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Original Article

Vasopressin inhibits endotoxin-induced upregulation of inflammatory mediators in activated macrophages

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ABSTRACT

Objectives: We sought to elucidate the effects of vasopressin on modulating the endotoxin-induced upregulation of inflammatory mediators.
Materials and Methods: A confluent murine macrophage-like cell line, RAW264.7 cells, were treated with lipopolysaccharide (LPS) (100 ng/mL) or with LPS plus vasopressin (10 pg/mL, 100 pg/mL, or 1000 pg/mL); the cells were denoted as the LPS group, the LPS-V(10) group, the LPS-V(100) group, and the LPS-V(1000) group, respectively. The respective control groups were run simultaneously. Vasopressin was administered immediately after LPS. The expression of inflammatory molecules was then assayed. The molecules that were assayed included the chemokine macrophage-inflammatory protein-2 (MIP-2); the cytokines tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6); nitric oxide (NO)/inducible NO synthase (iNOS); and prostaglandin E_2 (PGE_2)/cyclooxygenase-2 (COX-2).
Results: The differences between the LPS and LPS-V(10) groups in the concentration of inflammatory mediators were not statistically significant. By contrast, the LPS-V(100) and LPS-V(1000) groups were significantly lower than the LPS group in the concentration of MIP-2 ($p = 0.004$ and $p = 0.001$, respectively), TNF-α ($p = 0.045$ and $p = 0.007$, respectively), IL-1β ($p = 0.003$ and $p < 0.001$, respectively), NO ($p = 0.014$ and $p = 0.001$, respectively), iNOS mRNA ($p = 0.001$ and $p < 0.001$, respectively), PGE_2 ($p = 0.021$ and $p < 0.001$, respectively), and COX-2 mRNA ($p = 0.021$ and $p = 0.006$, respectively). The IL-6 concentration was moreover significantly lower in the LPS-V(1000) group than in the LPS group ($p < 0.001$), whereas the IL-6 concentration in the LPS-V(100) and the LPS groups was not significantly different.
Conclusion: In a dose-dependent manner, vasopressin inhibited the endotoxin-induced upregulation of inflammatory mediators in activated murine macrophages.

1. Introduction

It is well-established that the endotoxin-induced upregulation of inflammatory mediators such as the chemokines [e.g., macrophage-inflammatory protein-2 (MIP-2)]; the cytokines [e.g., tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6)]; nitric oxide (NO)/inducible NO synthase (iNOS); and prostaglandin E_2 (PGE_2)/cyclooxygenase-2 (COX-2) are crucial in mediating the systemic inflammatory response during sepsis [1–4]. In this response, septic patients have inappropriate vasodilatation and myocardial dysfunction that can result in hypoperfusion and eventually dysfunction of vital organs [5]. Previous data further indicate that modulating the systemic inflammatory response through inhibiting the endotoxin-induced upregulation of inflammatory mediators could reduce the pathological sequelae of sepsis, and thereby be a beneficial therapeutic strategy against sepsis [6,7].

Vasopressin is an endogenous nonapeptide synthesized in the paraventricular nuclei within the hypothalamus [8,9]. Vasoconstriction and antidiuresis are two well-known physiological functions of vasopressin [8,9]. Clinical observations reveal that septic patients tend to have low circulating concentrations of endogenous vasopressin [10–12]. A significant increase in sensitivity to exogenous vasopressin during sepsis has also been reported [13,14]. Previous data further reveal that exogenous vasopressin can restore aortic blood flow and preserve perfusion to vital organs [15,16].

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Based on these data, clinical guidelines now include exogenous vasopressin in the management of sepsis [17].

Previous data also have revealed that exogenous vasopressin decreases pulmonary inflammation in a rodent model of sepsis [18]. These data seem to indicate that the mechanisms underlying the observed beneficial effects of exogenous vasopressin during sepsis and vasopressin's effect on restoring organ perfusion may involve modulating the inflammatory response. To date, direct evidence depicting the effects of vasopressin in this regard are lacking. For further elucidation, we conducted this study with the hypothesis that vasopressin could inhibit the endotoxin-induced upregulation of inflammatory mediators in activated macrophages.

