The Effect of Hypertonic Saline on mRNA of Proinflammatory Cytokines in Lipopolysaccharide-Stimulated Polymorphonuclear Cells

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Background: Hypertonic saline is often used to resuscitate patients experiencing shock. In such conditions, polymorphonuclear cells and Toll-like receptors (TLRs) form an essential part of early induced innate immunity.

Objective: To investigate the immunomodulatory effect of hypertonic saline on polymorphonuclear cells by evaluating the changes in TLR-4 receptors and proinflammatory cytokines.

Methods: Polymorphonuclear cells were isolated from whole blood using Polymorphprep (Axis-Shield, Oslo, Norway). The isolated polymorphonuclear cells were plated at a density of 1 × 10^6 cells/mL in 6-well flat-bottomed culture plates and were stimulated with 1 μg/mL lipopolysaccharide or N-formyl-methionyl-leucyl-phenylalanine. The stimulated polymorphonuclear cells were cultured in hypertonic saline at 10, 20, or 40 mmol/L above isotonicity. After that, the changes in TLR-4 and cytokines were measured by quantitative real-time polymerase chain reaction and flow cytometry.

Results: The level of TLR-4 mRNA expression decreased after stimulation with N-formyl-methionyl-leucyl-phenylalanine, but hypertonic saline did not affect the TLR-4 mRNA expression. TLR-4 mRNA expression was clearly induced upon stimulation with lipopolysaccharide, and the addition of hypertonic saline restored TLR-4 mRNA expression in polymorphonuclear cells. The interleukin-1β mRNA expression was decreased in the hypertonic environment. On the other hand, the tumor necrosis factor-α value was not influenced by the addition of hypertonic saline.

Conclusions: Hypertonic saline has an immunomodulatory effect on polymorphonuclear cells through the TLR-4 pathway, and the interleukin-1β-associated pathway is influenced more by hypertonic saline than is the tumor necrosis factor-α-associated pathway.

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Introduction

Excess or prolonged inflammation can cause harm even though it is essentially beneficial to maintaining homeostasis. An excessive inflammatory response is referred to as systemic inflammatory response syndrome. Systemic inflammatory response syndrome can result in multiple organ failure, which is associated with a high death rate. The innate immune system is involved in the development of systemic inflammatory response syndrome.

Polymorphonuclear (PMN) cells form an essential part of early induced innate immunity. During the acute phase of inflammation, PMN cells are recruited to the site of inflammation and have been reported to be important in triggering organ damage after shock and resuscitation. Thus, PMN cells are the so-called first responders of inflammation. Toll-like receptors (TLRs) are also key components of the innate immune system. Ten human TLRs have been discovered so far, the targets of which are predominantly pathogen-associated molecular patterns. They are involved in multiple steps in the inflammatory reaction, eliminating invading pathogens and coordinating systemic defenses. TLR-1, -2, -4, -5, and -6 are expressed on the cell surface, where their major role is the recognition of bacterial products. TLR-3, -7, -8, and -9 are confined to intracellular compartments, where they specialize in viral detection or the recognition of nucleic acids. Chemokine receptor expression and function in neutrophils are regulated by TLR activation, which presumably facilitates recruitment and localization of these cells to sites of infection and inflammation.

Fluid resuscitation is essential in urgent situations such as trauma, sepsis, and hypovolemia. Prompt fluid resuscitation
administered to patients with such conditions can improve survival by shortening the duration of the shock period and decreasing the inflammatory response. For resuscitation, the proper selection of fluid is also important. Clinical resuscitation with lactated Ringer’s solution activates neutrophils; shed blood and hypertonic saline (HTS) do not. HTS is probably the most studied of all alternative fluid resuscitation strategies. HTS effectively raises blood pressure, improves microcirculatory blood flow, and protects tissues against reperfusion injury. Furthermore, the immunomodulatory effect of HTS has also been demonstrated in many studies. In our study, we investigated the immunomodulatory effect of HTS on PMN cells by evaluating the changes in TLR-4 receptors and proinflammatory cytokines.

