Mild Hypothermia Promotes Pro-Inflammatory Cytokine Production in Monocytes

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Abstract: Hypothermia is often associated with compromised host defenses and infection. Deteriorations of immune functions related to hypothermia have been investigated, but the involvement of cytokines in host defense mechanisms and in infection remains unclear. We have previously shown that mild hypothermia modifies cytokine production by peripheral blood mononuclear cells. In this study, the effects of hypothermia on the monocytic production of several cytokines and nitric oxide (NO) were determined. Monocytes obtained from 10 healthy humans were cultured with lipopolysaccharide (LPS) under hypothermic (33°C) or normothermic (37°C) conditions for 48 hours. We performed flow cytometric analysis for simultaneous measurement of interleukin (IL)-8, IL-1β, IL-6, IL-10, IL-12p70, and tumor necrosis factor (TNF)-α in culture supernatants. NO production was quantified as accumulation of nitrite in the medium by a colorimetric assay. Compared with normothermia, mild hypothermia raised the levels of IL-1β, IL-6, IL-12p70, and TNF-α produced by monocytes stimulated with LPS. On calculating the ratios of these elevated cytokines to IL-10, however, only IL-12p70/IL-10 and TNF-α/IL-10 ratios were significantly elevated under hypothermic conditions. In contrast, hypothermia did not affect NO production. This study demonstrates that mild hypothermia affects the balance of cytokines produced by monocytes, leading to a pro-inflammatory state. Specifically, monocytic IL-12 and TNF-α appear to be involved in the immune alterations observed in mild hypothermia. However, the clinical significance of these phenomena remains to be clarified.

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Mild hypothermia is utilized in the treatment of various clinical conditions, including severe brain injury and cardiac arrest.[1,2] It has been shown to decrease metabolic rate and oxygen consumption[3,4] and also to limit neurotransmitter release and neuronal degeneration caused by ischemia or oxygen deprivation.[5] Hypothermia, however, can adversely affect certain physiologic processes such as platelet function and blood coagulation and is often associated with depression of cardiovascular and respiratory function.[4] Moreover, several investigations have shown that the hypothermic condition might produce an immune-compromised state. Compared with normothermic patients, a significantly increased risk of infection has been revealed in mildly hypothermic patients with severe head injuries.[6,7] Perioperative hypothermia can also predispose patients to wound infections,[8] and in animal experiments, exposure to mild hypothermia enhances susceptibility to bacterial infection.[9]

Deterioration in immune function caused by hypothermia has been investigated, with several reports demonstrating that hypothermia decreases (a) neutrophil functions such as oxidative killing and phagocytic activities in vivo and in vitro[10,11] and (b) neutrophil count.[6] However, the involvement of cytokines in the host defense mechanism and in infection has remained unclear.

Beilin et al,[12] have demonstrated reduction of interleukin (IL)-1β and IL-2 production by peripheral blood mononuclear cells (PBMCs) in mild perioperative hypothermia. Moreover, two groups have recently shown the effects of mild hypothermia on cytokine production by PBMC cultures. In PBMCs cultured under such conditions, Russwurm et al,[13] described decreased production of IL-2, and Kimura et al,[14] observed delayed production of IL-1β, IL-6, and tumor necrosis factor (TNF)-α. Furthermore, we have revealed that mild hypothermia inhibits production of IL-10 and interferon (IFN)-γ from cultured PBMCs.[15] These cytokines are thus likely to be involved in the immune responses in mild hypothermia; however, no data are available regarding the effect of hypothermia on cytokine production by monocytes. Although cytokines are produced by both

monocytes/macrophages and lymphocytes, the former may be the main source of cytokines on initial contact with potentially pathogenic organisms. Lipopolysaccharide (LPS) stimulation of monocytes was found to lead initially to synthesis of the pro-inflammatory cytokines IL-1, IL-6, IL-8, and TNF-α and later to the synthesis of the anti-inflammatory cytokine IL-10; the latter cytokine down-regulates the former set of cytokines,[16] suggesting that monocytes have a critical role in immunomodulation.

