Exercise-recruited NK cells display exercise-associated eHSP-70

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ABSTRACT

It is hypothesized that increased plasma or serum concentrations of extracellular heat shock proteins (eHSP) serve as a danger signal to the innate immune system. Cellular binding of eHSP leads to activation of NK cells and monocytes, as measured by their increased cytokine production, mitotic division and killing capacity. We examined whether eHSP binds to NK lymphocytes in vivo in athletes performing endurance exercise in the heat. Eighteen trained male runners ran at 70% V̇O₂max at 35 °C and 40% relative humidity. Venous blood collected before, after and 1.5 h after exercise was analysed for leukocyte distribution, phenotype and eHSP70. NK cell-enriched samples were examined for co-localization of CD94 and eHSP70 expression. Plasma eHSP-70 concentration was measured by ELISA. Subjects ran for ~50 min, which elicited a reversible leukocytosis. NK cell count increased 83% (p < 0.01) immediately after exercise, then decreased to 66% of the resting level 1.5 h after exercise (p < 0.05). Plasma eHSP concentration increased 167% after exercise and remained elevated (by up to 71%) 1.5 h after exercise (p < 0.01). eHSP was expressed on both NK cells and monocytes at all times; the count of NK cells positive for eHSP doubled from 0.04 ± 0.02 10⁹/L (mean ± SD) to 0.08 ± 0.06 10⁹/L after exercise. In summary, exercise in the heat increased free plasma eHSP concentration, and the eHSP co-localized with CD94 on NK cells. These data confirm the link between exercise and activation of the innate immune system.

Keywords: innate immunity, cytokines, exercise, lymphocytes, natural killer cells, HSP

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INTRODUCTION

Investigations of the linkages between exercise and immune function have shifted from characterizing leukocytosis to investigating the interaction between various specific immune factors. The roles that these immune factors play are the focus of both mechanistic and clinical investigations. Exploring various candidate molecules has led to a contemporary paradigm in which exercise exerts an acute pro-inflammatory-like response (15). In contrast, long-term adaptation to exercise can generate a suppressed inflammatory profile especially when measured at rest. Regular exercisers have different resting levels of key blood markers of inflammation, suggesting a long-term effect of routine exercise on the pro- and anti-inflammatory balance (15). For example, serum markers such as C-reactive protein and white blood cell counts (WBC) typically are lower in routine exercisers (16). One recently identified mechanistic linkage between exercise and innate immunity is the highly inducible heat shock protein-70 (HSP70). Plasma or serum concentrations of HSP70 increases with exercise but the resting concentration is lower in trained individuals, at least during the initial adaptation to exercise in a hot, humid environment (13).

Heat shock protein-70 (HSP70) is one member of a family of highly conserved intracellular proteins synthesized by cells to resist stress-induced damage by assisting with the proper folding and transport of cellular proteins (12). When located in the extracellular space (and referred to as eHSP), these proteins activate both cellular and non-cellular components of innate immunity (1, 2). In plasma or serum, eHSP70 concentration increases during physical activity ~2-6 times in relation to both the intensity and duration of exercise (7). There is little consensus on which cell type(s) are the major source of the exercise-associated elevation in eHSP70 production. However, gene activation studies demonstrate an exercise-associated upregulation of eHSP70 expression in peripheral white blood cells (4, 6). Explanations for the increased eHSP70 levels associated with exercise include increased core body temperature (19), relative acidosis, and elevated serum corticosterone concentration (5). These and other factors probably contribute to the rise in eHSP70 production during exercise, particularly in hot and humid conditions.