Methods

Preparation of human PMNs

The study protocol and the written informed consent form were reviewed and approved by Korea University Guro Hospital (institutional review board No. KUGH 10157). Whole blood from 10 healthy volunteers was collected in EDTA tubes. A sterile processing environment was maintained with a clean bench, and 5 mL of each collected whole blood sample was separated in aliquots into 15-mL test tubes with 5 mL Polymorphprep (Axis-Shield, Oslo, Norway), followed by centrifugation for approximately 35 minutes at 500 g. Among the resulting cell layers after centrifugation, the PMN cell layer, located between the monocyte and red blood cell layers, was collected with a pipette. To remove the red blood cells remaining in the collected PMN cell sample, the sample was incubated with a 0.2% saline solution for 30 seconds, after which a 1.8% saline solution was added to create 0.9% normal osmotic pressure. The samples were centrifuged at 450 g for 10 minutes, followed by 2 washes with phosphate-buffered saline. The isolated PMN cells were incubated in Roswell Park Memorial Institute culture medium containing 10% fetal bovine serum, 1% penicillin-streptomycin, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, and 0.005% 2-mercaptoethanol. We confirmed cell densities of 1 × 10⁶ cells/mL and viabilities > 95% using trypan blue dye.

Stimulation of PMNs

Isolated PMNs were plated at a density of 1 × 10⁶ cells/mL in 6-well flat-bottomed culture plates and were stimulated with 1 μg/mL lipopolysaccharide (LPS) (Sigma-Aldrich, St Louis, Missouri) or 1 μM N-formyl-methionyl-leucyl-phenylalanine (fMLP) (Sigma Aldrich). LPS is an endotoxin found in the outer membrane of gram-negative bacteria and fMLP is a synthetic analogue of a chemotactic peptide derived from a variety of bacteria. We decided optimal concentration of each activator considering previous studies. HTS was added to PMN cells at the same time. The stimulated PMN cells were cultured in the presence or absence of HTS at 10 mM/L, 20 mM/L, and 40 mM/L above isotonicity. This resulted in sodium concentrations of 150 mM/L, 160 mM/L, and 180 mM/L respectively, which was measured by a GEM Premier 3000 blood gas analyzer (Instrumentation Laboratory, Lexington, Massachusetts). After that, PMNs were incubated for 2 hours. Incubation time was based on our preliminary study (supplemental data).

Isolation of mRNA and quantitative real-time polymerase chain reaction (qRT-PCR)

After 2 hours of incubation, total RNA was extracted from cells using an RNaseasy Mini Kit (Qiagen, Hilden, Germany) and treated with DNase (Turbo DNA-free kit; Invitrogen, Carlsbad, California). An amount of 200 ng total RNA from each sample was reverse transcribed into complementary DNA using a high-capacity complementary DNA reverse transcription kit. Expression of TLR-4, interleukin (IL)-1β, and tumor necrosis factor-α (TNF-α) mRNA was analyzed by qRT-PCR. The qRT-PCR reactions were performed using the ABI 7300 (Applied Biosystems, Foster City, California), and amplifications were done using the TaqMan Gene Expression Master Mix (Applied Biosystems, Foster City, California) with the following TaqMan probes (gene name, reference sequence number): GAPDH, Hs99999905_m1; TLR4, Hs00152939_m1; IL1B, Hs00174097_m1; and TNF, Hs01113624_g1. The thermal cycling conditions were 50°C for 2 minutes followed by an initial denaturation step at 95°C for 10 minutes, 45 cycles at 95°C for 15 seconds, and 60°C for 1 minute.

Flow cytometry analysis

Expression of cell surface TLR-4 was determined by flow cytometry analysis. The cells were washed with ice-cold phosphate-buffered saline, then resuspended in Flow Cytometry Staining Buffer (eBioscience, San Diego, California) and blocked for 20 minutes on ice. The cells were then stained with 5 μL (2 μg) phycoerythrin-conjugated anti–TLR-4 monoclonal antibody (clone HTA125; eBioscience) or 5 μL (0.5 μg) isotype control (PE-conjugated mouse IgG2a K; eBioscience) per test in the dark for 1 hour on ice following the manufacturer’s recommended protocol. After washing, the stained cells were resuspended in 100 μL staining buffer and analyzed by flow cytometry using Cytomics FC 500 (Beckman Coulter, Brea, California) and CXP software (Beckman Coulter). In each case, 100,000 cells were acquired.

