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Housing Temperature Influences the Pattern of Heat Shock Protein Induction in Mice following Mild Whole Body Hyperthermia

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Abstract

Purpose—Researchers studying the response of mice to stress generally use mice housed under standard, nationally-enforced conditions as controls. Few investigators are concerned whether basic, physical aspects of mouse housing could also be a source of stress, capable of influencing the subsequent impact of an experimentally applied stressor. We have recently become aware of the potential for standard housing conditions to influence important physiological and immunological properties in mice.

Materials and Methods—Here, we sought to determine whether housing mice at standard temperature (ST; 22°C) versus thermoneutral temperature (TT; 30°C) influences baseline expression of heat shock proteins (HSPs) and their typical induction following a whole body heating.

Results—There were not significant differences in baseline expression of HSPs at ST and TT. However, in several cases, we induction of HSP70, HSP110, and HSP90 in tissues of mice maintained at ST was greater than at TT, following 6 hours of heating which elevated core body temperature to 39.5°C. This loss of HSP induction was also obtained when mice housed at ST were given propranolol, a β -adrenergic receptor antagonist, used clinically to treat hypertension and stress.

Conclusions—Taken together, these data show that housing temperature has a significant influence on the expression of HSPs in mice after whole body heating and should be considered when stress responses are studied in mice.

Keywords

whole body hyperthermia; thermoneutrality; heat shock protein; cold stress

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Declarations of Interest

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Introduction

Stress proteins are a large class of molecules involved in numerous cellular processes that occur in both normal and pathological settings. These proteins function to maintain homeostasis as well as to prevent protein aggregation and misfolding in the face of environmental stress. The two major categories of stress proteins include the HSPs, which were first characterized by their induction following exposure of cells and organisms to increased temperature, and the glucose regulated proteins (GRP) which, in addition to responding to environmental stress stimuli, also sense nutrient deprivation (1–3). Moreover, these molecules are also induced by other forms of stress including changes in pH and increased levels of oxidative free radicals (4).

In addition to their roles in regulating the stress response, the HSPs also mediate numerous other cellular activities (5). Specifically, these proteins have been widely studied in inflammation, cellular metabolism, and tumorigenesis (6–10). Many of the molecular interactions involving HSPs have been investigated using *in vitro* culture systems. However, elucidation of *in vivo* HSP function relies heavily on the use of mouse models. Due to a high surface-area-to-volume ratio, mice have a remarkable capacity for heat exchange with their surrounding environment, allowing for rapid increases in their core temperature when heated (11). In a previous study, our group examined the expression of three members of the HSP70 superfamily– HSP70 (HSP72), HSP110, and GRP170 - in normal tissues after exposing 8 week old female BALB/c mice to several hours of whole body hyperthermia (WBH). This work helped elucidate the post-heating expression of HSP70 and HSP110 and showed that they were detectable at baseline levels in various tissues and organs prior to heating (12).

However, the rate of murine heat exchange also creates largely unrecognized problems for mice housed under standard conditions. In nearly all research facilities, mice are housed at standard, ambient temperatures between 20–26°C as directed by guidelines set forth by the National Research Council (13). This occurs despite the fact that their preferred, thermoneutral temperature, or the temperature at which basal metabolic rate is sufficient to maintain core temperature at 37°C, is approximately 29–31°C (14). Under standard housing temperatures, laboratory mice continuously lose heat to their environment and, thus, must expend more metabolic energy to generate sufficient heat to maintain their body temperature. The response to mild cold stress is regulated specifically by norepinephrine (NE), a stress hormone which can drive heat production through adaptive thermogenesis and other metabolic changes (15,16). This metabolic stress creates several important physiological changes in mice (14,17). Our lab has recently described the significant impact mild cold stress induced by cool housing temperature has on tumor growth and anti-tumor immunity (18,19) as well as therapeutic responsiveness (Eng et al., manuscript submitted).