The current study therefore aimed to examine whether mild hypothermia (33°C) during culture affects production of cytokines (particularly ratios of pro-inflammatory and anti-inflammatory cytokines) or of nitric oxide (NO), which is responsible for microbicidal activity against intracellular pathogens,[17] in monocytes. This study utilized a newly developed technique,[18] which uses uniform size microparticle-based flow cytometry to simultaneously measure a panel of six human cytokines from the same sample, thereby enabling calculation of cytokine ratios. The relative balance between pro-inflammatory and anti-inflammatory cytokines is thought to be an important marker of various diseases states[19–21] and may better reflect the net effect of cytokine responses than absolute levels of cytokines.

MATERIALS AND METHODS

Subjects

Ten healthy volunteers (five men and five women), aged from 20 to 40 years, were enrolled in this study. Subjects with a history of thyroid disease, renal disease, diabetes, recent fracture, or any disease known to affect cytokine production were excluded. This study was approved by the Institutional Review Board of Yamaguchi University Hospital, and informed consent was obtained from all subjects prior to the study.

Isolation and Culture of Monocytes

Monocytes were isolated and cultured according to the methods of Pacifici et al,[22] with certain modifications. In brief, heparinized blood (about 7 mL) was fractionated by Ficoll density gradient centrifugation using Lymphoprep (NYCOMED, Oslo, Norway). Mononuclear cells were removed from the interface, washed once with RPMI 1640 medium (Invitrogen Corp, Carlsbad, CA), and resuspended in RPMI 1640 medium containing 10% fetal bovine serum at a concentration of 1×10^6 cells/mL. Aliquots of 3 mL were transferred to an MSP-P monocyte-specific culture plate (JIMRO, Takasaki, Japan) and incubated for 2 hours at either 37°C or 33°C in a humidified atmosphere of 95% air/5% CO_2. After incubation, the nonadherent cells (enriched in lymphocytes) were removed from the plate, and the adherent mononuclear cells (enriched in monocytes) were washed once with RPMI 1640 medium. For the analysis of cytokine and NO production, monocytes were then incubated with or without 20 μg/mL LPS, an effective concentration for the production of cytokines,[23] at either 37°C or 33°C for 48 hours. Cell-free supernatants were stored at −80°C pending measurements. The adherent population was subsequently stained for monocyte/macrophage-specific α-naphthyl acetate esterase and found to consist of >97% monocytes. In the present procedure, monocytes comprised 25.8 ± 2.6% (mean ± SEM) of the entire original mononuclear population isolated by Ficoll density gradient centrifugation, as judged by staining with a monocytic marker, CD-14 antibody, and from the flow cytometric analysis. Cell viabilities following incubation at both temperatures were determined in duplicate by trypan blue dye exclusion. Percentage cell viability (with that at 37°C taken as 100%) was 95.1 ± 10.5% (mean ± SEM) at 33°C (no significant difference).

Flow Cytometric Analysis

Two-color flow cytometric analysis was performed using a FACSCalibur® flow cytometer and cytometric bead array (CBA) kit (human inflammation CBA; Becton Dickinson Immunocytometry Systems, San Jose, CA), according to the manufacturer's manual, as previously described.[18] In brief, CBA uses a series of beads with discrete fluorescence intensity. Each series of beads is coated with a monoclonal antibody (mAb) against a single cytokine (IL-8, IL-1β, IL-6, IL-10, IL-12p70, and TNF-α), so that the mixture of beads detects six cytokines in one sample. A secondary phycoerythrin (PE)–conjugated mAb stains the beads proportionally to the amount of bound cytokine. A cytokine standard provided with the kit and test samples (50 μL) of the monocytic culture supernatants were analyzed with the FACSCalibur® flow cytometer. Data acquired were analyzed using Becton Dickinson CBA software, and cytokine concentrations were determined from each cytokine standard curve.