2. Materials and methods

2.1. Cell culture and cell activation protocols

To facilitate our investigation, we employed RAW264.7 cells, which are an immortalized murine macrophage-like cell line that can readily express inflammatory mediators on exposure to endotoxin [19]. The RAW264.7 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Life Technologies, Grand Island, NY, USA) and incubated in a humidified chamber at 37°C in a mixture of 95% air and 5% CO_2. Prior to the experiments, the cells were plated on 6-well dishes ($1-2 \times 10^6$ cells per well; Corning, Acton, MA, USA). The culture medium was changed every day for a routine culture, and then changed 1 hour prior to each experiment. Cells under passage 20 were used for experiments. After reaching 80% confluency, the RAW264.7 cells were activated with Gram-negative endotoxins [lipopolysaccharide (LPS, 100 ng/mL) and *Escherichia coli* serotype 0127:B8 endotoxin; Sigma-Aldrich, St. Louis, MO, USA] to induce the upregulation of inflammatory mediators, as we have previously reported [20].

2.2. Experimental protocols

The RAW264.7 cells were randomized to receive phosphate-buffered saline (PBS) (Life Technologies); vasopressin (V; 1000 pg/mL; Life Technologies); LPS; or LPS plus vasopressin (10 pg/mL, 100 pg/mL, or 1000 pg/mL). The cells were designated as the PBS group, the V(1000) group, the LPS group, the LPS-V(10) group, the LPS-V(100) group, and the LPS-V(1000) group, respectively. Each group contained six culture dishes ($n = 6$). Vasopressin was administered immediately after LPS. After reacting with LPS for 24 hours (or for a comparable duration in groups without LPS), the cell cultures from each group were harvested. The vasopressin dosage range (10–1000 pg/mL) was determined on the basis of the plasma concentrations of patients receiving exogenous vasopressin for the treatment of sepsis [10].

2.3. Inflammatory mediator measurements

Freshly harvested culture media were analyzed by the respective enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA) for the concentrations of MIP-2, the cytokines (i.e., TNF-α, IL-1β, and IL-6), and PGE_2. Freshly harvested culture media were also analyzed for the concentrations of stable NO metabolites, nitrite, and nitrate by using a colorimetric assay kit (Cayman Chemical, Ann Arbor, MI, USA).

2.4. Analyses of iNOS and COX-2 transcriptional expression

The transcriptional expressions of iNOS and COX-2 were analyzed by reverse transcription and polymerase chain reaction (RT-PCR) [21,22]. The primer sequences and amplification protocols for iNOS, COX-2, and β-actin (as the internal standard) were adopted, based on previous reports [21,22]. RNA isolation, mRNA conversion to complementary DNA (cDNA) using reverse transcription (RT), and polymerase chain reaction (PCR) amplification were performed in accordance with previous protocols [21,22]. After separation, the PCR-amplified cDNA band densities were quantified by using densitometric techniques (Scion Image for Windows; Scion Corp., Frederick, MD, USA).

2.5. Statistical analysis

Data were analyzed by using one-way analysis of variance with the Tukey *post hoc* test to determine the between-group differences. Data are presented as the mean ± standard deviation. The significance level was set at 0.05. A commercial software package (SigmaStat for Windows; SPSS Science, Chicago, IL, USA) was used for data analysis.

3. Results

3.1. Chemokines and cytokines

Fig. 1 illustrates the chemokine and cytokine data. The concentrations of MIP-2, TNF-α, IL-1β, and IL-6 in the PBS and V(1000) groups were low. As we expected, the concentrations of MIP-2, TNF-α, IL-1β, and IL-6 were significantly higher in the LPS group than in the PBS group ($p < 0.001$ for all substances). The difference between the LPS-V(10) and LPS groups was not significant in the concentrations of MIP-2, TNF-α, IL-1β, and IL-6. However, the LPS-V(100) and LPS-V(1000) groups were significantly lower than the LPS group in the concentrations of MIP-2 ($p = 0.004$ and $p = 0.001$, respectively), TNF-α ($p = 0.045$ and $p = 0.007$, respectively), and IL-1β ($p = 0.003$ and $p < 0.001$, respectively). The IL-6 concentration was significantly lower in the LPS-V(1000) group than in the LPS group ($p < 0.001$), whereas the IL-6 concentration was not significantly different between the LPS-V(100) and LPS groups.

Our data also revealed that the MIP-2 concentration was not significantly different between the LPS-V(10), LPS-V(100), and LPS-V(1000) groups. The differences in the TNF-α and IL-6 concentrations were not significantly different between the LPS-V(10) and LPS-V(100) groups. However, the IL-1β concentration was significantly lower in the LPS-V(100) group than in the LPS-V(10) group ($p = 0.029$). The TNF-α, IL-1β, and IL-6 concentrations were moreover significantly lower in the LPS-V(1000) group than in the LPS-V(10) group ($p = 0.028$, $p < 0.001$, and $p < 0.001$, respectively). The IL-6 concentration was significantly lower in the LPS-V(1000) group than in the LPS-V(100) group ($p = 0.019$), whereas the TNF-α and IL-1β concentrations were not significantly different between the LPS-V(1000) and LPS-V(100) groups.

