Human monocyte stimulation by experimental whole body hyperthermia

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HUMAN MONOCYTE STIMULATION BY EXPERIMENTAL WHOLE BODY HYPERTHERMIA

STIMULIERUNG HUMANER MONOZYTEN DURCH EXPERIMENTELLE GANZKÖRPER HYPERTHERMIE

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Abstract

The thermal effect of fever, an evolutionarily conserved acute-phase response, has been associated with improved survival and shortened disease duration in infection. The molecular consequence of this beneficial fever response is poorly understood. To study an influence of hyperthermia on human monocytes, which are important in recognition and elimination of pathogens, twelve healthy volunteers were immersed in a 39.5°C hot water bath to increase their body temperature. The expression of the endotoxin receptor CD14 and the complement receptor CD11b increased after the hot water bath ($P<0.05$), whereas the expression of the selectin CD62L, which mediate the initial attachment of leukocytes at endothelium during inflammation, was downregulated after hyperthermia ($P<0.05$). Comparable changes in monocyte receptor expression were observed after an *in vitro* hyperthermia. Furthermore, 3h after *in vivo* hyperthermia the response of monocytes to endotoxin was enhanced in an *ex vivo* lipopolysaccharide-stimulation-assay as shown by increased TNF-α release ($P<0.05$). We conclude that the thermal effect of fever leads directly to an activation of monocytes which increases their ability to respond to a bacterial challenge.

Key words: CD14, LPS, fever, infection, hyperthermia

Abstract

Die Erhöhung der Körpertemperatur, eine evolutionär konservierte Akutphase Reaktion, verbessert das Überleben und verkürzt die Krankheitsdauer bei Infektionen. Die molekulare Auswirkungen dieser Fieberreaktion sind noch kaum beantwortet. Um den Einfluß von Hyperthermie auf humane Monozyten, die wichtig sind in der Erkennung und Eliminierung von Pathogenen, zu untersuchen, haben wir zwölf gesunde Probanden in ein 39,5°C heißes Wasserbad gelegt um ihre Körpertemperatur zu erhöhen. Die Expression des Endotoxinrezeptors CD14 und des Komplementrezeptors CD11b erhöht sich nach dem heißen Wasserbad ($P<0.05$), wogegen die Expression des Selektins CD62L, welches das erste Adhärieren der Leukozyten am Endothel während Entzündungen vermittelt, herunter reguliert wird ($P<0.05$). Vergleichbare Änderungen an der Monozytenrezeptor Expression wurde auch nach *in vitro* Hyperthermie induziert. Außerdem war, 3h nach *in vivo* Hyperthermie die Reaktion von Monozyten auf Endotoxin in einem *ex vivo* Lipopolysaccharid-Stimulation-Versuchs verstärkt, wie durch eine erhöhte TNF-α Freisetzung gezeigt ($P<0.05$). Wir schließen daraus, daß die Erhöhung der Körpertemperatur bei Fieber direkt zu einer Aktivierung der Monozyten führt und ihre Fähigkeit steigert auf einen bakteriellen Angriff zu reagieren.

Schlüsselwörter: CD14, LPS, Fieber, Infektion, Hyperthermie

INTRODUCTION

For thousands of years fever was considered a protective response, and physicians induced fevers to combat certain infections. When antipyretic drugs were introduced, physicians started to reduce fever, and induction of fever was virtually abandoned. However recent studies revealed further details on how tightly the process of fever is regulated and thus the concept of fever as a host defense response has reemerged (1). Numerous animal studies describe the protective effects of hyperthermia during infection, but the molecular consequence of this beneficial fever response is poorly understood.

When activated by invading microorganisms, tissue macrophages produce high amounts of interleukin-1 (IL-1) which acts as an endogenous pyrogen able to induce a febrile response (2, 3). IL-1 is presumed to reach the brain by bloodstream and thus to initiate the febrile response. The thermoregulatory center of the brain activates the brown adipose tissue finally leading to heat production (4). Elderly people do not increase their body temperature in response to infection to the same extend as children do. The reason seems to be based on reduced IL-1 release from monocytes in the elderly (5).

To date very little is known about the influence of elevated body temperature on leukocyte function. This is primarily due to the difficulty of separating the direct thermal effects from the myriad other physiological and neurological events that occur simultaneously with the fever response. Several groups used externally applied heat in an attempt to dissect on the impact of the thermal element of fever. It has been proposed that the protective effects of fever are due to reduced proliferation and survival of pathogens at elevated temperatures. However, a recent study involving a mouse model of infection revealed that the higher survival rate of the animals with elevated body temperature is related to increased clearance of bacteria and not to a decreased proliferation of pathogens (6).

