with *ad libitum* food and water at the Uniformed Services University Laboratory Animal Medicine facility.

We measured body core temperature (Tc) and locomotor activity (La) using a telemetry system (Mini Mitter Corp, Bend, OR). All experimental mice were surgically implanted with a transponder, Model G2 E-Mitter, as described previously [10]. At least 2 weeks were allowed for recovery. At the time of the experimental protocols, all mice were healthy as evidenced by body weight gains ($\geq$ pre-surgical levels), normal activity and no sign of infection.

2.2. Experimental procedure

Heat tests were conducted in an environmental chamber (Model 3950, Thermo Forma, Marietta, OH) in the mornings (~9:00 AM – 12:00 PM). Mice were placed in the chamber (~22 °C, relative humidity ~22-34%) for at least 4 hours on one occasion ~5 days before experimentation. One day before heat tests, mice were housed individually with fresh bedding in the chamber. Heat exposures began the following morning after stable baseline data were obtained. Food and water were removed from cages before heat exposure. The heat protocol used in this study was described previously [10]. Briefly, heat exposure was terminated at core temperature (T_c) of ~42.4 °C or 3 hours into heat exposure, whichever happened first. It took ~1 hour of heating to reach a predetermined chamber temperature of 39.5 °C. Mice were housed individually and real-time T_c and L_a were recorded continuously during experiments. Each animal underwent two heat tests separated by an interval of at least 7 days [10] to allow sufficient time for heat stress to recover [11]. We used this design to ensure the appropriate health status of each mouse and minimize experimental variations. The first heat test was used to screen for mice with severe hyperthermia ($T_c > 42.4$ °C) and extreme physical activity to be excluded from further experiments. Our inclusion criteria also required each mouse to have a normal weight gain between the two heat tests.

2.3. Collection and processing of blood and tissues

Collection of blood and tissues was performed under anesthesia ~18-22 hours following the final heat test. Blood, taken from a carotid artery, was collected in a heparin-primed tube and immediately centrifuged to obtain plasma. Animals were then sacrificed and tissues samples removed, cleaned in ice-cold phosphate buffered saline, rapidly frozen using dry

ice and stored at -80°C . Tissue samples were homogenized and centrifuged to obtain the supernatants for analyses [10]. The remaining pellets were evaporated to dryness and weighed for data correction.

2.4. Blood and tissue assays

Blood and tissue assays were performed to analyze markers for inflammation (interleukins 1β and 6: IL- 1β and IL-6), redox status (8-isoprostane, glutathione and superoxide dismutase or SOD) and stress (corticosterone and Hsp72). The following *Enzyme-Linked Immunosorbant Assay* (ELISA) kits sensitive to murine samples were used per manufacturers' instructions: plasma corticosterone (sensitivity: 30 pg/ml) and 8-isoprostane (sensitivity: 2 pg/ml) from Cayman Chemical (Ann Arbor, MI), plasma IL- 1β (sensitivity: 4.8 pg/ml) and IL-6 (sensitivity: 1.8 pg/ml) from R&D Systems (Minneapolis, MN), and plasma glutathione (sensitivity: 10 μM) and tissue Hsp72 (sensitivity: 0.2 ng/ml) from Enzo Life Sciences (Plymouth Meeting, PA). Plasma and tissue homogenate supernatant samples were measured in duplicate. The aspirating and washing cycles were completed using an automatic microplate washer (Tecan Group Ltd, Switzerland). Samples were analyzed using the Magellan Data Analysis System (Tecan, Austria) and normalized to dry tissue weight (dw). Intra- and inter-assay coefficients of variation for ELISA concentrations were less than 10% for each assay.

Plasma SOD capacity was determined based on inhibition of superoxide anion produced by xanthine oxidase [12]. Lucigenin (5 μM)-enhanced chemiluminescence was used to assess changes in xanthine oxidase-dependent superoxide. The reaction was initiated by adding xanthine (100 μM) to phosphate-buffered saline (PBS) containing

xanthine oxidase and plasma samples in a tube luminometer (Berthold AutoLumat Plus LB 953) at room temperature. The chemiluminescence signal was adjusted to background and continuously measured at 2Hz. All measurements were performed in duplicate. Inhibition of superoxide was calculated as relative reductions in chemiluminescence and the plasma SOD activity was calculated from a standard curve of inhibition of superoxide by Cu/Zn-SOD (Sigma –Aldrich, St. Louis, MO). All plasma samples were run in the same assays to avoid inter-assay variability.

