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Heat shock increases hydrogen peroxide release from circulating hemocytes of the snail *Biomphalaria glabrata*.

Euan R. O. Allan^{1,*}, Michael S. Blouin²

¹Department of Pathobiology, School of Veterinary Medicine, St. George's University, Grenada, West Indies

²Department of Integrative Biology, College of Science, Oregon State University, Corvallis, Oregon, USA

Abstract

Planorbid freshwater snails are important intermediate hosts for parasitic diseases caused by parasitic worms, most notably schistosomiasis. There are numerous reports of snails, specifically *Biomphalaria glabrata*, having compromised defences against schistosomes after being exposed to thermal stress. Environmental modifications to the defenses of schistosome transmitting snails could have negative ramifications for human disease risk in the context of climate change. Here the effects of heat shock on the production of hydrogen peroxide, a primary anti-microbial effector in many molluscs, were examined. The present findings show that heat shock increases NADPH oxidase 2 mRNA levels and hydrogen peroxide produced by snail hemocytes, and that both of these phenotypes could be reversed by an HSP-90 inhibitor. These findings indicate that snail defense systems are altered by heat shock at a molecular level in *B. glabrata*, and that snail immunity to many pathogens may be altered by the rapid variations in temperature that are associated with global climate change.

Keywords

Biomphalaria glabrata; NOX2; heat shock; hydrogen peroxide; hemocyte

1. Introduction

Freshwater planorbid snails are obligate intermediate hosts for various parasitic worms which can cause both human and veterinary diseases. Schistosomiasis is the most

*Corresponding Author euanroallan@gmail.com (EROA), Department of Pathobiology, School of Veterinary Medicine, St. George's University, Grenada, West Indies.

Euan Allan: Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Resources; Software; Supervision; Validation; Visualization; Roles/Writing - original draft; Writing - review & editing.

Michael Blouin: Funding acquisition; Project administration; Resources; Supervision; Roles/Writing - original draft; Writing - review & editing.

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detrimental disease caused by parasitic worms, because it can affect numerous mammalian species and is the cause of extensive human illness and hundreds of thousands of deaths per year [1]. The snail *Biomphalaria glabrata* (Bg) is a notable intermediate host of the schistosome parasites that cause schistosomiasis, and has a primary role the transmission of *Schistosoma mansoni* (Sm) in the Americas. Understanding the physiology and defense systems of schistosome transmitting snails, like *B. glabrata*, may be important for the development of methods to control snail populations and schistosome infection or transmission [2, 3].

The production of reactive oxygen species (ROS) by hemocytes in *B. glabrata* has been shown to be vital for snail defense against schistosome infection [4-11]. In hemocytes, NADPH oxidase 2 (NOX2) dependent ROS are a substrate for Cu/Zn superoxide dismutase's (SOD1) production of hydrogen peroxide, which is a potent anti-microbial effector and one of the primary mechanisms by which hemocytes kill schistosome larvae [6, 8, 12, 13]. Genetic or environmental factors that alter the efficiency of hydrogen peroxide production by hemocytes can modify the resistance of Bg to Sm infection, and thus may help to determine the magnitude of transmitted parasites to mammalian hosts [4, 6, 9]. It is important to note that the activity of intraphagosomal NOX2, and the subsequent enzymatic activity by antimicrobial enzymes including SOD1, can alter the efficiencies of many immune pathways and are completely essential for anti-microbial defenses in most organisms [12, 14]. Most importantly, superoxide, hydrogen peroxide, and hypochlorous acid are primary anti-microbial effectors, and any alteration to the activities of the enzymes that produce these compounds can have drastic effects on immune defense [14].

Recently, it has been shown that alterations in water temperature can modify the physiology of Bg and/or its ability to resist schistosome infection [15-21]. Specifically, heat shock of resistant Bg can increase their susceptibility to schistosome infection, though this phenotype is partially dependent on the Bg strain/genotype and duration of heat shock [15-17, 21, 22]. Heat Shock Proteins (HSP) are produced when cells experience a diverse array of environmental stressors. One such stress, transient elevated temperatures or 'heat shock', has been shown to induce the expression of numerous heat shock proteins, which trigger responses in multiple systems and activate numerous pathways. Generally, these responses promote stabilization of proteins, and help to regulate immune responses [23]. In the context of snails, heat shock of resistant snails increases the expression of HSPs, specifically HSP-70 and 90 [15, 17]. Chemical inhibition of HSP-90 was shown to prevent the reversal of the resistant phenotype in BS-90 snails [15]. These findings have implicated HSPs in schistosome defense, and also suggest that the increasing likelihood of heat shock, in the context of global climate change, may increase the susceptibility of Bg to schistosomes. No physiological mechanisms currently explain why Bg can become more susceptible to infection when they are exposed to elevated temperatures.