The release of HSP70 from cells is not always associated with cell death by necrosis; rather, cellular leakage of eHSP (caused by increased cell membrane permeability) from the intracellular to the predominant extracellular space (plasma or serum) probably accounts for the rise in eHSP concentration after exercise. Similar to other HSPs, plasma or serum eHSP70 concentration increases after exercise through complex interaction(s) between intracellular HSP production, extracellular release and eventual removal from the circulation (17). Hepatosplanchnic tissue releases HSP72 during semi-recumbent cycling, and the released HSP72 contributes to the exercise-induced increase in plasma HSP72 concentration (10). Regardless of the cell source and specific recirculation dynamics, circulating eHSP70 proteins influence immunity profoundly and serve as an endogenous alarm signal to the innate immune system.
If eHSP70 protein is a major signalling molecule mediating changes in innate immunity during exercise (10) circulating eHSP must initially bind to a marker on the cell membrane to elicit signal transduction events. In monocytes, this binding is through membrane-localized CD14. In an in vitro system in NK (natural killer) cells, eHSP70 binds to NK cell surface-bound CD94. When complexed with cell surface NKG2 chains, CD94 (a C-type lectin) is a key regulatory molecule in the activation of NK cells (21).

Cell-bound eHSP70 regulates several aspects of the cellular arm of innate immunity including upregulation of NK cell cytotoxicity (14) and stimulation of monocyte cytokine production of tumour necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6 (2). eHSP70 also communicates with the innate immune system through activation of non-cellular immune components such as complement (18). In vitro evidence suggests that eHSP70 augments both cellular and non-cellular aspects of innate immunity. Overexpression of eHSP protects against irreversible cellular damage in many organ systems, including cerebral ischaemia during heatstroke (11).

Few studies have examined whether the eHSP70 increases innate immunity in vivo through cell binding and more specifically through NK cell binding of CD94. The primary aim of this study was to investigate the interaction between serum eHSP70 concentration and eHSP70 expressed on NK cells (co-localized with CD94) and on monocytes (CD14) after prolonged exercise in a hot and humid condition. A secondary aim was to examine the relationship between cellular expression of eHSP70 and changes in unbound, circulating cytokine concentrations after exercise. We hypothesized that increased plasma concentration of eHSP during and after exercise would be reflected in increased binding to both NK cells and monocytes. We also expected to see eHSP co-localized with CD94 on the cell surface of NK cells.

METHODS

Subjects
Eighteen well-trained male athletes were matched for age, sport, and maximum volume of oxygen consumption (VO2max). The subject characteristics were (mean ± SD): age 33.4 ± 6.8 yr, height 1.82 ± 0.06 m, mass 74.0 ± 6.5 kg, body mass index 22.3 ± 1.9 kg·m⁻², sum of seven skinfolds 48.0 ± 12.6 mm, and VO2max 64.5 ± 6.8 (ml·min⁻¹·kg⁻¹). The study was conducted over six weeks during the Southern Hemisphere spring. The heat stress test involved running at 70% VO2max under ambient heat of 35 °C and relative humidity of about 40% until either core temperature (Tc) reached 39.5 °C, volitional fatigue, or 90 min total running time. Venous blood samples (10 mL into EDTA) were drawn before, after and 1.5 h after exercise.

Subjects with a prior history of heat injury or any immune-related conditions (e.g., hepatitis) were excluded from the study. Subjects reporting symptoms of constipation or with history of gastrointestinal (GI) surgery were also excluded.
The experimental procedures were approved by the Ethics Committees of the Australian Institute of Sport, Canberra, Australia, and The University of Queensland, Brisbane, Australia. Subjects provided written informed consent before testing. Another paper, reporting other aspects of the study and providing further details of the protocol has been submitted (12).

Methodology
Testing was conducted on a motorized treadmill completely enclosed within a large transparent tarpaulin tent (dimensions 10 m x 5 m x 3 m). The tent was heated to maintain an ambient temperature of 35 ± 1 °C and relative humidity of ~40 ± 2 %. The ambient temperature and relative humidity in the tent were monitored using a hand-held climatic logger (Kestrel 4000, Kestrel, Nielsel-Kellerman Inc, Minneapolis, USA), and these were maintained by regulating the air flow between the tent and surrounds (ambient laboratory conditions). The air content within the tent was maintained at 21% oxygen and 0.03% carbon dioxide, and balanced with nitrogen through an air mixture system (BOC Gases Australia Ltd, Canberra, Australia).