Hemolysis was assessed based on plasma lactate dehydrogenase (LDH) concentrations and colorimetric values. Plasma LDH was measured using an ELISA kit (sensitivity: 1.5 U/ml) from USCN Life Sciences Inc. (China). Direct absorbance at 414 nm of the plasma diluted in PBS was measured in a 96 well plate reader (Tecan Group Ltd, Switzerland) to quantitatively estimate free Hb content [13].

2.5. Blood hematocrit measurements

Packed red cell volume for each animal was determined by filling a micro-hematocrit capillary tube with blood, which was then sealed with a compound sealant. Hematocrit measurements were made with a CritSpin digital hematocrit reader following a centrifugation step with a CritSpin micro-hematocrit centrifuge per manufacturers' instructions (StatSpin, Norwood, MA).

2.6. Hemoglobin fractionation analysis

Each SCT mouse blood sample (10 μ l) was subjected to the separation and quantitation of normal (HbA) and variant hemoglobin species (HbS) using a commercial HPLC resolution assay (Ultra² Resolution System, Trinity Biotech Plc., Ireland). The HPLC assays were

performed by the Center for Sickle Cell Disease, Howard University Hospital (Washington, DC).

2.7. Data processing and statistical analysis

Data are expressed as mean $\pm$ SD. One-way ANOVA with Dunnett's post hoc tests and two-way ANOVA with Bonferroni's post hoc tests were used for comparison. The results were considered significant at $P \leq 0.05$.

3. Results

SCT mice were smaller in size than WT mice (SCT 22.1 ± 0.8 g versus WT 25.4 ± 0.4 g, $P < 0.001$). SCT and WT mice gained similar amounts of weight during the week between the two heat tests (SCT 1.2 ± 0.2 g versus 1.3 ± 0.3 g, $P > 0.05$). Four WT mice did not complete the first heat test due to a severe hyperthermic response (peak $T_c > 42.4$ °C) and were excluded from further tests. No significant differences were found in peak T_c or average L_a between SCT and WT mice during heat exposure (Table 1). There was no correlation between the measured HbS percentage and peak T_c values during heat exposure performed (Fig. 1). To examine how SCT and WT mice responded to similar thermal stress at local and systemic levels, we eliminated an additional 3 WT mice with a peak T_c greater than the highest value of SCT mice. Thus, SCT and WT mice selected for subsequent comparisons had a similar thermal response profile during heat exposure (Fig. 2.).

Hydration status was assessed by measuring body weight and blood hematocrit. Differences in body weight before and after the second heat test were measured in WT and SCT mice. Heat exposure did not result in a significant difference in relative changes in

body weight between WT and SCT mice ($-8.2 \pm 0.8\%$ versus $-7.2 \pm 0.6\%$, $P > 0.05$). Blood hematocrit in WT and SCT mice increased immediately following heat exposure, but returned to control levels ~20 hours later (Fig. 3.). SCT mice had a significantly lower hematocrit than their WT counterparts under control and post-heat conditions ($P < 0.05$).

As shown in Table 2, selected blood and plasma variables were compared between SCT and WT mice groups to assess severity of inflammation (IL-1 β , IL-6, corticosterone), oxidative stress (glutathione, 8-isoprostane, SOD) and hemolysis (LDH, absorbance 414nm). A two-way ANOVA revealed a main effect of heat for plasma IL-1 β , IL-6 and 8-isoprostane levels (P : 0.001-0.0001, $df = 1, 55$), but not for other variables. No effect as a result of being a SCT carrier was detected. That is, these plasma parameters in control and heat-exposed SCT mice were not significantly different from their WT counterparts.

A two-way ANOVA also revealed two main effects of SCT and heat on Hsp72 levels in each of the three tested organs - liver, heart and gastrocnemius muscle. Overall control and heat-exposed SCT mice had higher Hsp72 levels in these organs, compared to their WT counterparts. Heat exposure caused significant increases ($P < 0.0001$) in Hsp72 among all three organs analyzed (Fig. 4). The relative, heat-induced Hsp72 increases (differences between heat-exposed and control) in SCT mice were larger in liver ($268 \pm 7\%$), but smaller in heart ($146 \pm 10\%$) and gastrocnemius muscle ($138 \pm 8\%$), compared to WT mice (liver $217 \pm 15\%$, heart $279 \pm 22\%$, gastrocnemius muscle $422 \pm 32\%$, $P < 0.001$).