Given that Guadeloupean *B. glabrata* (BgGUA) snails do not exhibit modified infection phenotypes to schistosomes after heat shock [22], one would not expect that their primary immune defense (hemocyte produced ROS) would be altered by heat shock events. Contrary to this prediction, this study links one essential mechanism of hemocyte defense with heat shock exposure. The presented findings show that heat shock of BgGUA increases hydrogen

peroxide production by hemocytes in an HSP-90 dependent manner. These results also demonstrate that this elevated hydrogen peroxide production is likely the result of increased NOX2 mRNA levels, rather than increased SOD1 mRNA levels. Thus, there may be more substrate for SOD1 to convert to hydrogen peroxide rather than more SOD1 mRNA expression. Though elevated hydrogen peroxide is associated with schistosome resistance in Bg, and may help to explain the resilient infection phenotype in heat shocked BgGUA, extensive ROS production is a double-edged sword. Excessive ROS can contribute to dysregulation immune responses as well as bystander damage to self tissue. Therefore, these findings may also implicate heat shock with bystander damage during immune responses.

2. Materials and Methods

2.1 Animals and ethics.

BgGUA were collected in 2005 from Guadeloupe, and were housed and maintained identically as previously described [24-28]. In brief, snails were housed in 12-25-gallon glass tanks temperature in 26°C dechlorinated water. Experiments adhered to the Public Health Service Domestic Assurance for humane care and use of laboratory animals (PHS Animal Welfare Assurance Number A3229-01), as Animal Care and Use Proposal 4360; which was approved by the Oregon State University Institutional Animal Care and Use Committee.

2.2 Experimental protocol and sampling procedures.

Age matched (9-12 mm) BgGUA were removed from standard conditions (26°C) and placed in small tubs at 20°C (cold shock), 26°C (control), or 32°C (heat shock) for 6 h. Alternatively, snails were removed from standard conditions and pre-incubated with 100 nM Geldanamycin (GA; 1000x stock in Dimethyl sulfoxide (DMSO); Cayman-Chemical, Ann Arbor, MI; [15]), an HSP-90 inhibitor, or the DMSO carrier in water overnight prior to exposure to 26°C or 32°C for 6 h [22]. After temperature treatment, hemolymph was extracted at 26°C via the headfoot retraction technique as previously described [13]. In brief, snails from each treatment group were cleaned with 70% ethanol, hemolymph was extracted and pooled by treatment (from a minimum of 15 snails/treatment), shell debris was allowed to settle, and cells from each pooled group were counted (hemocytometer) and diluted to an equal concentration with pre-warmed Chernin's Balanced Salt Solution (CBSS; [7]) (20°C, 26°C, or 32°C) before plating (although cell numbers showed minimal variation). A separate aliquot of hemolymph from each temperature treatment was saved for qPCR analysis (see below). 100 µl aliquots of hemolymph were combined with pre-warmed CBSS, corresponding to the temperature at which the snails from each treatment were incubated, and allowed to settle and adhere in sterile 96-well black-walled/clear-bottom culture plates (Corning) for 100 min at 20°C, 26°C, or 32°C. After three successive washes with 200 µl pre-warmed CBSS, each well was replaced with 100 µl of pre-warmed (20°C, 26°C, or 32°C) CBSS or pre-warmed CBSS containing phorbol 12-myristate 13-acetate (PMA: 500 nM [4, 10]; Sigma-Aldrich, St. Louis, MO); or PMA with Diphenyleneiodonium (DPI: 0.5 µM [29]; Sigma-Aldrich, St. Louis, MO) [13]. All samples were incubated for 2 h at 20°C, 26°C, or 32°C before extracellular hydrogen peroxide release was measured. These processes were repeated on five separate occasions, for a total $n=5$.

2.3 Assessment of hydrogen peroxide production.

After cells were incubated for 2 h, as described above, 50 μ l of supernatant were removed from each well, from all temperature/compound treatments, and placed in a single culture plate. Thus, this final plate contained an aliquot of supernatant from cells incubated at each temperature, and exposed to each treatment. Hydrogen peroxide, and thus a proxy measure of the total oxidative burst of the hemocytes, was assessed by the Amplex Red Hydrogen Peroxide Assay Kit (Molecular Probes) according to the manufacturer's instructions (30 min post exposure to Amplex Red reagent at a constant 26 °C) [4, 10, 12, 30, 31]. DPI (direct inhibitor of NOX2) was used as a negative control for bursting cells. Data are shown as relative fluorescent units (RFU)/5000 cells, normalized to the 26 °C temperature control. Fluorescence was measured at 26 °C using a Molecular Devices Gemini XS Fluorescent Microplate Reader.

