Head-out immersion in hot water does not increase serum CXCL1 in healthy men

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ABSTRACT

Exercise-induced production of interleukin (IL)-6 results in the expression of chemokine CXC-motif ligand 1 (CXCL1) in mice. Recent studies described the increase in serum IL-6 levels during immersion of subjects in hot water. The present study investigated the effects of a 20-min head-out immersion in 42 °C water (hot-HOI) on serum concentrations of CXCL1 in eight healthy men. Venous bloods were taken at rest, immediately after hot-HOI, as well as 1, 2, 3, and 4 h after hot-HOI for measurements of serum concentrations of CXCL1, IL-6, tumor necrosis factor (TNF)-α, and high-sensitivity C-reactive protein (hsCRP), while assessing counts of blood cells (CBC) and monitoring core temperature (Tcore). Tcore and serum IL-6 increased during hot-HOI and remained high until 4 h after hot-HOI. However, serum CXCL1, TNF-α, hsCRP, and CBC remained constant throughout the experiment. In conclusion, the results from our study demonstrated that 20-min hot-HOI increased serum IL-6, but not CXCL1 in healthy men.

Keywords: IL-6; TNF-α; hsCRP; hyperthermia; myokine

Introduction

During systemic inflammatory state such as sepsis, serum levels of tumor necrosis factor (TNF)-α markedly increase just after an increase in serum interleukin (IL)-6. In contrast, the marked increase in serum level of IL-6 is not preceded by a rise in TNF-α during exercise[1]. Keller et al. suggested that the releasing of IL-6 from contracting skeletal-muscle fibers is mainly caused by IL-6 gene transcription[2]. Therefore, IL-6 is considered a myokine[3,4]. Estimates from recent proteomic studies predict that the number of myokines would be more than 600 and belonging to different families[5].

What is the mechanism of exercise muscle-induced production of IL-6? While the exact stimulus is still being investigated, evidence suggests that local heating can act as a stimulus to express IL-6 mRNA in skeletal muscle of mouse, i.e. the skeletal muscle acts as a “heat stress sensor”[6,7]. In addition, other studies showed that hot water immersion significantly increases IL-6 in humans, and concluded that skeletal muscles secrete IL-6 in response to exercise and heat exposure[8].

Human chemokine CXC-motif ligand 1 (CXCL1) is a 6.4-kDa polypeptide, classified as a chemokine that plays a role in recruiting leukocyte in inflammatory and immune reactions[6]. However, Nedachi et al. demonstrated that CXCL1 also works when released from contracting skeletal muscles[10]. Mice, in which the tibialis cranialis muscle over-expresses CXCL1, have lower visceral and subcutaneous fat mass, and the CXCL1-dependent decrease in adipose tissue mass coincides with improvements in glucose tolerance and insulin sensitivity in the whole
body\textsuperscript{[11]}. Other studies have shown that hepatic expression of CXCL1 mediates the liver-protective potential of IL-6, and that CXCL1 is involved in wound healing, acts as a protectant against multiple sclerosis, and has potential neuroprotective effects\textsuperscript{[12-14]}.

The overexpression of IL-6 in murine muscles is followed by marked increase in serum level of CXCL1 and mRNA expression of CXCL1 in the liver\textsuperscript{[15]}. In an experimental study involving wild-type mice, 1 h of swimming exercise induced increases in serum IL-6 immediately after exercise, and in serum CXCL1 2 h after exercise, whereas the same exercise did not modulate liver CXCL1 mRNA expression in IL-6 knockout mice\textsuperscript{[15]}. The results suggest a tight muscle–liver crosstalk during exercise, in which exercise-induced IL-6 induces a production of CXCL1 in the liver\textsuperscript{[15]}.

Based on the above findings, we hypothesized that heat stress-induced IL-6 increases CXCL1 expression in humans. To test our hypothesis, we measured in the present study serum CXCL1 levels before, during and after 20-min head-out immersion in 42 °C water (hot-HOI) in a group of normal adults.