3.2. NO, iNOS mRNA, PGE_2, and COX-2 mRNA

Fig. 2 illustrates the data on NO, iNOS mRNA, PGE_2, and COX-2 mRNA. The concentrations of NO, iNOS, PGE_2, and COX-2 were significantly higher in the LPS group than in the PBS group ($p < 0.001$ for all substances). The differences in the concentrations of NO, iNOS, PGE_2, and COX-2 similarly were not significantly different between the LPS-V(10) and LPS groups. By contrast, the LPS-V(100) and LPS-V(1000) groups were significantly lower than the LPS group in the concentrations of NO ($p = 0.014$ and $p = 0.001$, respectively); iNOS ($p = 0.001$ and $p < 0.001$, respectively); PGE_2 ($p = 0.021$ and $p < 0.001$, respectively); and COX-2 ($p = 0.020$ and $p = 0.006$, respectively). The differences in the concentrations of NO, iNOS, PGE_2, and COX-2 moreover were not significantly

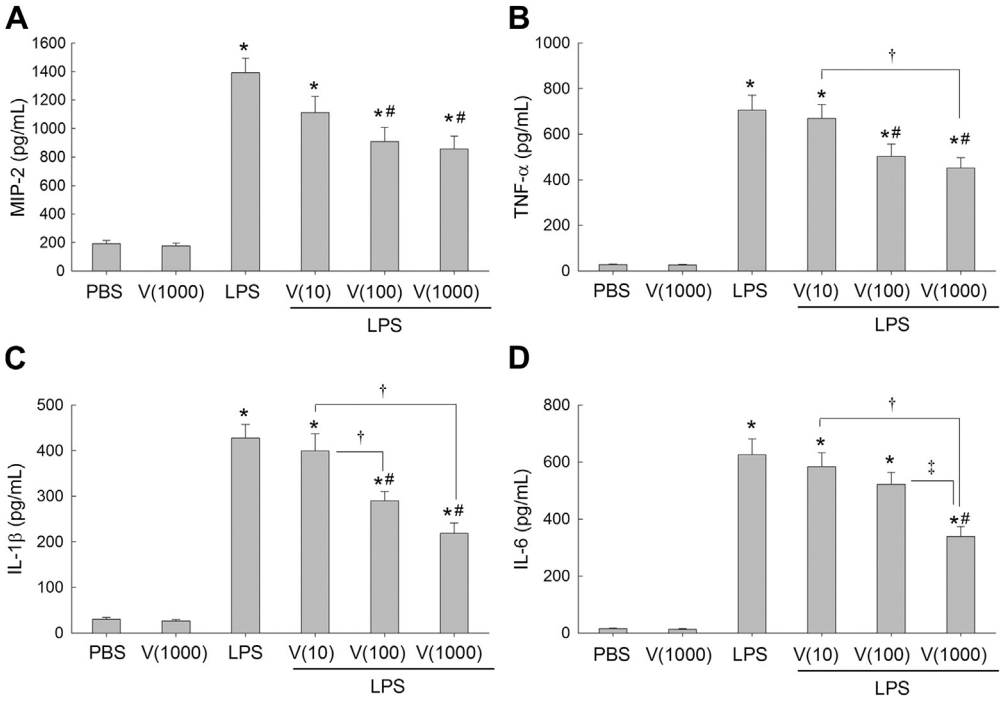

Fig. 1. The concentrations of (A) macrophage inflammatory protein-2 (MIP-2), (B) tumor necrosis factor-α (TNF-α), (C) interleukin (IL)-1β, and (D) IL-6 in murine macrophage RAW264.7 cells. The data are derived from 6 culture dishes from each group and expressed as the mean ± standard error. LPS = the lipopolysaccharide (100 ng/mL) group; LPS-V (10) = the LPS plus vasopressin (10 pg/mL) group; LPS-V(100) = the LPS plus vasopressin (100 pg/mL) group; LPS-V(1000) = the LPS plus vasopressin (1000 pg/mL) group; PBS = the phosphate-buffered saline group; V(1000) = the vasopressin (1000 pg/mL) group. * $p < 0.05$, vs. the PBS group. # $p < 0.05$, vs. the LPS group. † $p < 0.05$, the LPS-V(100) or LPS-V(1000) group vs. the LPS-V(10) group. ‡ $p < 0.05$ the LPS-V(1000) group vs. the LPS-V(100) group.