2.4 qPCR of HSP-90, SOD1, and NOX2 in BgGUA hemolymph.

Quantitative RT-PCR (qPCR) was used to assess mRNA levels of SOD1 and NOX2 in pooled BgGUA hemolymph extracted from snails that were exposed to 20°C, 26°C, or 32°C for 6 h; or 32°C pre-incubated with Geldanamycin prior to temperature exposure. RNA extraction, cDNA synthesis, and qPCR were conducted as previously described, and all samples were stored at -80 °C [12, 28, 30-32]. Briefly, total RNA from hemolymph (extracted as described above) was extracted using the Direct-Zol RNA miniprep kit (Zymo Research) and quantified (all samples contained between 50ng/ μ l and 200ng/ μ l total RNA) using a Nanodrop (ThermoFisher). RNA quality was assessed via A_{260}/A_{280} ratios, which were between 1.8–2.2 for all samples; cDNA was synthesized using iScript Reverse Transcriptase Supermix for RT-qPCR (BioRad), and qPCR was performed using 300 nM of high efficiency primers (all primers had a single melt curve, and had efficiencies between 90-100%) [15, 28, 33, 34]. HSP-90 (F: 5'- TGTGCGCAGAGTGTTTCATCATGG-3'; R: 5'- CTCCTGTGAGGCTTCAATGAGTC-3')[15], SOD1 (F: 5'- GGTGATGATGGTGTGCTGA-3'; R: 5'- GATACCAATGACACCACAAGCTAA-3') [34] and NOX2 (F: 5'- TCACCAGTCGAGAGTGTGTTGAA-3'; R: 5'- TGCCACTCTAGGCGTGAGAT-3') [33] were designed and used as previously described. *β actin* (F: 5'-GCTTCCACCTCTTCATCTCTTG-3'; R: 5'- GAACGTAGCTTCTGGACATCTG-3') was used as an internal control for mRNA expression, and did not vary across treatments (SD of delta Ct was examined from numerous genes examined by RNA sequencing in a previous study [28, 35, 36]. The following PCR conditions were applied (using an Applied Biosystems 7500 fast qPCR thermocycler): 95°C for 5 min; 40 cycles of 95°C for 15s and 60°C (58°C for NOX2) for 15s [15, 28, 33, 34]. All mRNA levels were normalized to *β actin*, and presented relative to the 26°C control temperature.

2.3 Statistical analyses.

Each replicate (n) for hemocyte experiments represents a single sampling of the base population, which occurred on a single day and used a single Amplex Red or qPCR assay [13, 29, 31]. Statistical analyses were completed by one-way ANOVA (Barlett's test for equal variance showed no differences in any groups) with a Tukey post-test ($p < 0.05$). An

additional two-way ANOVA was completed on samples exposed to temperature and Geldanamycin treatments. Analyses were completed using GraphPad Prism software (La Jolla, CA, USA).

3. Results

3.1 BgGUA hemocytes exhibit elevated hydrogen peroxide production after heat shock.

PMA induces the canonical hydrogen peroxide pathway beginning by activating protein kinase C and mitogen-activated protein kinases [10, 37]. Subsequently, NOX2 is activated and produces superoxide which is then converted to hydrogen peroxide by SOD1. These findings verify that PMA-induced hydrogen peroxide production can be inhibited by DPI exposure in BgGUA hemocytes, and that PMA stimulation is sufficient for hemocyte hydrogen peroxide production (Figure 1a) [4, 9, 10, 13, 38]. PMA stimulated hydrogen peroxide production, but not baseline fluorescence observed with DPI inhibition, is significantly altered by 6 h temperature treatments (Figure 1b-c). Hemocytes from snails exposed to 6 h at 20°C produced ~25% less hydrogen peroxide than the 26°C controls, while 32°C heat shocked snails has hemocytes that produced ~25% more hydrogen peroxide after stimulation with PMA (Figure 1b). Heat shock was verified by measurement of HSP-90 mRNA expression (Figure 1d).

3.2 Heat shocked increases hydrogen peroxide production in BgGUA, independent of SOD1 mRNA expression, via HSP-90-dependent increases in NOX2 mRNA levels.