Monocytes play an important role in the unspecific immune response. Since monocytes have a many CD14 and CD11b molecules on their surface they can recognize and phagocytose lipopolysaccharides (LPS) and LPS-bearing bacteria (7). Concomitantly, during infection the adhesion molecule, CD62L (L-selectine), mediates the initial rolling of monocytes and neutrophils on the endothelium for further migration into the tissue (8). In the present study we investigated whether an *in vivo* as well as an *ex vivo* hyperthermia model influences the expression of CD14, CD11b and CD62L on peripheral blood monocytes.

METHODS

Study design and protocol

The study was designed as a prospective, randomized, cross over, controlled clinical trial in six healthy men and six healthy women aged 22-29 years. They were immersed in a hot water bath (water temperature 39.5°C) for 2h. Tympanic temperature was recorded using an electronic thermometer (Braun AG, Kronberg, Germany). One week later volunteers served as their own controls, being immersed in a thermoneutral water bath (36°C). Blood samples were taken before the experiment and when the body temperature reached its highest level, as well as 1, 2, 4, 8, and 22h after the end of the treatment. Blood was drawn into EDTA-vacutainer-tubes, in a total amount of 110mL, and analyzed within 30 minutes. The protocol was accepted by the local ethics committee of Vienna.

Ex vivo heat treatment

Whole blood of healthy volunteers was heat treated in a shaking 39°C hot water bath for 25 minutes. Meanwhile the control group was incubated at 37°C.

Ex vivo whole-blood LPS stimulation

Blood obtained from healthy volunteers of the water bath experiments was collected in vacutainer tubes containing 0,072ml 7,5% EDTA. Whole blood was then incubated in sterile polystyrene tubes (NUNC, Roskilde, Denmark) for 3h at 37°C in a 1:1 dilution with RPMI medium alone or RPMI medium containing LPS (final concentration 1 µg/ml; Escherichia coli serotype 055:B5, Fa SIGMA).

Analysis of cell surface protein expression

Expression of cell surface proteins was detected by flow cytometry using specific antibodies. Whole blood (50µl) was incubated with antibodies directed against CD14 (6µl, clone RM052) from Becton Dickinson, (San Jose, CA, USA) and CD62L (7µl, clone Dreg56), CD11b (7µl, clone bear 1) or CD114 (7µl, clone LMM775) for 20 minutes at 4°C in the dark. All other antibodies were purchased from Immunotech (Marseille Cedex 9, France). After the depletion of red blood cells by brief ice-cold hypotonic lysis in FACS™ Lysing Solution (Becton Dickinson, San Jose California, USA) the cell preparations were resuspended in phosphate buffered saline containing 1% paraformaldehyde. Negative control staining was performed with appropriate fluorescence labeled isotype IgG antibodies (6 µl, clone 679.1Mc7, Immunotech Marseille Cedex 9, France). Stained cells were analyzed using a flow cytometer (FACScan, Becton Dickinson, San Jose, CA, USA). 15,000 events were

acquired and gating based on forward and side scatter was performed to differentiate granulocytes, monocytes and lymphocytes. Instrument settings are fixed for all different donors. Experiments using the monocyte specific marker CD14 revealed that the applied monocyte gate contained at least 92% monocytes in all samples. The contaminating cells were mainly lymphocytes. The density of cell surface molecules is expressed in mean fluorescence intensity (MFI).

Determination of plasma TNF-α levels

TNF-α levels in plasma of the LPS - stimulated whole blood were determined by an enzyme immuno assay (EIA) with sensitivity of 5pg/ml (Amersham, Buckinghamshire, England). Results were then corrected for leukocytosis and calculated for $3x10^5$ TNF-α releasing leukocytes/µl.

Blood cell counts

Differential blood cell counts were measured with a routine coulter counter (model STKS, Hialeah, FL, USA).

Statistical analysis

Wilcoxon's test for paired samples was used to determine differences between heat-treated and untreated groups. Values were considered significant when $P < 0.05$.

RESULTS

Influence of a hot water bath on body temperature

To analyze the thermal effects of fever we induced whole body hyperthermia by submerging healthy volunteers in a hot water bath. As shown in figure 1, a 36°C warm water bath for 2h had no effect on the body temperature. In contrast, a 39.5°C hot water bath led to an increase of body temperature to 39°C within 40 minutes. After the end of the hyperthermic exposure the body temperature decreased immediately to normal values. Thus, a 39.5°C hot water bath seems to be a valid model for whole body hyperthermia, whereas the 36°C water bath reflects an isothermal situation and serves as a regular control.