After the baseline blood sample was obtained the subject was given about 10 min to perform his own stretching exercises before entering the tent. Once inside the tent and on the treadmill, the subject was given 2 min to adjust to the predetermined speed associated with an exercise intensity of 70% VO2max. Water was consumed ad libitum throughout the test and the volume was recorded.

Tc was measured with an ingestible temperature sensor pill (HQI Inc, Palmetto, FL) swallowed at least 4 h before the heat stress test to allow enough time to transit down the GI tract. The temperature sensor is not digestible or degradable, and is expelled from the body through the digestive tract. Three days after the subject had swallowed the sensor pill, the absence of a signal from the sensor was checked using an analogue receiver to confirm it had been expelled. Heart rate (HR) during exercise testing was monitored continuously with a heart rate meter (Polar, Kempele, Finland).

Blood Analysis
Blood was drawn at each sample time directly into sterile EDTA tubes. Aliquots of fresh whole blood were separated for haematological studies (cell counts) and flow cytometry. The remaining whole blood was centrifuged and the plasma was separated and frozen at ~80 °C in pyrogen-free microtubes (Eppendorf, Hamburg, Germany). These aliquots were subsequently assayed in batches to measure a range of immune-related analytes.

Blood Cells
Complete blood cell counts were performed with a haematology analyser (Advia 120, Bayer Diagnostics, Berkshire, UK). Lymphocyte phenotyping was performed using fresh whole blood and completed within 5 h of collection using a Coulter Epics XL Flow Cytometer (Fullerton, USA). Mononuclear cells (lymphocytes and monocytes) were stained for flow cytometry by incubating whole blood with relevant antibody cocktails comprising three different antibody conjugates...
individually labelled with fluorescein isothiocyanate (FITC), or phycoerythrin (PE) or cyanin–phycoerythrin (PC5); the emission peaks were measured at 525, 575 and 670 nm, respectively. This approach allowed us to simultaneously discriminate three surface markers per cell and to gate cells into categories of interest.

Fluorescently labelled antibodies were used to identify NK cells and monocytes: anti-CD3 FITC, anti-CD3 PC5, anti-CD14 PE, anti-CD19 PE, anti-CD94 PE, anti-CD16 PC5 and anti-CD56 PC5 (Beckman Coulter, Fullerton, USA). We also measured cell surface-bound HSP using anti-HSP72 FITC (Stressgen Biotechnologies, Victoria, Canada). The relevant isotype controls included anti-mouse IgG isotype FITC (Beckman Coulter), anti-mouse IgG isotype PE (Beckman Coulter) and anti-mouse IgG isotype PC5 (Beckman Coulter). All of these antibodies were used according to the manufacturers’ recommendation (usually 20 μL antibody per 100,000 cells) and at cell densities of about 1 x 10⁶ nucleated cells per mL.

Mean fluorescence intensity (MFI) of < 1 was considered to indicate non-specific, cellular autofluorescence. MFI was determined for each individual fluorescent conjugate, thereby providing an indirect indication of the number of copies of each marker found on the cell-surface. Following incubation with antibody cocktails, Optilyse C lysing solution (Beckman Coulter) was used to remove contaminating red blood cells. A total of 10,000 nucleated cells per sample were counted for phenotyping purposes.

Immunofluorescence microscopy of enriched NK lymphocytes was used to investigate the co-localization of the NK marker CD94 and eHSP70. The NK cells were separated from peripheral blood mononuclear cells (PBMC) using Ficoll-Paque Plus (Amersham Biosciences, Piscataway, USA), followed by washing. NK cells were enriched further from PBMC using RosetteSep™ NK enrichment cocktail (StemCell Technologies, Alameda, USA). RosetteSep™ contains an antibody mixture that, during incubation, creates tetrameric cross-linkages between the unwanted PBMC and remaining red blood cells, thereby increasing the density of unwanted cells through the formation of cell rosettes. When subsequently placed on a density gradient, rosetted cells are separated from non-rosetted cells (NK cells). NK purification was evaluated using flow cytometry (CD3−, CD19− and CD16\56+). After confirming NK cell enrichment, the cells were incubated the with antibody cocktails including fluorescently conjugated primary antibodies (anti-HSP70 and anti CD94), and co-localization was investigated using immunofluorescence microscopy (Nikon Eclipse TE300, inverted microscope, Nikon Ltd., London, UK). Prolong Antifade Slide mounting fluid (Molecular Probes, Invitrogen, Eugene, USA) was used to extend the visible image time and images were analysed using METAMORPH, (West Chester, USA) quantitative imaging system software.