Environmental factors, such as temperature, have been known to enhance or blunt HSP induction in response to severe stress stimuli in various organisms including fish (20,21) and lizards (22). Since stress proteins, such as HSPs, play such prominent roles in maintaining normal cellular homeostasis, we wondered whether their expression in response to hyperthermia changed as a result of the mild, but chronic cold stress that mice experience

from their standard housing conditions. Therefore, we tested whether housing mice at thermoneutrality (TT; 30°C) affects expression of HSPs following WBH compared with mice housed at standard temperature (ST; 22°C). The results obtained here suggest that unrecognized physiological stresses, like housing temperature, can influence the outcome of experimentally applied stressors. Thus, it is important that investigators who are studying stress responses in mice take these other factors into account when they assess stress protein induction.

Materials & Methods

Animals

6 week old female BALB/c mice were purchased from the National Cancer Institute. The mice were implanted with subcutaneous temperature probes (Bio Medic Data Systems) and maintained on *ad libitum* standard chow diets and water in temperature controlled vivarium set to either 22°C (ST) or 30°C (TT) for 2 weeks prior to whole body hyperthermia.

Hyperthermia

Fever range whole-body hyperthermia was induced for 6hrs in mice using a Wisconsin Oven incubator. Prior to hyperthermia, mice were injected with 1mL of saline. Mice were then heated at 38.5°C until core temperature reached 39.5°C. Afterwards, incubator temperatures were adjusted in order to maintain core temperatures between 39.5 to 40°C. Following 6hrs of heating, mice were either euthanized at 0hrs or were housed back in ST or TT-maintained vivarium for 24 or 48hrs.

Tissue Collection

Immediately following heating, both control groups and 0hr post-whole body hyperthermia groups were sacrificed, while the remaining groups were returned to the same housing temperatures for 24 and 48hr time points. Tissues were harvested and immediately snap frozen in liquid nitrogen. Frozen samples were stored at -80°C until they were processed.

ELISA

Whole blood was collected with 0.5M EDTA by cardiac puncture at the time of sacrifice. Samples were centrifuged at 16,100rcf in a table top centrifuge to separate the plasma fractions. Plasma samples were snap frozen and stored at -80°C until they were processed. ELISAs were performed to assess the levels of plasma norepinephrine (Rocky Mountain Diagnostics) and plasma HSP70 (R&D Systems). Experimental reactions were read at 450nm on a Biotek Synergy HT plate reader.

Western Blot

Protein lysates were prepared and quantitated as previously described (Ostberg et al, 2002). Samples were run on 10% acrylamide gels and transferred to PVDF membranes (EMD Millipore). Membrane blots were blocked with 1% nonfat milk in TBS for 1hr. Antibodies to HSP70 (HSP72/HSPA1A) (Stressgen), HSP90 (Cellsignaling), and HSP110 (Stressmarq) were diluted in TBS-T (1:1000) and blots were incubated overnight at 4°C. Membranes

were incubated with anti-rabbit Dylight 790 (Jackson) and anti-mouse Dylight 680 (Jackson) secondary antibodies diluted in TBS-T (1:15000) and then developed by Odyssey Scanner (Li-Cor). Fluorescence intensity was assessed with Image Studio Lite Software (Li-Cor) was normalized to GAPDH (GeneTex).

***In vivo* Propranolol Studies**

Four groups of 6 week old female BALB/c mice were acclimated to either ST or TT for 2 weeks as previously described prior to heating. Additionally, four groups of mice were simultaneously acclimated to ST or TT and treated once daily with 10mg/kg of propranolol (Sigma Aldrich) by intraperitoneal injections.

Results

Norepinephrine levels serum levels

Previous work has established that housing mice at prescribed sub-thermoneutral ambient temperatures does not affect their normal body temperature although it does increase the amount of metabolic effort needed to maintain a normal body temperature. To confirm that mice in our study housed at ST or TT did not affect body temperature, we first measured the core temperatures of healthy mice housed at these two temperatures. As predicted from previous work (18,19), the body temperature of the mice did not differ while being housed at ST versus TT (Figure 1A). However, previous studies have also demonstrated that even moderately cool temperatures are sufficient to induce stress in mice (23–25). Since the response to mild cold stress is regulated specifically by norepinephrine (NE), we next assessed the levels of NE in the serum of mice housed at ST and TT to determine if baseline expression differed in these two groups. Naïve BALB/c mice were first acclimated for two weeks at ST or TT. Analysis of the plasma revealed that norepinephrine levels were significantly higher in mice at ST compared with mice at TT, supporting that animals housed at the standard temperature conditions were, in fact, cold stressed (Figure 1B).