Influence of *in vivo* hyperthermia on monocyte count

In response to a number of different insults monocyte counts can be changed within hours. Therefore we investigated whether hyperthermia affects the total number of monocytes in the blood flow. We analyzed white blood cell counts at different time points using a quantitative hematology analyzer. The results are shown in

Table 1. During the 2h of hyperthermic exposure monocyte counts remain unaffected. However, the number of monocytes increases 6h after the end of hyperthermia by 75% and is reduced to baseline levels at 22h (a slight decrease at this time point is not statistically significant). Analyzing other white blood cell populations revealed that they react differently. Whereas neutrophil counts increase remarkably with a peak at 3h after hyperthermia ($P < 0.001$), lymphocyte count decreases slightly during hyperthermic exposure reaching a minimum after 3h and returning to normal values after 6h.

Position of Table 1

Influence of *in vivo* hyperthermia on cell surface molecule expression

To investigate the immunomodulatory effects of hyperthermia, we monitored the expression of cell surface proteins which are involved in monocyte activation and adhesion to the endothelium, namely CD14, CD11b, and CD62L. As shown in Figure 2, CD14 expression on monocytes increased during hyperthermia and reached the highest level at 6h after the end of the hot water bath. Similarly, CD11b expression in the heat-treated group was significantly increased showing a peak at 6h ($P < 0.02$) after the end of hyperthermic exposure. In contrast, CD62L decreased rapidly during hyperthermia and showed the lowest level 6h after the end of heat-treatment. These results demonstrate that the expression of cell surface proteins on monocytes of heat-treated volunteers changes clearly during the investigated period. Small alterations could be also observed in the unheated controls. In a second control group without water bathing we saw the same variations of CD14, CD11b and CD62L (data not shown). We constitute the alterations of these receptors with circadian rhythm. However, in the hyperthermia group these changes were much more pronounced.

Influence of *ex vivo* hyperthermia on cell surface molecules

As described above, *in vivo* hyperthermia leads to changes in the expression of monocyte cell surface molecules. However, the data provide no evidence that these changes are due to a direct thermal effect. It is well known that hyperthermia induces a response of various systems in the human body including the endocrine network (9) and cytokines (10) which may also lead to the changes observed on monocyte surface expression. To exclude such indirect effects of hyperthermia we took blood from healthy volunteers, exposed it to an *ex vivo* hyperthermia and analyzed the expression of the same cell surface proteins. We found that 25 minutes at 39°C were sufficient to observe: The expression of CD14 as well as of CD11b was significantly increased after hyperthermia (see Fig. 3). In contrast, CD62L expression was downregulated.

Influence of *in vivo* hyperthermia on *ex vivo* LPS-stimulated TNF-α release

Since, as we have proven, CD14 is heat-induced, and CD14 is the most prominent LPS-receptor on monocytes we must ask whether heat-treatment affects the LPS-sensitivity of monocytes. In order to answer this question, we determined the TNF-α release following LPS stimulation of whole blood from heat-exposed volunteers. 3h after hyperthermic exposure, TNF-α release ($P < 0.05$) was significantly higher in the 39.5°C treated group than in the normothermic controls (Fig. 4). After 24h TNF-α release in the heat-treated group returned to baseline. We found no detectable plasma levels of TNF-α after the 39°C hot water bath and in the control group (data not shown).

DISCUSSION

This study shows that sustained elevated body temperature modifies the phenotype and function of peripheral blood monocytes. Hyperthermia induces a strong increase in CD14 expression, a downregulation of CD62L expression and an enhanced expression of CD11b *in vivo*. These changes correlate with an increased responsiveness of monocytes to LPS. Similar alterations of these cell surface proteins were observed immediately after *an in vitro* heat treatment of isolated whole blood. These data indicate that hyperthermia directly affects the expression of receptors on monocytes and not by endocrine or other mediators.

Monocytes have an important function in the clearance of circulating endotoxin. It has already been shown that activation of monocytes and the concomitant specific changes in monocyte receptor expression such as that occurring after a hot water bath can help overcome severe infection. For example, pretreatment with the monocyte activator MTP-PE (muramyl tripeptide phosphatidylethanolamine), which leads to similar changes in the cell surface protein expression (11,12), induces a more rapid clearance of bacteria and dramatically reduces mortality in a porcine model of septicemia (13).