Since a significant effect of heat shock on hemocyte hydrogen peroxide production after PMA stimulation was observed, it was prudent to examine mRNA levels of SOD1 and NOX2 in hemolymph extracted from snails exposed to all three temperature treatments as well as snails exposed to heat shock after being pre-treated with the HSP-90 inhibitor (Geldanamycin). SOD1 mRNA levels in were unaffected by temperature treatments (Figure 2a). Intriguingly, NOX2 mRNA levels were significantly elevated, by ~1.75-fold, in hemolymph from 32°C treated snails, but not if the snails were treated with Geldanamycin (Figure 2b).

Some recent evidence suggests that HSPs, including HSP-90, can modify NOX2 dependent ROS production [39, 40]. Since hemocyte hydrogen peroxide production is dependent on NOX2 generated ROS, HSP-90 was inhibited to determine if HSP-90 activity was involved in the increase in hydrogen peroxide after heat shock of BgGUA. Geldanamycin is an HSP-90 inhibitor that has been previously shown to bind HSP-90 and block some of the physiological effects of HSP-90 and heat shock in Bg [15, 41]. Snails pre-treated with Geldanamycin, before heat shock treatment, do not exhibit elevated hydrogen peroxide compared to 26°C controls (Figure 2c). Together, these findings directly implicate HSP-90 activity in the hemocyte hydrogen peroxide pathway via some interaction with NOX2 mRNA expression.

4. Discussion

The interplay between global climate change and infectious disease risk is complex and will likely be very disease specific. Mathematical models of schistosome infection currently

postulate that warming trends may increase global schistosomiasis risk primarily because they suggest that there will be an increase in the range of intermediate snail species (like Bg) outside of their current control areas [42-44]. Additionally, direct experimental approaches, addressing the hypothesis that climate change could affect schistosome transmission, suggest that resistant snails could become more susceptible in warmer or less stable conditions as a result of increased HSP expression [15, 17]. Heat shock and HSPs have been implicated in snail-schistosome defense and snail physiology, but mechanistic explanations for any heat shock-induced effects are still lacking and the observed phenotypes are inconsistent across strains/conditions [15-18, 21, 22]. The present study hypothesized that BgGUA hemocyte responses would not be altered by heat shock due to the robustness of BgGUA's schistosome infection phenotype [22]. Contrary to this hypothesis, the findings provide further evidence that HSP-90 activity, as a result of heat shock, can alter the physiology of Bg by modifying its immune response. These results show that heat shocked BgGUA hemocytes have elevated NOX2 mRNA levels and produce more hydrogen peroxide after stimulation with PMA. These findings also support the conclusion that these effects are likely to be dependent on HSP-90 activity, and a subsequent HSP-90 dependent increase in NOX2 mRNA levels, because HSP-90 inhibition prevents elevated NOX2 mRNA levels, and exacerbated hydrogen peroxide production by heat shocked hemocytes. Thus, it is reasonable to postulate that heat shocked BgGUA hemocytes produce more hydrogen peroxide as a result of increased NOX2 mRNA levels stemming from HSP-90 activity. These results support the idea that heat shock and schistosome related immune defenses are intrinsically linked [15, 17, 18, 39].

It is not surprising that hemocytes from snails kept at sub-optimal temperatures, in this case 20°C, produced less hydrogen peroxide despite having normal mRNA levels of NOX2 and SOD1. This is likely because ectothermic animals can experience cold-induced slowdowns in metabolism and mitosis [16, 45]. Additionally, cold shock can inhibit HSP proteins in other species and therefore, the cold shock treatment could also limit the effects of elevated HSP expression on NOX2 [46]. Conversely, one may not expect heat shock to increase hydrogen peroxide in BgGUA because this antimicrobial mediator is essential for schistosome defense, and the susceptibility of BgGUA to schistosome infection is unaltered by heat shock in previous work [22]. Given that BS90 snails can become more susceptible to schistosomes after heat shock, that resistant snails generally produce more hydrogen peroxide, and that hydrogen peroxide is essential for schistosome killing [9, 15]; one could expect heat shock to either compromise hydrogen peroxide production, or cause extensive damage to infected snails increasing their mortality to infection. Instead, there is a marked increase in hydrogen peroxide production in heat shocked BgGUA hemocytes, and this increase is independent of temperature alone because it could be prevented by the addition of HSP inhibitors. These snails, unlike BS90 snails under specific conditions, also show no changes in susceptibility or mortality during post-heat shock schistosome challenge [15, 17, 21, 22]. It is possible that BgGUA's heat-stable infection phenotype is the result of the protective effects of elevated ROS or an ability to mitigate damage to its own tissues from elevated ROS. Therefore, one could speculate that the discrepancies in observed infection phenotypes may be partially explained by differential effects of HSP on NOX2 expression and ROS production. Regardless of these speculations and susceptibility discrepancies, these

findings are the first to provide a possible molecular mechanism underlying some changes in immune responsiveness in snails as a result of climatic events.

