Title:

PASSIVE HEAT STRESS REDUCES CIRCULATING ENDOTHELIAL AND PLATELET MICROPARTICLES

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Running Title:

Circulating Microparticles in Heat Stress

What is the central question of this study?

Does passive heat stress of +2°C esophageal temperature change concentrations of circulating arterial endothelial- and platelet-derived microparticles in healthy adults?

What is the main finding and its importance?

Concentrations of both circulating endothelial- and platelet-derived microparticles were markedly decreased in heat stress. Reductions in circulating microparticles may indicate favorable vascular changes associated with non-pathological hyperthermia.

Abstract

Interest in circulating endothelial- (EMPs) and platelet- (PMPs) derived microparticles has increased due to their potential pathogenic role in vascular disease and as biomarkers for vascular health. Hyperthermia is commonly associated with a proinflammatory stress, but may also provide vascular protection when the temperature elevation is non-pathological. Circulating microparticles may contribute to the cellular adjustments and resultant vascular impacts of hyperthermia. Here, we determined whether circulating concentrations of arterial EMPs and PMPs are altered by passive heat stress (+2°C esophageal temperature). Ten healthy young men (age: 23±3 yr) completed the study. Hyperthermia was achieved by circulating ~49°C water through a water perfused suit that covered the entire body except the hands, feet, and head. Arterial (radial) blood samples were obtained immediately prior to heating (normothermia) and in hyperthermia. Average esophageal temperature in normothermia was 37.2±0.1°C and at hyperthermia 39.1±0.1°C. Concentrations of both circulating EMPs and PMPs were markedly decreased in hyperthermia. Activation-derived EMPs were reduced by ~30% (from 61±8 to 43±7 MP/μL; $p<0.05$), and apoptosis-derived EMPs were reduced by ~45% (from 46±7 to 23±3 MP/μL; $p<0.05$). Likewise, circulating PMPs were reduced by ~75% in response to hyperthermia (from 256±43 to 62±14 MP/μL). These beneficial reductions in circulating EMPs and PMPs in response to a 2°C increase core temperature may in part underlie the reported vascular improvements following therapeutic bouts of physiologic hyperthermia. *(Maximum 250 words)*

Introduction

The molecular and cellular changes in heat stress are integrative and complex. Initially, heat stress incites an acute phase cytokine response that protects and repairs tissues, but can progress into a proinflammatory and procoagulative state when severe (Bouchama & Knochel, 2002). Tissue injury in heat stroke (clinically defined as a core temperature above 40°C with the presence of central nervous system dysregulation) consequently arises not only from the direct cytotoxic effect of heat, but also from progressive inflammatory and hypercoagulant responses of the host (Bouchama & Knochel, 2002; Leon & Bouchama, 2015). The cellular changes associated with progressive heat stress may involve contribution from circulating microparticles.

Microparticles are small (100 to 1000 nm) membrane vesicles that are released into the circulation by various cell types (e.g. endothelial cells, platelets, leukocytes and monocytes) in response to a myriad of physiologic and pathologic processes, indicative of cellular activation or apoptosis. Clinical interest in circulating microparticles, particularly those derived from endothelial cells (EMPs) and platelets (PMPs), has increased due to their potential pathogenic role in vascular disease and procoagulant states (Stepien *et al.*, 2012; Barteneva *et al.*, 2013), and as biomarkers of vascular health (Horstman *et al.*, 2004; Martinez *et al.*, 2005). Important to the cellular process of progressive heat stress, elevations in circulating microparticles as well as coagulation markers have been reported in a baboon model of heat stroke (Gilbert *et al.*, 1991). It is currently unknown, however, whether circulating microparticles increase in response to physiologic levels of heat stress, i.e. core temperature no greater than 40°C, in healthy adult humans.

Accordingly, the experimental aim of this study was to determine the effects of passive heat stress on circulating arterial concentrations of EMPs and PMPs in healthy adults. Due to the known hypercoagulant state associated with severe pathological hyperthermia, it was

hypothesized that passive heat stress of +2°C core temperature would cause an increase in circulating EMPs and PMPs. Whole-body heating inducing a +2°C rise in core body (esophageal) temperature was achieved using a warm water perfused suit. Circulating concentrations of EMPs and PMPs were determined from arterial blood samples taken in normothermia (before heating) and in hyperthermia.

Materials and Methods

Ethical Approval

All experimentation was completed at the Centre for Heart Lung and Vascular Health, University of British Columbia, Kelowna. The ethical committee of the University of British Columbia approved the study (H15-00166). The study conformed to the standards set by the Declaration of Helsinki. All subjects provided informed written consent before experimentation.