Enzyme-Linked Immunosorbent Assay

The concentration of IFN-γ in the monocytic culture supernatants was measured in duplicate using an enzyme-linked immunosorbent assay (ELISA) kit (Bio-Sourse Europe S.A., Nivelles, Belgium).

Quantification of Cytokines

We determined the number of monocytes per mL by multiplying the number of mononuclear cells by the percentage of monocytes in the entire original mononuclear population. To normalize the results according to the number of monocytes per mL, amounts of IL-8, IL-1β, IL-6, IL-10, IL-12p70, and TNF-α were expressed as pg per 10^6 monocytes and the amount of IFN-γ was expressed as IU per 10^6 monocytes.[22]

Nitrite Assay

NO production was quantified as nitrite accumulation in the culture medium by a colorimetric assay with Griess reagent (Sigma, St. Louis, MO), as described by Green et al.[24] In brief, 100 μL aliquots of supernatant were added to an equal volume of Griess reagent, and the mixtures were then incubated for 15 minutes. In this system, nitrite ions react with 1% sulfanilamide in 5%

ortho-phosphoric acid/0.1% *N*-1 naphthylethylenediamine dihydrochloride to yield an azo-chromophore, the absorbance of which was measured at 550 nm using a 96-well microplate reader. Data were expressed as μM.

Statistical Analysis

Data were expressed as mean ± SEM. Group mean values were compared using the Wilcoxon matched-pairs signed-ranks test. Probability (P) values less than 0.05 were considered to indicate significant differences.

RESULTS

Effects of Mild Hypothermia on LPS-Induced IL-8, IL-1β, IL-6, IL-10, IL-12p70, and TNF-α Production by Monocytes

In preliminary studies, we determined TNF-α and IL-6 production by cultured monocytes using ELISA. TNF-α is known to be released earlier than IL-6. We confirmed that the production of both cytokines gradually increased until 48 hours of culture and accordingly decided on this as the incubation period, although we did not perform kinetic analysis of any other cytokines. In addition, we conducted ELISA measurement of levels of cytokines (IL-1β, IL-6, IL-10, and TNF-α) produced by unstimulated monocytes, as a time control (48 hours). In unstimulated monocytes, cytokine production was almost undetectable in both hypothermic and normothermic conditions (eg, IL-6, pg/mL [mean ± SEM]; 1.5 ± 0.3 [33°C] vs 4.0 ± 1.3 [37°C]; n = 6), with no significant differences in levels between the two groups after normalization for number of monocytes (ie, IL-6, pg/10^6 monocytes [mean ± SEM]; 5.4 ± 1.2 [33°C] vs 12.8 ± 3.8 [37°C]; n = 6, $P = 0.094$). We therefore did not further determine cytokine production by unstimulated monocytes.

Levels of cytokine production in the supernatants of monocytes cultured for 48 hours with LPS at both 33°C and 37°C are shown in Table 1. Significantly higher levels of IL-1β, IL-6, IL-12p70, and TNF-α were produced by monocytes cultured under mild hypothermia compared with those cultured under normothermic conditions. This difference was not apparent for IL-8 or IL-10.

TABLE 1. Cytokine Production in Monocytes Stimulated with LPS under Both Hypothermic and Normothermic Conditions

Cytokines (pg/10^6 Monocytes)	Normothermic Condition (37°C)	Hypothermic Condition (33°C)
IL-8	576,917 ± 150,932	586,422 ± 113,814
IL-1β	7021 ± 2469	15,932 ± 5468*
IL-6	140,370 ± 42,429	200,065 ± 44,298†
IL-10	1108 ± 325	984 ± 254
IL-12p70	86 ± 31	464 ± 137*
TNF-α	6351 ± 2238	12,767 ± 3923*

Data are expressed as mean ± SEM, n = 10.
Statistical significance: *$P < 0.05$, †$P < 0.01$ compared with normothermic conditions.