Heat shock protein expression in visceral organs and the brain

Next, we sought to investigate if chronic stress from sub-thermoneutral housing temperatures could affect the induction of HSPs when body temperature was raised to approximately 39–40°C. As demonstrated above, the body temperatures of mice is the same when they are maintained for extended time periods at ST or TT. After 2 weeks of being housed at either condition, we heated mice for 6hrs (well known to induce HSPs) and then harvested tissues 0, 24 or 48hrs following heat treatment. Control mice did not receive WBH and were held at either ST or TT until euthanized. Tissues were analyzed for the expression of total HSP70, HSP90, and HSP110. We found no significant differences in baseline HSP expression between mice housed at ST and TT in the brain, lung, and heart (Figure 2A–C). Confirming our previous findings (12), the expression of the HSPs significantly increased in certain tissues immediately and 24hrs after heat treatment when mice were maintained at ST (Figure 3). However, the induction of HSP70, HSP90 and HSP110 following WBH was lower in the lungs (Figure 3A, 3D–F), heart (Figure 3B, 3G–I) and brain (Figure 3C, J–L) of mice housed at TT compared to ST. Of note, we did not observe significant differences in the expression of HSP70, HSP90, or HSP110 in the spleen or kidney of mice at ST and TT

following WBH (Supplemental Figure 1). Additionally, we examined the concentration of plasma HSP70 in mice housed at ST and TT following WBH. Interestingly, plasma HSP70 levels in mice housed at TT increased more rapidly following heating compared with mice housed at ST; however, while the differences were statistically significant, the change in heat shock protein levels were relatively minimal (Supplemental Figure 2).

Effect of β -blockers on heat shock protein expression

As previously reported, maintenance of body temperature depends heavily on the biochemical process of thermogenesis. Under conditions of cold stress, rodents increase systemic production of the catecholamine, norepinephrine, to drive UCP1-mediated heat production in brown adipose tissue (24,26,27). To determine if the differences observed in HSP expression were driven by catecholamine levels, we pre-treated mice housed at ST and TT with the β -blocker, propranolol, for 2 weeks during the standard acclimation period prior to WBH. We hypothesized that if HSP expression was being altered by mild cold stress, then addition of a β -blocker to mice housed at ST should make their HSP response more similar to that seen at TT. Notably, pre-treatment with β -blockers reduced the overall induction of HSPs in the lungs and hearts, similar to that seen in untreated mice housed at TT given WBH (Figure 4). However, β -blocker use did not influence HSP expression in mice housed at TT, strongly suggesting that β -adrenergic signaling in tissues due to sub-thermoneutral housing temperatures is regulating the heat shock response (Figure 4C–H).

Discussion

The use of mouse models in biomedical research has led to a massive breadth of knowledge about physiological interactions in complex living organisms. Many of these studies have further fueled significant clinical advances, particularly in the development and testing of novel therapeutics. However, the growing reality that the housing conditions of mice in research facilities could impact experimental results has become a major concern (17,28). Since these suboptimal conditions can induce baseline physiological stress in mice, the impact on outcome of both systemic and cellular stress responses could be influenced by housing conditions and is generally underappreciated by investigators using these models.

In our study, we explored whether the stress imparted on mice by suboptimal housing temperature in research facilities impacts expression of HSPs in response to mild (fever-range) whole body hyperthermia. Our data demonstrate that mice housed at standard temperatures have significantly elevated levels of norepinephrine, the key catecholamine involved in regulating adaptive thermogenesis. Evidence from previous studies has shown that norepinephrine can directly modulate the production and even secretion of HSPs in various tissue types (29–31).