Data and statistical analysis

One-way ANOVA and the post hoc test were used for statistical analysis with the SPSS software package (version 13.0, IBM-SPSS Inc, Chicago, Illinois). A P value < 0.05 was considered to indicate statistically significant differences.

Results

The effects of HTS on TLR-4 in PMN cells with LPS and fMLP stimulation

As shown in Figure 1, TLR-4 mRNA expression was clearly induced upon stimulation with LPS. The mean (SD) TLR4/GAPDH-negative gene expression was 2.06 (0.40) in the only LPS-stimulated PMN cells. HTS mildly increased the mean (SD) TLR4/GAPDH-relative gene expression to 2.36 (0.54) at 10 mM/L above isotonicity, but without a statistically significant difference. HTS decreased the mean (SD) TLR4/GAPDH-relative gene expression to 1.75 (0.27) at 20 mM/L above isotonicity and 1.25 (0.22) at 40 mM/L above isotonicity. One-way ANOVA showed the difference in the mean values among 5 groups (P < 0.001). The TLR4/GAPDH-relative gene expression at 40 mM/L above isotonicity was significantly different from that of only LPS-stimulated PMN cells and 10 mM/L above isotonicity (P < 0.05) (Figure 1A). On the other hand, the mean (SD) TLR4/GAPDH-relative gene expression was decreased in PMN cells stimulated with fMLP (0.99 [0.52]). The mean (SD) TLR4/GAPDH-relative gene expression was 1.00 (0.60) at 10 mM/L above isotonicity, 0.89 (0.45) at 20 mM/L above isotonicity, and 0.74 (0.26) at 40 mM/L above isotonicity without statistical difference (Figure 1B).

Flow cytometry was used to examine the changes in cell surface TLR-4 protein levels on LPS-stimulated PMN cells. The result of flow cytometry was in agreement with that of qRT-PCR. The number of TLR-4-positive cells increased in the LPS-stimulated
PMN cells. The addition of HTS decreased the proportion of TLR-4-positive cells in LPS-stimulated PMN cells. The mean (SD) percentage of cells stained with anti–TLR-4 monoclonal antibody was 22.2% (3.8%) in the control group, 41.3% (6.0%) in the LPS-stimulated group, 25.7% (5.9%) at 10 mmol/L above isotonicity, 22.9% (4.5%) at 20 mmol/L above isotonicity, and 22.7% (6.1%) at 40 mmol/L above isotonicity (Figure 2).

The effect of HTS on proinflammatory cytokines in LPS-stimulated PMN cells

The IL-1β/GAPDH mRNA expression decreased to 1.09 ± 0.31 at 20 mmol/L above isotonicity and 0.93 ± 0.55 at 40 mmol/L above isotonicity (p < 0.05). The IL-1β/GAPDH mRNA expression increased to 2.66 ± 1.54 at 10 mmol/L above isotonicity, however, without statistical difference. On the other hand, the TNF-α/GAPDH mRNA expression was not influenced by adding HTS. The TNF-α/GAPDH mRNA expression was 1.41 ± 0.24 at 10 mmol/L above isotonicity, 1.38 ± 0.25 at 20 mmol/L above isotonicity and 1.42 ± 0.24 at 40 mmol/L above isotonicity (Figure 3).

Discussion

Isotonic crystalloid has been commonly used in fluid resuscitation in shock due to trauma or sepsis. However, it has unavoidable limitations such as an inflammatory response caused by administration of a large volume. In contrast, HTS has been reported to be a fluid therapy method that confers many advantages, including a rapid increase in plasma that can be achieved by a relatively small resuscitation volume. Although there is still controversy regarding the feasibility of initial treatment using HTS, the clinical aspects of the usefulness of HTS have been studied. Several studies have been performed on the effectiveness of small volume resuscitation, which is defined as a rapid infusion of HTS in combination with dextran or hetastarch.