Subjects

Ten healthy young men (age: 23 ± 3 yr) were studied. All subjects were non-obese (body mass index: 23.0 ± 2.0 kg/m^2) normotensive (blood pressure: 118/70 ± 6/7 mmHg), normoglycemic (<99 mg/dL), non-smoking and free of overt cardio-metabolic and respiratory disease.

Experimental Protocol

Subjects arrived at the laboratory after a minimum 4-hour fast and 12-hour abstinence from alcohol and caffeine containing beverages. Under local anesthesia (1% lidocaine) and ultrasound guidance, a 20-gauge arterial catheter (Arrow, Markham, Ontario, Canada) was placed in the right radial artery. For measures of arterial blood pressure the radial catheter was attached to a pressure transducer positioned at the height of the right atrium (TruWave transducer; Edwards Lifesciences). Heart rate was obtained from the R-R intervals measured from a three-lead ECG. Finally, a thermocouple probe (RET-1, Physitemp Instruments, Clifton, NJ) was inserted 40 cm past the nostril into the esophagus for measures of core temperature.

Following arterial cannulation and insertion of the esophageal thermometer, subjects were fitted into a tube-lined suit (Med-Eng, Ottawa, ON, Canada) that covered the entire body except the head, feet and hands. After 15 minutes of supine rest, a baseline blood sample was collected from the radial artery. Thereafter, the tube lined suite was perfused with 49°C water until the subjects' reached an esophageal temperature of +2°C above baseline, an absolute core temperature of 39.5°C, or at the subject's volitional thermal tolerance, at which point a second blood sample was collected.

Microparticle Isolation and Identification

Microparticle isolation and identification was based on accepted methodology, as described by (Jenkins *et al.*, 2013; Durrer *et al.*, 2015). Briefly, arterial blood was collected into sodium citrate containing tubes and centrifuged at 1,500 x g for 10 minutes at room temperature, thereafter platelet poor plasma was collected and stored at -80°C for batch analysis. For the characterization and quantification of circulating MP subspecies, platelet poor plasma was thawed and centrifuged at 13,000 x g for 2 minutes yielding platelet free plasma, and 100μL was transferred to a TruCount tube (BD Biosciences, New Jersey, USA). EMP phenotype was determined using markers indicative of endothelial activation (CD62E) and apoptosis (CD31/CD42b) (BioLegend, San Diego, California). PMP phenotype was determined by using markers indicative of platelet activation (CD62P). Samples were incubated with the flourochrome labeled antibodies for 20 minutes in a dark at room temperature. Following incubation, samples were fixed with 2% paraformaldehyde (ChemCruz Biochemicals, Santa Cruz, California) and diluted with RNAse free PBS. Microparticle size threshold was established using Megamix-Plus SSC calibrator beads (Megamix-Plus SSC beads, Biocytex, Marseille, France), and only events <1 μm in size and positively expressing markers of endothelial activation (CD62E+) and apoptosis

(CD31$^+$/CD42b$^-$) and platelet activation (CD62P) were counted. Samples were analyzed using BD Biosciences FACSAria I High Speed Cell sorter and flow cytometer (University of Colorado Anschutz Medical Campus Allergy and Clinical Immunology/Infectious Disease Flow Core). Importantly, the flow cytometry used has a forward and side scatter sensitivity that has been clinically validated for identification of 0.5 μm beads. The use of MegaMix Plus-SSC fluorescent beads, with varied diameters (0.16, 0.20, 0.24 and 0.5 μm), ensures the FACSAria is capable of analyzing MPs at its inferior limits. The concentration of EMPs and PMPs were determined using the formula: ([number of events in region containing MPs/number of events in absolute count bead region] x [total number of beads per test/total volume of sample]) (Nielsen *et al.*, 2014).

Statistical Analysis

All data are denoted as mean ± SD. After testing the data for normality with repeated Shapiro Wilks *W* tests, differences from baseline to heat stress were assessed using two-tailed paired Student's t-tests. Significance was determined at an alpha of $p < 0.05$.

Results

The passive heating protocol resulted in an increased esophageal temperature from 37.2±0.2°C to 39.1±0.4°C. Total heating time (i.e., time elapsed from baseline to heat stress) was 56±4 min. Only three of the subjects requested to terminate the heating before reaching an esophageal temperature of +2°C. Whole body heating resulted in a significant increase in heart rate (from 65±10 to 120±14 bpm) and a reduction in mean arterial blood pressure (from 91±7 to 78±10 mmHg).