The function of CD14 is controversial. Since CD14-deficient mice were protected against LPS-mediated shock, there must be several approaches to block CD14. In a recent study it was found that the intestinal mucosa of anti-CD14-treated rabbits exhibited a 50-fold increase in bacterial invasion and more severe tissue injury than controls (14). Furthermore, the reduced responsiveness of monocytes to LPS, like TNF-α release, which is found in patients in the late septic phase (15) as well as in healthy volunteers after LPS infusion (16), is associated with a low level of CD14 in all subjects and increased endotoxin levels in the septic patients (17). In our study upregulated CD14 expression also correlates with the increase in LPS-induced TNF-α release. Similar increase

in LPS-induced TNF-α release was seen in mice exposed to febrile temperatures (18, 19). Unfortunately the CD14 expression was not investigated in that animal study. Cultivation of human peripheral blood mononuclear cells at 39°C, however, had no influence on TNF-α release after endotoxin challenge (20). In pilote experiments we also not measured an increase of LPS-induced TNF-α release of in vitro heat-treated blood samples. High expression of CD14 seems, therefore, to be crucial for this monocyte function especially in the in vivo setting.. This may reflect a helpful response of monocytes to hyperthermia which leads to an improved LPS neutralizing capacity.

The increase of CD11b expression on monocytes of heat-exposed volunteers supports this assumption. This protein is a complement receptor which mediates the phagocytosis of opsonized bacteria. It has been known since 1979 that febrile temperature increases phagocytosis (21). We suppose that this enhanced phagocytosis is at least partly due to a hyperthermia-induced increase in CD11b expression. Although CD11b also plays an important role in monocyte adhesion to the endothelium, the increase in CD11b expression does not seem to mediate monocyte adhesion and extravasation, because an increased monocyte count was detected in circulation after the hot water bath. This may be explained by the simultaneous decrease in CD62L levels. The high constitutive expression of CD62L is essential for rolling and subsequent extravasation of leukocytes. G-CSF, an immunstimulatory cytokine also induce a strong decrease in CD62L expression and an increase in CD11b expression. In a human model of endotoxemia, pretreatment with GCSF induces a strong upregulation in TNF-α release, but also a strong decrease in adhesion of granulocytes to the lung (22). Whole body hyperthermia induce similar changes in CD11b and CD62L expression on neutrophils (data not shown). We focussed on monocytes, because they have a very high expression of the LPS receptor CD14 and they are the major source of TNF-α after endotoxin challenge (23).

The effects of hyperthermia on CD14, CD62L and CD11b, described here for the first time, suggest that fever has a regulatory capacity involving peripheral monocytes distant from the site of infection: on one hand these monocytes increase their ability to recognize and respond to pathogens and on the other hand they may exhibit reduced extravasation which helps to avoid extensive migration of monocytes into the tissue and thus keeps a high number of monocytes in the blood stream. As systemic response, the thermal component of fever directly leads to an increase in pathogen eliminating capacity (6, 24) in the blood which helps to prevent the spreading of a local infection. The ability of hyperthermia to warn the peripheral immune system distant from the site of primary infection suggests that fever has a messenger role for the immune system: This is a new aspect of fever research.

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FIGURE LEGENDS

Figure 1:

Body temperature during hot water bath: Tympanic temperature of healthy volunteers (n = 12) during and after a 36°C (○) or 39.5°C (■) water bath (WB). Data are expressed as mean ± SEM.

Figure 2

Influence of *in vivo* hyperthermia on monocyte cell surface protein expression: Healthy volunteers (n = 12) were immersed in a 36°C (○) or 39.5°C (■) warm water bath. Cell surface proteins on peripheral blood monocytes were measured by flow cytometry. **(a) CD14, (b) CD11b and (c) CD62L.** All values are expressed as means ± SEM. *P < 0.05 from control group

Figure 3

Influence of *ex vivo* hyperthermia on monocyte cell surface protein expression: Whole blood from healthy volunteers (n = 5) was heat treated for 25 min in a shaking 39.5°C water bath, meanwhile control group was incubated at 37°C. Cell surface protein expression was measured by flow cytometry. **(a) CD14, (b) CD11b, (c) CD62L.** Values are expressed as means ± SEM. *P < 0.05 from control group

Figure 4

TNF-α release after *ex vivo* LPS stimulation in whole blood: Whole blood from healthy volunteers after the 36°C (○) or 39.5°C (■) warm water was stimulated for 3h with LPS (1µg/ml). Results are expressed as means ± SEM. *P < 0.05 from control group

TABLE LEGENDS

Table1:

Effect of *in vivo* hyperthermia on leukocyte count: Healthy volunteers (n = 12) were immersed in a 36°Cor 39.5°C warm water bath. Count of neutrophils, lymphocytes and monocytes in peripheral blood was measured before, during and after heat treatment. Data are expressed as mean ± SEM. *P < 0.05 from control group