HTS has also been studied experimentally for the treatment of sepsis and has been reported to have some promising beneficial effects. From a clinical perspective, Hannemann et al conducted the first study on the effect of small volume resuscitation in severe sepsis. Oliveira et al reported that an HTS/dextran solution improved cardiovascular performance and resuscitated severe sepsis. Oliveira et al reported that an HTS/dextran solution improved cardiovascular performance and resuscitated severe sepsis.
production of chemokines. \(^{26}\) However, reports showed that TNF-α and causes the generation of superoxide anions and stimulates the fMLP binds G-protein receptor on cell surfaces that can recognize LPS. fMLP binds G-protein receptor on cell surfaces and causes the generation of superoxide anions and stimulates the production of chemokines. \(^{26}\) However, a report showed that the TNF-α and IL-1β mRNA expression induced by fMLP is not different from that induced by LPS in human peripheral blood monocytes. \(^{27}\) So we investigated if TLR-4 expression on PMN cells can be influenced by fMLP stimulation. As shown in Figure 1, the level of TLR-4 mRNA expression decreased after stimulation with fMLP. Varying the osmolarity did not affect the TLR-4 mRNA expression. In light of all this, we concluded that fMLP does not involve the TLR-4 signaling pathway and HTS has no association with fMLP-stimulated PMN cells. PMN cells were stimulated with LPS for further analysis.

The increased TLR-4 mRNA expression with LPS stimulation was suppressed after adding HTS, according to the qRT-PCR results. From the flow cytometry results, the number of cells expressing TLR-4 on the membrane surface also decreased after adding HTS. The HTS changed the IL-1β mRNA expression on LPS-stimulated PMN cells similar to the pattern of change in TLR-4. However, HTS did not affect the level of TNF-α mRNA expression. We believe that HTS has an immunomodulatory effect on PMN cells through the TLR-4 pathway. In addition, HTS appears to have more influence on the IL-1β-associated intracellular signaling pathway than the TNF-α-associated intracellular signaling pathway. Hatanaka et al. \(^{28}\) also reported that sodium chloride does not significantly affect the production of TNF-α and inhibits the production of IL-1β in LPS-stimulated monocytes by the enzyme-linked immunosorbent assay.

Gundersen et al. \(^{29}\) pointed out that the question of clinical relevance for HTS exists because its reported in vitro effects have been difficult to demonstrate in vivo. In vitro studies frequently apply osmolarity > 350 mOsm/kg. In the same manner, mild hyperosmolarity increased the TLR-4 mRNA expression in qRT-PCR in this study, although the result was not statistically significant, and also showed that a high osmolarity has a greater immunosuppressive effect in qRT-PCR.

When interpreting the results of this study, there are several limitations. Our study was done in only PMN cells and did not include interaction with other factors such as the endothelium. The numbers of measured immune parameters were limited and PMN cells were collected only from healthy donors. Further research should be carried out to investigate the effect of HTS on intracellular signaling pathways. In addition, the result of our study was descriptive and did not provide any details of how HTS modulates TLR-4 expression.

Conclusions
HTS has an immunomodulatory effect on PMN cells through the TLR-4 pathway, and the IL-1β-associated pathway is influenced more by HTS than is the TNF-α-associated pathway.

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Conflicts of Interest
The authors have indicated that they have no conflicts of interest regarding the content of this article.
Appendix A. Supplemental data

TLR4/GAPDH relative mRNA expression in nonstimulated polymorphonuclear (PMN) cells (n = 5). TLR4/GAPDH-relative expression was measured at 30 minutes, 2 hours, 4 hours, 8 hours, and 24 hours in nonstimulated polymorphonuclear cells. Expression of TLR4/GAPDH was analyzed by quantitative real-time polymerase chain reaction. Cell viability was checked by trypan blue using hemocytometer. Mean (SD) cell viability was 96.8% (0.8%) after 4 hours of incubation. However, it was decreased to 78.8% (3.1%) after 8 hours of incubation.

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.curtheres.2014.06.003.

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