**Materials and methods**

**Subjects**

Eight healthy males voluntarily participated in the present study. Table 1 lists the characteristics of these subjects. None of the subjects were on any medications at the time of the study.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Healthy males (mean±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>25.9±3.5</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>173.3±4.0</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>70.1±12.0</td>
</tr>
<tr>
<td>BMI (kg/m(^2))</td>
<td>23.3±3.6</td>
</tr>
</tbody>
</table>

**Experimental protocol**

The subjects were informed to refrain from strenuous physical activity and alcohol on the day before the study and to refrain from taking any fluids or foods from 2200 h the night before the study, except for tap water, until the completion of the study. They reported to the laboratory at 0800 h on the experiment day. Each subject wore a swimming trunk at the time of the study. A copper-constantan thermocouple probe was inserted into the esophagus and its tip placed at the atrium level to monitor body core temperature ($T_{\text{core}}$) throughout the study. A heparinized indwelling catheter was placed into the right antecubital vein. Before the onset of the study, the subjects sat in a room outside of the water tank for 1 h as the control period. After confirmation of stable $T_{\text{core}}$, measurements were started during the 10-min rest in the sitting position outside the tank. Then, the subject walked into and sat in the hot (42 °C) water tank, immersing the entire body, except the head, into the water. Measurements were taken after 20 min, then the subject left the tank to sit nearby for 4 h and measurements were repeated during this period. Thus, the entire measurement was of 270 min duration. Venous blood samples were collected just before hot-HOI (pre-HOI), 30 min (at end of HOI), 90 min (1 h post-HOI), 150 min (2 h post-HOI), 210 min (3 h post-HOI), and 270 min (last measurement) from the onset of the study. Blood samples (6 mL each) were withdrawn through the intravascular catheter and were used for the measurements of IL-6, CXCL1, TNF-α, high sensitivity C-reactive protein (hsCRP), hematocrit, and counts of blood cells.

**Analysis of blood samples**

Total blood cell counts were assessed by using a cell counter. Hematocrit was determined by centrifugation. Other venous blood samples were drawn into pre-chilled serum venipuncture tubes. The tubes were centrifuged at 3500 rpm for 10 min at 4 °C, and then the serum was separated and stored at −80 °C until analysis. IL-6 was measured by enzyme immunoassay (ELISA) for IL-6 (R&D Systems, Minneapolis, USA) with assay sensitivity of 0.039 pg/mL and intra- and inter-assay coefficients of variability (average CV of different concentrations) of 7.4% and 7.8%, respectively. CXCL1 was assayed using ELISA for CXCL1 (R&D Systems) with assay sensitivity of <10 pg/mL and intra- and inter-assay coefficients of variability of 2.9% and 5.2%, respectively. TNF-α was analyzed using ELISA for TNF-α (R&D Systems) with assay sensitivity of 0.106 pg/mL and intra- and inter-assay coefficients of variability of 5.4% and 8.3%, respectively. IL-6, CXCL1, and TNF-α immunoassays were performed in duplicate by investigators blinded to the study design.

**Statistical analysis**

All results were represented as mean±SEM except when noted otherwise. The differences between two parameters were compared with one-way analysis of variance, followed by multiple
comparisons at various time points. When the F-value was significant ($p < 0.05$), the time period and the study condition comparisons were made using Fisher’s LSD test.

**Ethics statement**

The study protocol was approved by the Ethics Review Committee of Wakayama Medical University and conformed to the Declaration of Helsinki. A signed informed consent was obtained from each subject after a complete explanation of the purpose and risks of the present study.

**Results**

$T_{\text{core}}$ significantly increased immediately after HOI, and was still higher at 1 h post-HOI, 2 h post-HOI, and 3 h post-HOI, but recovered to pre-HOI on the last measurement (Figure 1). Table 2 lists the blood cell counts at the six different time points. Hemoglobin, hematocrit, erythrocyte count, and monocyte count remained constant throughout the study. Serum IL-6 concentrations were significantly higher at 1 h post-HOI, 2 h post-HOI, and 3 h post-HOI, and recovered to pre-HOI level in the last measurement (Figure 2A). Serum concentrations of CXCL1 (Figure 2B), TNF-α (Figure 3A), and hsCRP (Figure 3B) remained unchanged throughout the experiment.