**Plasma cytokines and HSP**
Plasma HSP70 concentration was measured with a StressXpress ELISA kit from Stressgen Bioagents (Victoria, BC, Canada). Plasma IL-6 concentration was
measured with the Quantikine HS kit from R&D Systems (Minneapolis, USA), and IL-10 concentration with the OptEIA kit from BD Biosciences (San Diego, USA). The ELISAs were performed according to the manufacturer’s instructions using a microplate reader (VERSAMAX, Molecular Devices, Sunnyvale, USA).

Statistical Analysis
Measures of centrality and spread are shown as the mean value ± SD. Differences between groups and sample times are expressed as a percentage with 90% confidence intervals, p values and Cohen effect size (3). The criteria for evaluating effect size were: <0.2, trivial; 0.2–0.6 small; 0.6–1.2, moderate; and > 1.2, large. P < 0.05 was accepted as significant.

RESULTS

Total white blood cell counts, subtyping (phenotyping) and eHSP70 cellular binding

Total white blood cell counts showed the expected exercise-induced reversible leukocytosis. Total white blood cell counts (mean x 10⁹.L⁻¹ ± SD) showed a moderate increase from from 7.07 ± 1.18 before exercise to 8.85 ± 1.57 after exercise (ES, 0.71 ± 0.56, ± 90% confidence limits; p < 0.01). In contrast, there only a small but variable change from before exercise to 1.5 h after exercise 9.11 ± 2.58 x 10⁹.L⁻¹ (ES -0.28 ± 0.77). The absolute lymphocyte count was 2.61 ± 0.54 x 10⁹.L⁻¹ before exercise, and the count increased to 3.09 ± 0.87 x 10⁹.L⁻¹ after exercise (ES, 0.51 ± 0.50, p=0.001). Lymphocyte counts were below baseline levels by 1.5 h after exercise (1.97 ± 0.38 x 10⁹.L⁻¹, ES,-0.05 ± 0.8, p=0.003).

Absolute NK lymphocyte counts (defined as CD3−, CD16/56+) contributed substantially to the observed changes in lymphocyte count, increasing from 0.39 ± 0.15 x 10⁹.L⁻¹ before exercise to 0.79 ± 0.32 x 10⁹.L⁻¹ after exercise (ES, 0.78 ± 0.39, p < 0.05). Lymphocyte count decreased to 0.12 ± 0.05 x 10⁹.L⁻¹ 1.5 h after exercise. NK cell count decreased markedly from before to

Figure 1. Percentage of NK lymphocytes, percentage of NK cells expressing CD94, and percentage of NK cells co-expressing CD94 and HSP70. Data are presented as mean ± SD.
1.5 h after (ES, 0.68 ± 0.41, p < 0.05). As a proportion of total lymphocyte count, absolute NK cell count represented 15.6% ± 6.1% before exercise, 22.5% ± 6.6% after exercise, and 6.6% ± 1.8% 1.5 h after exercise (Figure 1).

The proportion of CD94+ NK cells did not change substantially from before to after exercise or to 1.5 h after exercise (Figure 1). Similarly the number of CD14+ monocytes was constant at all times: 0.39 ± 0.11 \times 10^9 \text{L}^{-1} before exercise, 0.43 ± 0.12 \times 10^9 \text{L}^{-1} after exercise, and 0.44 ± 0.12 \times 10^9 \text{L}^{-1} 1.5 h after exercise.

The absolute counts of CD14+ monocytes and CD94+ NK cells expressing eHSP were similar before and 1.5 h after exercise (0.2–0.4 \times 10^9 \text{L}^{-1}). However, after exercise the number of CD94+ NK cells expressing eHSP doubled from 0.04 ± 0.02 to 0.08 ± 0.06 \times 10^9 \text{L}^{-1} (ES, 0.81 ± 0.46, P < 0.05; Figure 2).