In other species, active HSP-90 has been shown increase cytosolic superoxide, the substrate for SOD1, by directly increasing NOX2 expression [39, 40]. The results in this study support these findings because heat shocked hemocytes have elevated NOX2 mRNA and hydrogen peroxide, despite having normal mRNA levels of SOD1. These findings suggest that the increased NOX2 mRNA levels in heat shocked of BgGUA hemocytes, likely provides more superoxide for SOD1 to convert to hydrogen peroxide. This process is not initiated in the presence of Geldanamycin which blocks the effects of HSP-90 [15, 41]. Therefore, heat shocked hemocytes pre-treated with Geldanamycin do not have elevated NOX2 mRNA levels and do not produce more hydrogen peroxide, making the observed phenotype HSP-90 dependent. The requirement of HSP-90 for this phenotype reflects the HSP-90 dependence shown in other studies describing heat shock phenotypes in Bg, and further implicates HSP-90 in immune response of schistosome compatible snails [15]. These findings also implicate heat shock in immune defenses against many potential pathogens in snails, not just schistosomes, because ROS/hydrogen peroxide are key anti-microbial factors in all animals. Future work should examine the production of ROS in the context of schistosome infection and heat shock using multiple strains of both Bg and Sm to determine if these findings are mirrored *in vivo*.

The present study has shown that heat shock of BgGUA exacerbates hydrogen peroxide by hemocytes in an NOX2 and HSP-90 dependent manner. Characterizing the physiological ramifications of global climate change on the physiology ectothermic animals, like trematode transmitting snails, is important from both an ecological and epidemiological context as it can provide mechanism for broader phenotypes observed in nature. Understanding how the warming climate in the tropics can interact with the immune system of intermediate hosts of human diseases may provide important information for modeling future disease dynamics, and these novel phenotypes should be considered in these models. Finally, given the importance of numerous molluscs to food systems, fisheries economies, and aquaculture the present study provides a novel interaction between heat shock and important immune pathways intrinsic to mollusk immunodefense. Understanding how environmental conditions could compromise food production by altering immune responses to problematic and opportunistic pathogens may be important given increasing global climatic variation.

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Highlights:

- Heat shock increases NOX2 mRNA expression in snail hemocytes
- Heat shock increases hydrogen peroxide production by snail hemocytes
- Heat shock induced hydrogen peroxide production is HSP-90 dependent

Figure 1. Hydrogen peroxide production by BgGUA hemocytes extracted from snails after cold or heat shock.

(A) Stimulation of BgGUA hemocytes with PMA (500 nM), PMA (500 nM) + DPI (0.5 μ M), or CBSS alone from snails kept at 26°C ($n = 5$). (B-C) Hemocytes recovered from BgGUA exposed to 20°C (cold shock), 26°C (control), or 32°C (heat shock) for 6 h and stimulated with (B) PMA (500 nM) or (C) PMA (500 nM) + DPI (0.5 μ M) prior to hydrogen peroxide assessment ($n = 5$). Data (mean $\pm$ SD) are shown as RFU/5000 cells presented relative to the 26°C temperature control. (D) HSP-90 mRNA levels in hemocytes recovered from BgGUA temperature treatments listed above. mRNA levels are normalized to β -actin, presented relative to the 26°C (control), and shown as mean $\pm$ SD. Significant differences from control (PMA+DPI or 26°C) (one-way ANOVA $p < 0.05$) are denoted by an asterisk (*).

Figure 2. Elevated hydrogen peroxide production by BgGUA hemocytes is dependent on HSP-90-induced increases in NOX2, but not SOD1, mRNA levels.

(A) SOD1 and (B) NOX2 mRNA levels in hemocytes recovered from BgGUA exposed to 20°C (cold shock), 26°C (control), or 32°C (heat shock) for 6 h; as well as 32°C exposed snails that were pre-treated with Geldanamycin (GA) ($n = 5$). mRNA levels are normalized to β -actin, presented relative to the 26°C (control), and shown as mean $\pm$ SD. (C) Hemocytes recovered from BgGUA pre-incubated with GA (100 nM) or carrier overnight and then exposed to 26°C (control), or 32°C (heat shock) for 6 h; and stimulated with PMA (500 nM) prior to hydrogen peroxide assessment ($n = 5$). All data (mean $\pm$ SD) are shown as RFU/5000 cells presented relative to the 26°C temperature control. Significant differences (one and two-way ANOVA $p < 0.05$) are denoted by an asterisk (*).