**Discussion**

This is the first study that examines the serum level of CXCL1 to hot-HOI in young and healthy men. The major findings of this study are the followings: 1) hot-HOI resulted in increased levels of serum IL-6; 2) no changes in serum CXCL1 levels were noted; and 3) the levels of $T_{\text{core}}$ and IL-6 returned to baseline at 4 h after hot-HOI. Thus, hot-HOI activated the releasing of IL-6 but did not stimulate the releasing of CXCL1. The increase in serum IL-6 was not induced by dehydration during hot-HOI because hematocrit and erythrocyte count did not change after hot-HOI, compared with the baseline.

It is well known that expressions of mRNA for $\text{IL-6}$ and other proinflammatory cytokines, such as $\text{TNF-α}$ and $\text{IL-β}$, are mainly regulated by the toll-like receptor (TLR) signaling cascade, which results in nuclear translocation and activation of

![Figure 1. Core temperatures before and after head-out immersion (HOI) in hot water (42°C). The left side of the plot represents data before HOI. Time 0 represents the onset of the study. The time between the two vertical dashed lines represents the time spent in hot-HOI. Data are mean ± SEM. *$p < 0.05$, compared with before immersion.](image)

| Table 2. Hemoglobin, hematocrit and blood cell count values measured during the study |
|---------------------------------|----------------|----------------|----------------|----------------|----------------|
|                                | Pre-HOI        | Immediately after immersion | 1 h post-HOI | 2 h post-HOI | 3 h post-HOI |
| Hemoglobin concentration (g/dL) | 15.0 ± 0.3     | 15.4 ± 0.3           | 15.4 ± 0.3    | 15.0 ± 0.5    | 15.5 ± 0.3    |
| Hematocrit (%)                  | 44.2 ± 0.8     | 45.6 ± 0.9            | 45.7 ± 0.8    | 44.4 ± 1.3    | 45.9 ± 0.9    |
| Erythrocyte count (× 10$^9$/μL) | 49.2 ± 1.1     | 50.7 ± 1.2            | 50.9 ± 1.2    | 49.4 ± 1.6    | 51.2 ± 1.2    |
| Monocyte count (10$^2$/μL)      | 1.9 ± 0.4      | 1.4 ± 0.2             | 1.8 ± 0.3     | 1.9 ± 0.3     | 2.1 ± 0.2     |

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NF-κB during systemic inflammatory state such as sepsis. In contrast, exercise-induced increase in serum IL-6 appears in advance of a rise in TNF-α \(^1\). The stable levels of TNF-α, hsCRP, and monocyte count throughout the present measurement indicate the lack of inflammatory response during hot-HOI. Therefore, the increase in serum IL-6 is unlikely to be induced through TLR receptor signaling cascade. On the other hand, the simultaneous recovery of \(T_{core}\) and IL-6 strongly suggests that \(T_{core}\) is a strong activator for releasing IL-6.

Previous studies demonstrated the interactions between \(T_{core}\) and IL-6 production during exercise. Rhind \textit{et al.} reported that the decrease in \(T_{core}\) during endurance exercise is associated with attenuation of the IL-6 response\(^{10}\). Starkie \textit{et al.} reported the augmentation of the circulating IL-6 response when endurance exercise is performed in a hot environment, and concluded that releasing IL-6 from contracting skeletal muscles is a temperature-related phenomenon\(^{17}\). Another study described the upregulation of IL-6 in skeletal muscle following exposure to heat, both \textit{in vitro} and \textit{in vivo}\(^6\)\(^{16}\). Our results of changes in IL-6 level after hot-HOI strongly suggest that the increase in \(T_{core}\) is an independent factor responsible for the increase in serum IL-6. The significant rise in IL-6 level after hot-HOI corresponds to the findings of previous study\(^8\). Interestingly, hyperthermia is reported to induce the release of IL-6 from skeletal muscle tubules in mice\(^6\). Considered together, it seems that

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**Figure 2.** Serum IL-6 (A) and CXCL1 (B) levels measured before and after head-out immersion (HOI) in hot water (42°C). The left side of the plot represents data before HOI. Time 0 represents the onset of the study. The time between the two vertical dashed lines represents the time spent in hot-HOI. Data are mean ± SEM. *\(p < 0.05\), compared with before immersion.
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skeletal muscles are one of the organs which produce IL-6 at least after hot-HOI.