4. Discussion

The present study provides controlled, experimental evidence showing no thermoregulatory dysfunction in SCT mice exposed to passive, moderate heat, compared to WT mice. Our results also reveal no abnormal inflammatory or stress-related responses that suggest disruption of physiological homeostasis in SCT mice under heat stress. Despite their normal thermal homeostasis, SCT mice heterozygously expressing human HbS were associated with significantly elevated Hsp72 levels in all tested tissues under basal and post-heat conditions, compared to normal wild-type mice. We are unaware of any studies examining tissue Hsp72 levels in human subjects with SCT.

Disagreement remains among investigators over whether SCT should be considered completely benign and asymptomatic [5, 14]. Previous publications have linked SCT to various medical complications based on case reports or using uncontrolled observational studies as evidence [15-17]. Recent concerns focus primarily on whether SCT carriers are vulnerable to injuries under extreme conditions, such as hypoxia and intense activity. High ambient temperature has been raised as one of potential risk factors contributing to exercise-related injuries in SCT individuals [18]. Here we demonstrate that compared to normal mice, SCT mice did not experience thermoregulatory dysfunction, when exposed to passive, moderate heat. These results suggest that hyperthermia alone may not be an immediate concern for SCT mice exposed to acute heat stress, although they should not be used to suggest normal thermoregulation for individuals with SCT performing physical activity in hot climates.

Overexpression of cellular Hsp72, the inducible form of Hsp70, is commonly found under various stress conditions [19-21]. As molecular chaperones, Hsp70 proteins assist in

a wide range of housekeeping functions, including the folding and assembly of newly synthesized proteins and the refolding of misfolded and aggregated proteins [19]. They serve as a defense mechanism that protects cells against stress insults. Reduced expression of cellular Hsp72 is found in many chronic metabolic disorders. For example, humans [22, 23] and animals [24] with type 2 diabetes have been shown to have reduced expression of Hsp72 in skeletal muscles. In the present study, both control and heat-exposed SCT mice showed higher Hsp72 levels in liver, heart and skeletal muscle tissues, compared to their normal counterparts. Our samples were likely collected during Hsp72 accumulation plateaus in tissues following heat exposure [11, 25]. Our study revealed no evidence that heat exposure caused pathophysiological stress in SCT mice, as no changes were found in several key markers for heat injury including glutathione [26]. To our knowledge, no information on the tissue Hsp72 in human SCT carriers is currently available. Future studies are needed to examine whether the transgene itself affects heat shock signaling.

In addition to the increased expression of Hsp72 in tissues, our results also revealed altered homeostasis of blood hematocrit in SCT mice, as compared to the control mice. Specifically, SCT mice had lower hematocrit levels than normal mice. The lower blood hematocrit in SCT mice did not present itself due to increased hemolytic activity, as evidenced by their plasma LDH and colorimetric values being similar to normal mice under both basal and heat-exposed conditions. The homeostatic regulation of body fluids in SCT mice also seemed to be normal. Heat exposure induced relatively similar increases in hematocrit in WT and SCT animal groups, which returned to control levels ~20 hours after heat exposure. We are not aware of studies that link SCT to changes in hydration status.

The present study has several limitations that need to be acknowledged. First, the mouse model used in this study may not mimic human SCT carriers resulting from hemoglobin polymerization. Second, our study showed that WT mice exhibited a wider range of peak T_c in response to heat exposure than SCT mice. We previously reported similar variations in heat-induced hyperthermia in WT mice [10, 27]. However, due to the relatively small sample size, these results should not be used to infer a better ability to maintain thermal homeostasis during passive heat exposure by SCT than by WT mice. Finally, the SCT mouse model of heat hyperthermia should not be used to address concerns about the effects of high ambient temperatures on human SCT individuals during exercise. Rather, our results demonstrate the integrity of thermoregulatory function in SCT mice during passive heat exposure.