Circulating arterial concentrations of EMPs and PMPs were markedly affected by passive heat stress. Changes in activation- and apoptosis-derived EMPs are shown in Figure 1. Heat stress resulted in a ~30% reduction in activation-derived EMPs (from 61±26 to 43±21 MP/μL; $p < 0.05$) and a ~45% reduction in apoptosis-derived EMPs (from 46±23 to 23±9 MP/μL; $p < 0.05$). In addition, circulating PMPs were significantly reduced (~75%) in response to heat stress (from 256±135 to 62±46 MP/μL; Figure 2).

Discussion

The novel finding of the present study is that passive heat stress (2°C rise in esophageal temperature) reduces circulating arterial concentrations of EMPs and PMPs in young healthy men. To our knowledge, this is the first study to determine the effects of passive heat stress on circulating microparticles. These surprising findings potentially represent novel favorable effects that buttress the vascular benefits previously reported with non-pathophysiological hyperthermia (Umscheif *et al.*, 2010; Ohori *et al.*, 2012; Laukkanen *et al.*, 2015; Brunt *et al.*, 2016; Romero *et al.*, 2017).

The notion of therapeutic benefits from controlled hyperthermia is not new (Lee Titsworth *et al.*, 2014), but has recently gained interest in large part following a 30-year prospective study (Laukkanen *et al.*, 2015) indicating that lifetime sauna use is associated with lower rates of cardiovascular related deaths. In addition, Brunt et al., (Brunt *et al.*, 2016) recently reported that 8-weeks of passive whole-body heat therapy (hot water immersion until a rectal temperature of 38.5°C, 4-5 times per week) reduced resting systolic and diastolic blood pressure, arterial stiffness, carotid intima media thickness, and improved flow mediated dilation of the brachial artery in young adults. Remarkably, these vascular changes were comparable to improvements generally associated with aerobic exercise training (Wilson *et al.*, 2016). Moreover, the improvements in vascular function were observed as early as 2-weeks into the heat intervention suggesting rapid endothelial adaptation to this stimulus. An acute vascular improvement (increased macro- and micro-vascular dilator function) with a single heat stress bout has also been shown in older men (Romero *et al.*, 2017). The results presented herein compliment and significantly extend these findings by demonstrating that acute passive heat stress induces marked reductions in circulating arterial EMPs (~35%) and PMPs (~75%). Elevated EMPs and PMPs are established biomarkers of endothelial damage (Horstman *et al.*, 2004; Viera *et al.*, 2012;

Melki *et al.*, 2017), arterial stiffness (Chen *et al.*, 2012) and systemic vascular dysfunction (Amabile *et al.*, 2005). Reductions in circulating EMPs and PMPs may be a consequence, and an indicator, of favorable endothelial and platelet responses to physiological heat stress.

Endothelial cells release phenotypically and quantitatively distinct microparticles dependent upon cell activation or apoptosis (Jimenez *et al.*, 2003), and both forms provide insight regarding the status of the endothelium serving as early physiological signals of vascular distress (Yong *et al.*, 2013). Indeed, both activation- and apoptosis-derived EMPs are thought to be causative agents in vascular pathology, contributing to vascular dysfunction, inflammatory processes and, ultimately, atherogenesis. For example, EMPs have been implicated in promoting endothelial oxidative stress (Burger *et al.*, 2011), diminishing nitric oxide bioavailability (Jimenez *et al.*, 2003), enhancing cell adhesion molecule expression (Burger *et al.*, 2011), and impairing endothelial vasomotor function (Brodsky *et al.*, 2004). In the present study, passive heat stress reduced circulating arterial concentrations of both activation- and apoptosis-derived EMPs in healthy young men, potentially reflecting an advantageous endothelial response to the heating stimulus.

Platelet-derived microparticles also adversely affect endothelial function and contributes to a proatherogenic environment by promoting inflammation and thrombosis (Melki *et al.*, 2017). Released as a result of platelet activation, PMPs can, in turn, induce endothelial cell activation and apoptosis and trigger various coagulation pathways. For example, PMPs have been shown to stimulate endothelial cells to release inflammatory cytokines such as interleukin(IL)-6 and IL-8 and express various proatherogenic adhesion molecules such as intracellular adhesion molecule-1, vascular cell adhesion-1 and E-selectin (Barry *et al.*, 1998; Nomura *et al.*, 2001; Mause *et al.*, 2005).