The proportion of CD14+ monocytes expressing eHSP and the proportion of CD94+ NK cells expressing eHSP and CD94+ did not change substantially at any time (Figure 2).

At rest, the MFI was usually in range 5–6 for CD14+ monocytes, for CD94+ NK cells as well as for those cells that were also positive for eHSP (double positives). After exercise, in addition to the increased cellular expression of eHSP (in relative and absolute terms), the MFI of CD14+ monocytes and of CD94+ NK cells expressing HSP also increased. These MFI values increased by 20–30% during exercise. This magnitude of increase was not statistically significant due to large between-subject variation and small sample size. Overall, the MFI values remained elevated by about 20–30% above resting values 1.5 h after exercise, suggesting that exercise increased the copy number of both eHSP and the cell surface molecules to which it was bound (CD14 and CD94).
The purity of the NK-enriched samples for the co-expression studies was 88%. These cell suspensions were analysed for CD3 (T lymphocytes) and CD19 (B lymphocytes) (Figure 3). Co-localization of CD94 and eHSP70 on the surface of NK cells was indicated by immunofluorescence microscopy. When stained with single fluorescent antibodies, cell surface staining with green (FITC) fluorescence indicated eHSP localization, whereas red (PE) fluorescence indicated expression of CD94. When stained with the two combined (mixed) antibodies, orange-coloured cell surface fluorescence indicated co-localization of HSP and CD94 to the same sites on the cell-surface. In all cases, this fluorescent staining was blocked by pre-incubating with unlabelled antibodies. The immunofluorescence microscopy indicated that 30-40% of CD94+ NK cells were also HSP+. This proportion was greater than that detected using flow cytometry (where values were usually < 30%).

**Plasma eHSP.** Plasma HSP70 concentration increased from 2.04 ± 0.86 ng·mL⁻¹ before exercise to 5.45 ± 1.66 ng·mL⁻¹ after exercise (p < 0.001) and then decreased to 3.49 ± 1.26 ng·mL⁻¹ 1.5 h after exercise (p = 0.0006).

**DISCUSSION**

Exercise in a hot humid condition produced sufficient cell stress to increase plasma HSP70 concentration. eHSP was detected bound to circulating NK lymphocytes and monocytes. This is the first *in vivo* report showing that eHSP binds to NK cells and that the binding to NK cells co-localizes with the NK activation molecule CD94. Although eHSP was found bound to cells before exercise, the increases in MFI and the absolute number of eHSP+ NK cells in the circulation suggest that acute exercise activates this branch of the innate immune system. These data complement in vitro evidence of activation of immunostimulatory cells in response to eHSP binding. Our findings should be extended in future stud-
ies to more precisely quantitate the cell surface density of CD94 (and CD14) using specific commercially available microbeads. When such beads are run at identical FACs machine settings alongside of the cells of interest, a more accurate quantitation of cell-marker density can be made on a per cell basis. Such an approach would extend our observations of increased MFI.

Our flow cytometry measurements indicated that exercise did not substantially alter the proportion or absolute count of eHSP+ monocytes, whereas exercise doubled the absolute count of eHSP+ NK cells. Krause and co-workers examined the potential biological significance of such NK–eHSP binding events (9). Autologous NK cells (from patients with cancer) stimulated by incubation with HSP in vitro had increased tumour-killing activity when readministered to the patients. In our study, NK cells were presumably stimulated in a similar manner after binding eHSP in vivo during exercise. By inference from the findings of Krause and co-workers (9), the binding of eHSP+ to NK cells that we report here during exercise, may have increased NK tumour-killing activity as the NKs exited the circulation and entered the tissues following exercise. This augmentation of NK cell activity may improve immunosurveillance.