In conclusion, our results provide experimental evidence of no thermoregulatory dysfunction in SCT mice during moderate heat exposure sufficient to disrupt basal thermal homeostasis. SCT mice heterozygously expressing human β S globin gene are associated with increased basal and acute stress levels of tissue Hsp72 but not systemic inflammatory and stress markers. Further studies are needed to determine whether the altered tissue Hsp72 profiles in SCT mice are due to the heterozygous gene or have any physiological significance. Cellular stress proteins in human SCT carriers may merit investigation to validate this mouse model as a physiologically relevant phenotype that can be used to assess stress vulnerability in SCT individuals.

Author contributions

Y.C. designed the project, performed the experiments, analyzed the data and wrote the manuscript.

A.I. performed the experiments, analyzed the data and helped prepare the manuscript.

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Table 1. Body core temperatures (Tc) and locomotor activity (La) of WT and SCT mice

	basal Tc (°C)	Heat test		
		peak Tc (°C)	peak Tc range (°C)	La (counts/min)
WT (n = 18)	36.2 ± 0.2	40.8 ± 0.6	40.2 - 42.0	5.4 ± 1.5
SCT (n = 16)	36.0 ± 0.2	40.8 ± 0.4	40.2 - 41.4	5.3 ± 2.1

Peak Tc values were calculated as the averages of Tc during the last 3 minutes of heat exposure.

Table 2. Comparison of blood and plasma variables in WT and SCT mice

	<u>Control</u> ¹		<u>Heat exposed</u>	
	WT	SCT	WT	SCT
IL-1 β (pg/ml)	6.9 $\pm$ 0.3	6.7 $\pm$ 0.2	10.1 $\pm$ 0.2*	10.1 $\pm$ 0.3*
IL-6 (pg/ml)	6.2 $\pm$ 0.3	5.7 $\pm$ 0.2	8.8 $\pm$ 0.3*	8.5 $\pm$ 0.3*
Corticosterone (ng/ml)	27.6 $\pm$ 3.0	28.1 $\pm$ 1.4	27.9 $\pm$ 1.0	32.9 $\pm$ 1.1
Glutathione (μ M)	90.3 $\pm$ 1.4	92.2 $\pm$ 3.0	91.9 $\pm$ 1.9	91.8 $\pm$ 2.0
8-isoprostane (ng/ml)	2.0 $\pm$ 0.2	2.4 $\pm$ 0.2	4.2 $\pm$ 0.2*	3.7 $\pm$ 0.3*
SOD ² (units/ml)	23.7 $\pm$ 1.0	25.9 $\pm$ 0.9	25.9 $\pm$ 1.5	24.1 $\pm$ 2.9
LDH ³ (units/ml)	150.1 $\pm$ 2.0	160.4 $\pm$ 4.6	156.9 $\pm$ 2.4	154.2 $\pm$ 2.1
Absorbance 414nm	0.081 $\pm$ 0.033	0.108 $\pm$ 0.028	0.089 $\pm$ 0.014	0.087 $\pm$ 0.012

1. Not exposed to heat;
2. Superoxide dismutase;
3. Lactate dehydrogenase.

**P*: 0.001-0.0001, overall effect of heat exposure (two-way ANOVA, *df* = 1, 55).

Figure legends

Fig. 1. Scatter plot analysis for peak Tc and HbS content in SCT mice. Peak Tc of each animal (n = 15) was calculated as the averages of Tc during the last 3 minutes of heat exposure.

Fig. 2. Body core temperature of WT and SCT mice and corresponding ambient temperature. Core temperature values are presented as mean (solid line) $\pm$ SD (dashed line). WT: n = 15; SCT n = 16.

Fig. 3. Blood hematocrit levels in WT (open bar) and SCT mice (solid bar) under control condition and, 0 and 20 hours following heat exposure. The control samples were obtained from mice that were not exposed to heat (WT n = 8, SCT n = 8); the post-heat samples (WT n = 8, SCT n = 7) were obtained following the second heat test. * $P < 0.001$, compared to control; # $P < 0.001$, compared to WT.

Fig. 4. Effects of heat exposure on Hsp72 levels in liver, heart and gastrocnemius muscle in WT (open bar) and SCT (black bar) mice. For control groups: WT n = 8, SCT n = 8; for heat exposed groups: WT n = 15, SCT n = 15. * $P < 0.0001$, compared to control; # $P < 0.0001$, compared to WT.

Fig. 1

Fig. 2

Fig. 3

Fig. 4