In a previous study examining the effects of methamphetamines on the induction of HSP expression in the brain, the authors reported greater increases in HSP70 expression in mice housed at thermoneutrality compared to those housed at the standard room temperature (32). However, they reported greater expression of HSP70 was seen in the brains of mice housed at thermoneutrality. These discrepancies in the expression of HSPs with our findings may come about due to the significantly higher body temperature induced by methamphetamines

(33). Following injection of methamphetamine, the authors of this study observed rectal temperatures climb to 43°C within 90 minutes. This suggests that at these much higher temperatures, where protein aggregation and greater cellular damage can occur, that HSP induction in thermoneutral adapted mice would be potentially greater than animals housed at standard temperatures and function as a protective mechanism. Perhaps more similar to our studies, mild heating (resulting in a 2°C increase in core temperature) five days prior to 42°C heat shock blunted HSP72, an important regulator of stress induced apoptosis, expression in mice (34). These findings correlate with our own data, suggesting that the diminished HSP induction in mice housed at TT following WBH may also be a result of heat acclimation. A second possibility for the lack of induction of HSPs in mice, even though their core body temperature is elevated by WBH, may be due to the effects of adrenergic receptor signaling on HSP induction. Several studies in various mammals and vertebrates have shown that circulating catecholamines can enhance the expression of HSPs in various tissues through currently unknown mechanisms (35–37).

In particular, work in trout and porcine models have shown that HSP induction by stressors such as high temperatures or hypoxia, respectively, can be mitigated or even abrogated by use of β 1 and β 2-adrenergic receptor antagonists (35,37). Overall, both our work, and the previous reports show the importance of fully understanding the role of ambient temperature in data interpretation of HSP expression studies.

In addition to changes in HSP expression in the brain, we observed the greatest shifts in HSP expression in the hearts and lungs of mice housed at ST versus TT. Since heart and lung tissues are highly innervated by sympathetic fibers, it is not surprising that baseline systemic stress could affect responses to heat stress in these cells. These findings are in line with previous studies showing that expression of HSP70 in brown adipose tissues could be induced by β -adrenergic receptor activation (38). In our studies, the differences were readily abrogated by prior administration of the β -blocker, propranolol, suggesting that β -adrenergic receptors play an important role in regulating HSP expression under mild stress conditions, as well. Furthermore, these findings also highlight the fact that even moderate cold stress could significantly alter the induction of HSPs, which could have important scientific implications for modeling pathologies including myocardial infarctions, infections, and even malignancies.

Much more investigation will be required to fully tease out the implications of housing temperature on HSP biology, including study of additional and a wider range of temperatures used to induce thermal stress. Furthermore, the work presented here does not extend to different murine strains. Other immunocompetent strains including C3H and C57BL/6 will need to be examined to determine how HSP expression is impacted by housing temperature. Other important work should be directed at immunocompromised mice such as SCID and NUDE which are often used for tumor xenograft experiments. We expect particularly insightful findings from NUDE and other hairless mice (i.e. SKH1) as they may be even more sensitive to cool housing temperatures. Moreover, very little is still known about whether the α -adrenergic receptors also play a role in regulating the induction of HSPs in response to stress. These receptors have a greater binding affinity for norepinephrine, but are primarily located at peripheral vascular sites and the genitourinary

tract (39,40). Thus, these receptors may also contribute to the differences in HSP expression in cold stressed mice at these different tissue sites. A recent study showed that HSP70 inhibited the inflammatory response through IL-10 driven downregulation of CCAAT/enhancer-binding proteins (41). As these transcription factors are involved in the regulation of various cellular functions including proliferation, differentiation and cytokine production, activation of this pathway may have wide-ranging off target effects. Because HSPs may serve as targets for therapeutic intervention, it will ultimately be important to examine the impact of ambient temperatures on HSP expression in mouse models for diseases, including cancer (42–44), autoimmunity (45) and infection (46).