CD94 is an important molecule in the regulation of NK cell activation. Exercise did not substantially alter the proportion of CD94+ NK lymphocytes, which is consistent with a previous report from our laboratory (20). In contrast, exercise increased the absolute count of CD94+ NK lymphocytes. Exercise can also increase CD94 gene expression in PBMC (6). This increase may relate to conversion of CD94− NK cells to CD94+ NK cells during exercise. We speculate that if a similar exercise-associated increased CD94 gene expression occurred in our study, it is possibly reflected by those NK cells that were mobilized during exercise (originally CD94−) that then became CD94+. Alternatively, increased CD94 gene expression may be reflected in the 20–30% increase in MIF for CD94 that we observed from before to after exercise.

Exercise increases CD94 as well as CD14 gene expression (6). In our study, exercise did not substantially alter the proportion or the absolute count of monocytes expressing both CD14 and eHSP. As with the NK cells however, increased CD14 gene expression might be reflected in the increase in mean fluorescence intensity for CD14 noted after exercise. Increases in mean fluorescence intensity for CD14 and CD94 may reflect increased expression of these receptors on individual positive cells. The altered receptor expression may increase the number of potential eHSP docking sites, thereby upregulating the potential for activation of innate immune cells during exercise.

The changes in plasma HSP70 concentration were transient. Our resting and post-exercise values were similar to those reported in another study (7). In contrast, Pockley and co-workers reported much higher at rest values (1131 ± 254 ng.mL−1) than seen in our study (17). This disparity may be attributable to differences in the age of participants or methodologies to measure plasma HSP70 concentration (an in-house ELISA compared with a commercially available ELISA). The transient increase in plasma HSP70 concentration after exercise is well docu-
mented and is related to both exercise intensity and duration. Numerous factors including increased Tc, metabolic acidosis, activation of the hypothalamic–pituitary–adrenal axis, oxidative stress, and changes in blood glucose availability influence the plasma levels of the HSP70 in response to exercise.

The relatively modest numbers of HSP+ NK cells and monocytes following exercise were surprising, particularly considering the increase in plasma HSP70 concentration and the relative availability of CD94 and CD14 binding sites. One possible explanation is that extracellular HSP is cleared efficiently from the circulation following exercise in well-trained athletes, possibly through a rise in circulating antibodies to HSP. The source of eHSP may also influence the binding of HSP to NK cells and monocytes. Expression of HSP on NK cells and monocytes might reflect intracellular synthesis and transport of HSP to the surface of these cells, as opposed to binding of eHSP produced by other cells. Regardless of the precise mechanism, combined in vitro and in vivo evidence suggests that HSP cell-binding events probably increase the activity of cells of the innate immune system. Future investigators should extend these findings using FACs methodologies (in cell sorting mode). Enriching (post exercise) for HSP+ NKs and comparing them to HSP- NKs ex vivo, could address issues about if HSP binding changes relative cytotoxicity and cell cytokine production. This experimental approach would also address the concerns that some in vitro assessments may be artifactual due to LSP contamination of exogenous HSP (8).

Plasma IL-6 and IL-10 concentrations increased after exercise, but the time course of changes differed between IL-6 and IL-10. Unlike IL-6, IL-10 concentration remained elevated 1.5 h after exercise and these data have been presented in more detail elsewhere (12). Although exercise did not significantly alter the plasma concentrations of IL-1β, IL-2 and TNF-α, the increases in plasma eHSP-70, IL-6 and IL-10 concentrations as well as changes in immune cell numbers indicate that exercise in the heat induced a transient acute inflammatory-like response, followed by a counteracting anti-inflammatory response.

**CONCLUSIONS**

NK lymphocytes and monocytes expressed eHSP-70 on their cell surface both at rest and following exercise. Our study provides the first in vivo evidence of HSP70 expression on these cell types. The findings of in vitro studies indicate that eHSP-70 cell binding enhances NK proliferation, cell killing activity, and monocyte cytokine production. Binding of HSP to NK cells and monocytes in vivo may also influence numerous other cellular and non-cellular components of innate immunity. Immunofluorescence microscopy of enriched NK lymphocytes demonstrated that eHSP co-localized with the NK activation-linked molecule CD94.
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