TABLE 2. Cytokine Balance in Monocytes Stimulated with LPS under Both Hypothermic and Normothermic Conditions

Cytokine Ratios	Normothermic Condition (37°C)	Hypothermic Condition (33°C)
IL-8/IL-10	745 ± 203	806 ± 215
IL-1β/IL-10	7.0 ± 1.8	15.8 ± 3.8
IL-6/IL-10	167 ± 42	275 ± 84
IL-12p70/IL-10	0.09 ± 0.02	0.59 ± 0.11*
TNF-α/IL-10	6.3 ± 1.7	12.5 ± 3.3*

Data are expressed as mean ± SEM, n = 10.
Statistical significance: *$P < 0.05$ compared with normothermic conditions.

Effects of Mild Hypothermia on LPS-Induced IFN-γ Production by Monocytes

No significant difference in IFN-γ production by monocytes was apparent between hypothermic and normothermic conditions (4.1 ± 2.0 vs 1.7 ± 0.3 IU/10^6 monocytes, respectively).

Ratios of Production of IL-8, IL-1β, IL-6, IL-12p70, and TNF-α to IL-10 by Monocytes

We calculated ratios of pro-inflammatory cytokines to IL-10 produced by monocytes. At 33°C, IL-12p70/IL-10 and TNF-α/IL-10 ratios were significantly increased (Table 2).

Effects of Mild Hypothermia on LPS-Induced NO Production by Monocytes

No significant difference in monocytic nitrite production was apparent between hypothermic and normothermic conditions (1.9 ± 0.1 vs 2.2 ± 0.4 μM, respectively).

DISCUSSION

In the current study, mild hypothermia (33°C) raised the production of IL-1β, IL-6, IL-12p70, and TNF-α from cultured monocytes and increased the ratios of IL-12p70/IL-10 and TNF-α/IL-10, indicating that mild hypothermia might shift the balance of inflammatory mediators toward dominance of pro-inflammatory cytokines. This shift is in accordance with that demonstrated by our previous studies in PBMCs.[15] The present results indicate that hypothermia per se may cause physiologic stress. Indeed, elevated pro-inflammatory cytokine levels have been observed in patients with accidental hypothermia.[25]

Host defense and immune functions in response to various challenges such as infection and traumatic injury are certainly thought to involve a mechanism whereby pro-inflammatory and anti-inflammatory reactions exhibit mutual modulation. The two types of cytokine mediating these reactions are produced mainly by monocytes/macrophages and act as initiators during the reaction process. Among these, IL-10 is an anti-inflammatory and immunosuppressive cytokine that affects the function of monocyte/macrophage cell lineages and limits

the production of pro-inflammatory cytokines.[16] In addition, the relative balance between pro-inflammatory and anti-inflammatory cytokines has been used to characterize a variety of diseases states[19–21] and is often considered more important than the absolute concentrations of cytokines in an individual. Based on these observations and considering infection to be likely during hypothermia unless the balance between these cytokines is favorable to host defenses, we calculated the ratios of pro-inflammatory cytokines to IL-10. The present study demonstrated that only IL-12p70/IL-10 and TNF-α/IL-10 ratios in monocytes were increased, suggesting that IL-10 does not inhibit IL-12p70 and TNF-α production and that these cytokines are effective in immune modulation in hypothermia.

IL-12p70 is a heterodimeric cytokine composed of a heavy chain (p40) and a light chain (p35) produced primarily by monocytes/macrophages and dendritic cells.[26] In addition to its pro-inflammatory role, IL-12 acts as an immunomodulatory cytokine in inducing IFN-γ production, contributing to cell-mediated immunity.[26] Recent studies show that IL-12p70 production by monocytes is impaired after traumatic or thermal injury[27] and that IL-12 therapy improves survival after subsequent sepsis, presumably via induction of pro-inflammatory cytokines.[27,28] Moreover, after major traumatic injury, normal or elevated IL-12p70 levels are associated with survival, whereas decreased IL-12p70 levels correlate with the development of adverse clinical outcomes.[29] These studies suggest that IL-12 is critical for maintaining postinjury immune function. Therefore, elevation of IL-12p70 may be a beneficial component of vital reactions, including host responses against infection in hypothermia.