Conclusion

In summary, the findings presented here highlight the surprising influence that mild chronic stress can have on basic physiological responses. In particular, this work demonstrates that stress induced by housing temperature has a significant, yet underappreciated, effect on HSP induction in mice following WBH. Our data indicate that the metabolic impact of housing temperature should be considered when any type of stress response is being studied in mice. Overall, it would be prudent for investigators working on HSPs or any stress proteins in animal models to perform these studies at more than one ambient temperature to fully gauge the complete range of responses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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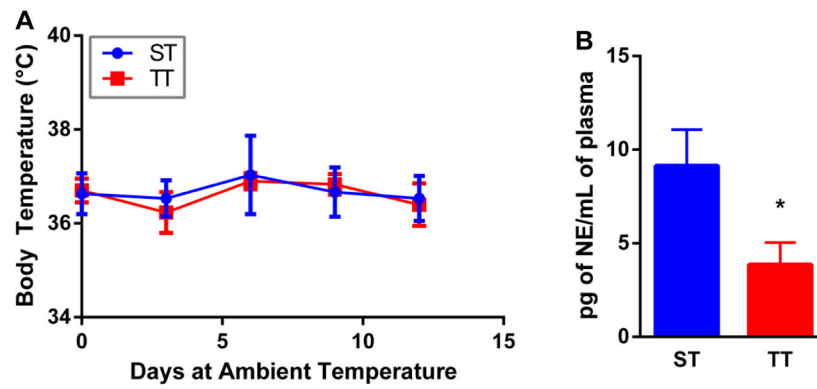


Figure 1.

Body temperature does not change in mice housed at ST and TT, but plasma norepinephrine levels are increased in mice maintained at ST. (A) core body temperature of mice housed at ST and TT for 12 days. No significant differences by two-way Anova; $n = 3$. (B) Plasma levels of norepinephrine from mice acclimated to standard room temperature and thermoneutrality for 14 days. $*p < 0.05$ by Student's t test; $n = 8$.

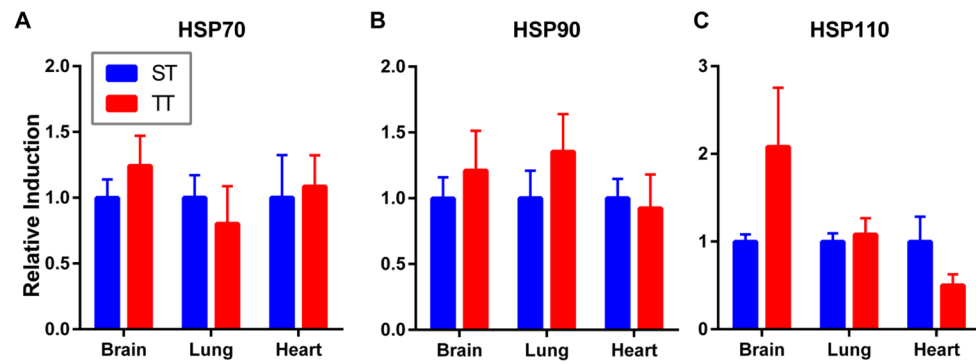


Figure 2.

Housing mice at thermoneutrality does not significantly alter baseline expression of HSPs. Brain, lung, and heart expression of (A) total HSP70 (B) HSP90 (C) HSP110 of mice housed for 14 days at standard temperatures and thermoneutrality. No statistically significant differences by Student's t test. $n = 3-4$ /experiment, experiments were performed twice and data were combined.