Serum CXCL1 protein levels and CXCL1 mRNA expression levels in muscle and liver increase in swimming mice\textsuperscript{[15]}. Importantly, the induction of CXCL1 expression is highest in the liver; the liver being the main source of releasing CXCL1 after exercise\textsuperscript{[15]}. The same study demonstrated that IL-6 is released from the muscle-regulated liver CXCL1 expression\textsuperscript{[15]}. One hour of swimming exercise would induce a 5-fold increase in serum IL-6 in wild-type mice immediately after exercise, and the increase was still seen at 2 h post-exercise, which was coupled with an increase in serum CXCL1\textsuperscript{[15]}. In another study of mice, 30 min of treadmill running induced a 2.8-fold increase in serum IL-6, together with an increase in serum CXCL1\textsuperscript{[10]}. In the present study, hot-HOI induced a 2-fold increase in serum IL-6 but no change in CXCL1. Therefore, the stability of CXCL1 and increase in IL-6 level suggest that hyperthermia per se contributed to the decrease in CXCL1 expression.

Previous studies described a close relationship between increased expression of IL-6 mRNA in the skeletal muscle in a hot environment and those of heat shock protein (HSP)-72 mRNA, and that treatment with KNK437, a heat shock factor (HSF) inhibitor, significantly reduced IL-6 mRNA expression\textsuperscript{[6]}. The main pathway involved in regulation of releasing IL-6 in various stressed

Figure 3. Serum TNF-α (A) and hsCRP (B) levels before and after head-out immersion (HOI) in hot water (42 °C). The left side of the plot represents data before HOI. Time 0 represents the onset of the study. The time between the two vertical dashed lines represents the time spent in hot-HOI. Data are mean ± SEM.
conditions, including hyperthermia, activates mitogen-activated protein kinases (MAPK), or more specifically, stress-activated protein kinases (SAPK) such as c-jun N-terminal kinase (JNK)\[^{18}\]. JNK increases a transcription of IL-6 through the downstream activation of c-fos and c-jun, which dimerize and bind to the activator protein-1 (AP-1) regulatory element on the IL-6 promoter. Welc \emph{et al.} examined the heat-induced transcriptional control of IL-6 in C2C12 muscle fibers, and demonstrated that the regulation of releasing IL-6 in hyperthermia is directly controlled by HSF-1 and AP-1 signaling\[^{7}\]. Nedachi \emph{et al.} have recently succeeded in establishing an advanced \textit{in vitro} muscle exercise model using highly developed C2C12 myotubes that possess electric pulse stimulation (EPS)-evoked vigorous contractile activity\[^{10}\]. They found marked upregulation of CXCL1 expression in contractile C2C12 myotubes after 24-h EPS. Moreover, they strongly suggested that the NF-κB signaling pathway is directly involved in CXCL1 expression in response to EPS-evoked contractility\[^{19}\]. However, NF-κB signaling was reported to be downregulated by hyperthermia in mouse myoblast cells\[^{20}\]. These results of the above studies might explain the lack of change in serum CXCL1 level and the increase in serum IL-6 level after hot-HOI.

The other possible explanation for the lack of change in serum CXCL1 level after hot-HOI would be related to an insufficient expression level of IL-6; even serum IL-6 significantly increased after hot-HOI. Furthermore, there was no contraction of skeletal muscles and/or lower energy expenditure because sitting would be associated with the lack of altering in the serum CXCL1 level\[^{15}\]. However, it is not clear the reason why serum CXCL1 remained unchanged after hot-HOI.

**Conclusion**

The present study demonstrated that 20 min of hot-HOI did not affect CXCL1 expression in young healthy men, but increased serum IL-6 levels, probably through the increase in core body temperature.

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**Conflict of interest**

The authors declare no potential conflict of interest with respect to the research, authorship, and/or publication of their article.

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