A reduction in PMPs with heat stress suggests a reduction in platelet activation, a finding consistent with *in vitro* studies (Pasha *et al.*, 1995; Etulain *et al.*, 2011). For example, Etulain *et al.* (Etulain *et al.*, 2011) reported that platelet adhesion, aggregation, and ATP release were diminished when exposed to a higher temperature (38.5 to 42°C) microenvironment. Mechanistically, reduced platelet activation with heat exposure is attributed to decreased p38 signaling (Etulain *et al.*, 2011). Given that p38 mitogen-activated protein kinase also causes a release of pro-inflammatory EMPs (Curtis *et al.*, 2009), it stands to reason that reduced p38 activity with heat stress may contribute to the reduction in both PMPs and EMPs reported in this study. In fact, heat stress (Liu *et al.*, 2016b), and heat stress pretreatment (Liu *et al.*, 2016a) inhibits lipolysaccharide-induced apoptosis via inhibiting the p38-signaling pathway. Decreased EMPs in passive heat stress may also result from changes in blood flow shear patterns. That is, passive heat stress increases antegrade, and decreases retrograde shear rate (Romero *et al.*, 2017). Because an acute reduction in antegrade and increased retrograde shear is associated with marked increases in EMPs (Jenkins *et al.*, 2013), it is conceivable that vice versa is true – i.e. increased antegrade and decreased retrograde shear could cause a reduction in EMPs.

In addition to reduced microparticle release, passive heat stress may also increase microparticle clearance. Clearance occurs predominantly by phagocytosis, endocytosis, micropinocytosis, and membrane fusion (Ayers *et al.*, 2015). The cells and organs involved include the epithelial cells, endothelial cells and macrophages, and lungs, spleen and liver, respectively (Ayers *et al.*, 2015). A heat-induced increase in microparticle clearance at the liver and spleen is unlikely due to the known hyperthermia-induced reductions in splanchnic blood flow (Deschamps & Magder, 1994). However, candidate mechanisms favoring heat-induced microparticle clearance include macrophage phagocytosis and uptake at endothelial cells. For example, microparticle clearance via macrophages may be increased in heat stress from augmented T cell activity

(Ostberg *et al.*, 2001). Meanwhile, microparticle uptake at endothelial cells may be increased via proliferation of the developmentally regulated endothelial cell locus 1 (DEL-1) from up-regulated vascular endothelial growth factor (VEGF) signaling (Kanamori *et al.*, 1999; Aoki *et al.*, 2005).

It is important to emphasize that the degree of acute heat stress employed in the present study represents a physiological (non-pathological) challenge exclusive to healthy young men; as such the results of our study should be viewed within this context. More severe levels of hyperthermia (core temperature exceeding 40°C) may result in elevated concentrations of circulating EMPs and PMPs. Indeed, lipid membranes are vulnerable to high temperatures (Balogh *et al.*, 2013), as excessive heat can increase the flux of extracellular Ca^{2+} into the cytosol via cyclic nucleotide gated Ca^{2+} channels (Finka *et al.*, 2012; Balogh *et al.*, 2013). An increased trans-membrane cation flux disrupts the enzymatic activity of the lipid transporters aminophospholipid translocase (flippase), floppase and scramblase, and activates cytoskeletal proteases (e.g. calpains). This in turn can destabilize the cell membrane and cause microparticle release (Roseblade *et al.*, 2013). Future studies are needed to determine circulating microparticle concentrations with varying degrees of hyperthermia. Given the adverse vascular and hemostatic consequences of heat stroke, microparticles may be important etiologically. Moreover, it is entirely unknown if microparticle concentrations are reduced with smaller core temperature elevations generally attained in therapeutic hyperthermia (i.e. +1 to 1.5°C core temperature). The time course of reduced microparticle concentrations following a return to normothermia, and with long term (weeks) bouts of repeated hyperthermia (heat acclimation) also remains unknown. Lastly, a limitation of this study is the lack of any functional vascular measure, which should be assessed in future study.

In conclusion, the results of the present study indicate that acute passive heat stress of +2°C core temperature lowers circulating arterial concentrations of EMPs and PMPs in young

healthy adult men. Reductions in circulating EMPs and PMPs may contribute to the vascular benefits associated with whole-body heat therapy. These results are exclusive to healthy young men, and future studies are needed to determine whether they extend to women, middle-aged and older adults, as well as in disease populations.

Author Contributions

Conception / design of the work was performed by ARB, PNA, and CAD. Acquisition, analysis / interpretation of data was performed by ARB, TDB, JGH, MS, RLH, DF, and JD. All authors (ARB, PNA, TDB, JGH, MS, RLH, DF, JD, and CAD) were involved with drafting the work or revising it critically for important intellectual content. All authors (ARB, PNA, TDB, JGH, MS, RLH, DF, JD, and CAD) also approved the final version of the manuscript; agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved; and qualify for authorship. All those who qualify for authorship are listed.

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Figure Legends

Figure 1. Effect of passive heat stress on circulating arterial concentrations of activation-derived (panel A) and apoptosis-derived (Panel B) EMPs. Values are mean±SD, * $p < 0.05$.

Figure 2. Effect of passive heat stress on circulating arterial concentrations of PMPs. Values are mean±SD, * $p < 0.05$.