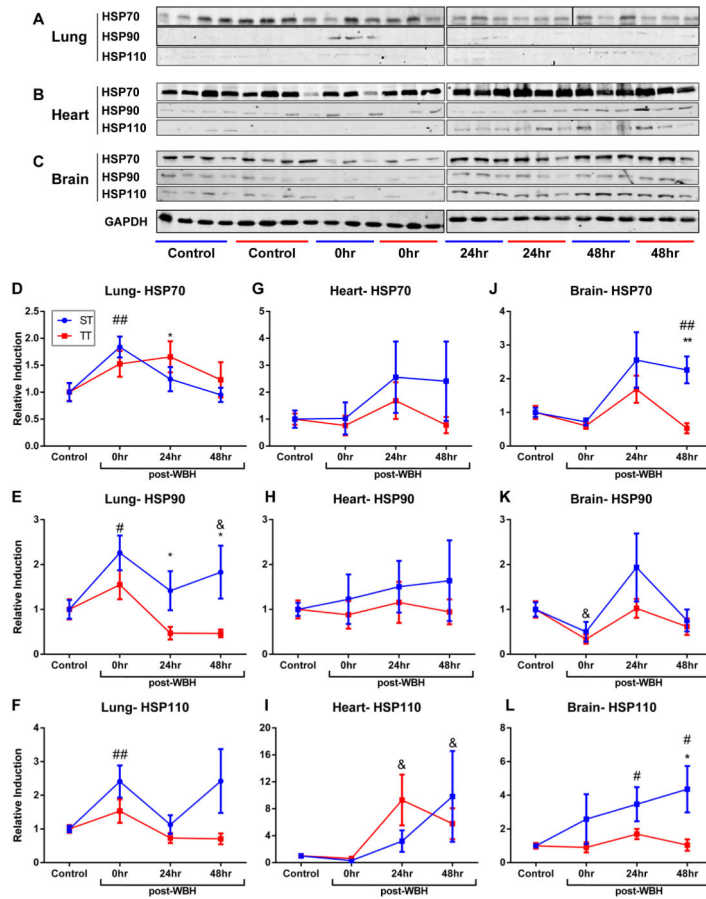


Figure 3.

The expression of heat shock proteins is decreased in visceral organs of mice housed at TT compared to mice housed at ST after WBH. (A) Lung, (B) heart, and (C) brain expression of HSP70, HSP90 and HSP110 following 6hrs of whole body hyperthermia. (D–L) Fluorescence intensity of heat shock protein expression relative to unheated ST or TT controls. * $p < 0.05$, ** $p < 0.01$ (ST versus TT), # $p < 0.05$, ## $p < 0.01$ (heated ST versus unheated ST control), & $p < 0.05$ (heated TT versus unheated TT control) by Student's t test; $n = 3-4$ /experiment, each experiment was performed twice and combined.

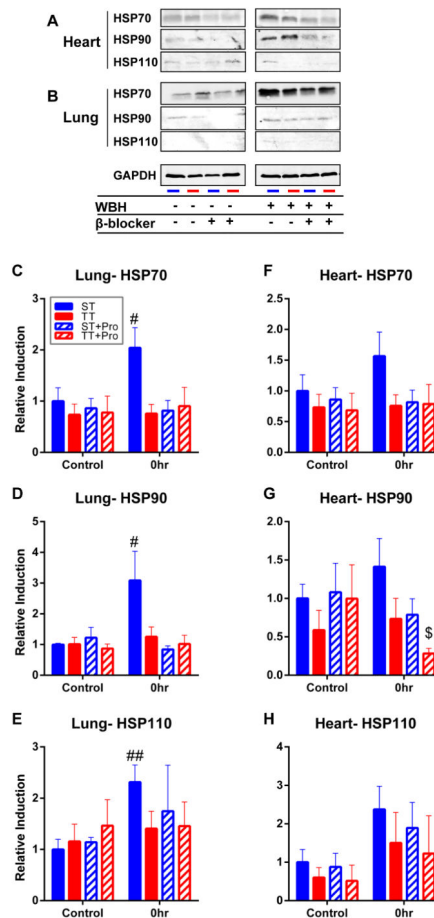


Figure 4.

The induction of heat shock proteins is abrogated by propranolol in mice housed at ST after WBH. **A)** Lung and **B)** heart expression of HSP70, HSP90 and HSP110 following 6hrs of whole body hyperthermia with and without the β -blocker, propranolol. **C–H)** Densitometry quantifications of heat shock protein expression relative to unheated ST only, TT only, ST + propranolol, and TT + propranolol. ## $p < 0.01$ (heated ST versus unheated ST control), \$ $p < 0.05$ (heated TT+ propranolol compared to unheated TT+ propranolol) by Student's t-test; $n = 3-4$ /experiment, each experiment was performed twice and combined.