TNF-α is well known as a key mediator in the optimal activation of host defenses in response to infection.[30,31] In addition, TNF-α receptor 1-deficient mice show increased susceptibility to infection by *Listeria monocytogenes*.[32] Elevation of TNF-α seems therefore to act as a host defense mechanism against micro-organisms even in mild hypothermia.

Taken together, these findings for monocytes as well as PBMCs[15] support reports that no significant increases in infection in patients with hypothermia are observed,[1,33,34] although conflicting data have been reported in this regard.[6,7]

We should be aware, on the other hand, that cytokines can have adverse as well as beneficial consequences. When the expression of pro-inflammatory cytokines is dysregulated, cellular injury, shock, and even death can result.[35] In patients with traumatic or ischemic brain injury, increased levels of pro-inflammatory cytokines are related to poorer clinical outcome.[36,37]

We have previously shown that the same hypothermic conditions (33°C) decreased the production of IL-10 and IFN-γ in PBMCs stimulated with phytohemagglutinin (PHA), a T-lymphocyte activator.[15] However, in the current experiment in monocytes, production of neither of these cytokines was found to be decreased at 33°C. These data suggest that lymphocytes are more susceptible to hypothermia than monocytes, resulting in diminished IL-10 and IFN-γ production by PBMCs (presumably mainly by T-lymphocytes) but not by monocytes. This interpretation is supported by the finding that cytokine production was unchanged in PBMCs stimulated with LPS under hypothermic conditions, although a decrease has been observed in cytokine production by PHA-stimulated PBMCs.[13] Hence, when these two studies are taken together, monocytic secretory activity appears to be unimpaired in hypothermia. Indeed, the present study revealed that IL-12p70 and TNF-α production by monocytes is increased under hypothermic conditions. This increased pro-inflammatory cytokine production is in accordance with findings that in vitro hypothermia augments TNF-α and IL-1β production by monocytes[38] and TNF-α, IL-1β, and IL-6 production by LPS-stimulated PBMCs.[39] Monocytes may thus act as the initial source of cytokines on contact with potentially pathogenic organisms in hypothermia. Another factor to be considered is that the interaction between monocytes and lymphocytes might have influenced the pattern of cytokine production, as previously reported.[12–15]

Pro-inflammatory cytokines and LPS are thought to stimulate the expression of inducible NO synthase and hence the production of NO in macrophages and mononuclear cells.[40,41] In this study, the pro-inflammatory state did not appear to induce monocytic NO production, in accordance with findings of failed release of NO by cytokines in monocytes.[42] One possible mechanism behind this finding could be that cytokine-mediated induction of NO production occurs after a certain time lag and was therefore not detected under the current experimental time frame. NO is responsible for microbicidal activity against intracellular pathogens[17]; however, the fact that NO remained unchanged indicates that this activity is, at the least, not defective in hypothermia.

There are several limitations to our study. We were unable to determine in vivo levels of the same cytokines in plasma or serum during hypothermia. The results might have differed if we had harvested monocytes from hypothermic patients. Furthermore, extrapolation of results from monocytes in vitro to the effects of hypothermia in a complete organism, in which many other cell types and hormones are involved, should always be made with great caution, as previously stated.[15] In addition, it is well recognized that an early release of pro-inflammatory cytokines may lead to a delayed release of anti-inflammatory cytokines including IL-10. It is conceivable, therefore, that such early cytokine release and elevated ratio of pro-inflammatory to anti-inflammatory cytokine at that time might have impacted later cytokine release and ratios in this study. This response should be borne in mind when interpreting our data.

In conclusion, mild hypothermia (33°C) increases IL-12p70/IL-10 and TNF-α/IL-10 production ratios in monocytes, suggesting that monocytic IL-12 and TNF-α may be involved in the immune alterations observed in mild hypothermia, and that the pro-inflammatory state

induced might be beneficial to host responses. However, the clinical significance of these phenomena remains to be clarified.

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