different between the LPS-V(100) and LPS-V(10) groups. The concentrations of NO and COX-2 in the LPS-V(1000) and LPS-V(10) groups were not significantly different. However, the concentrations of iNOS and PGE_2 were significantly lower in the LPS-V(1000) group than in the LPS-V(10) group ($p < 0.001$ and $p = 0.045$, respectively). The iNOS concentration was significantly lower in the LPS-V(1000) group than in the LPS-V(100) group ($p = 0.009$). By contrast, the concentrations of NO, PGE_2, and COX-2 were not

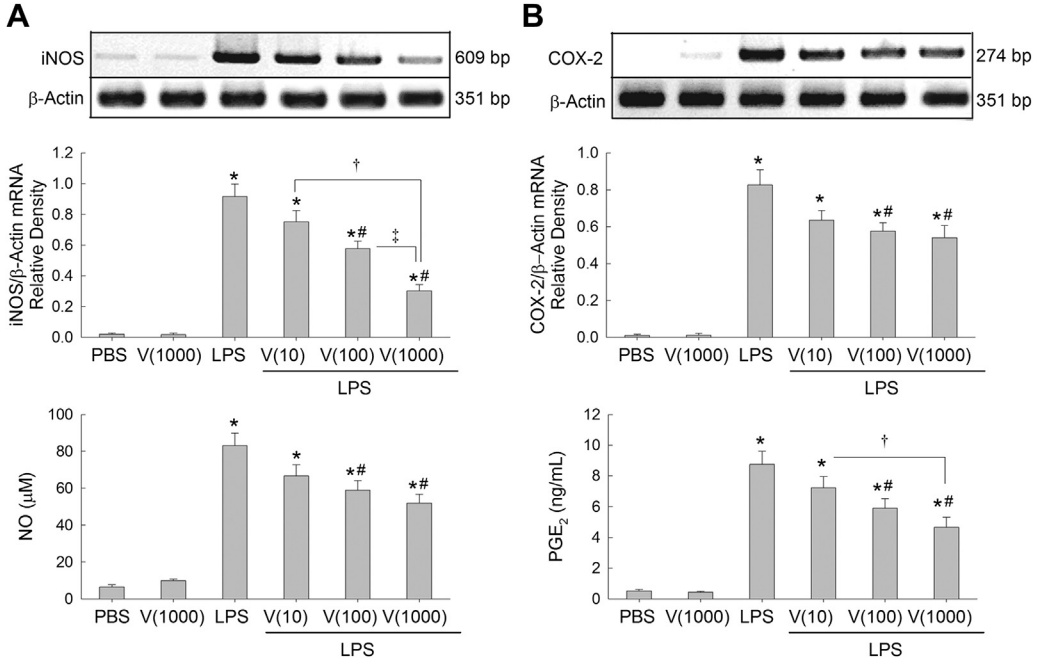

Fig. 2. The concentrations of (A) nitric oxide (NO) and (B) prostaglandin E_2 (PGE_2), and representative gel photography and densitometric analysis of inducible NO synthase (iNOS) and cycloosygenase-2 (COX-2) mRNA in murine macrophage RAW264.7 cells. The expression of iNOS and COX-2 mRNA were analyzed by using reverse transcription and polymerase chain reaction (RT-PCR). The data are derived from six culture dishes from each group and expressed as mean ± standard deviation. LPS = the lipopolysaccharide (100 ng/mL) group; LPS-V (10) = the LPS plus vasopressin (10 pg/mL) group; LPS-V(100) = the LPS plus vasopressin (100 pg/mL) group; LPS-V(1000) = the LPS plus vasopressin (1000 pg/mL) group; PBS = the phosphate-buffered saline group; V(1000) = the vasopressin (1000 pg/mL) group. *$p < 0.05$, vs. the PBS group. #$p < 0.05$, vs. the LPS group. †$p < 0.05$, the LPS-V(100) or LPS-V(1000) group vs. the LPS-V(10) group. ‡$p < 0.05$ the LPS-V(1000) group vs. the LPS-V(100) group.

significantly different between the LPS-V(1000) and LPS-V(100) groups.

4. Discussion

Data from the current study, in concert with data from previous studies [1–4], confirmed that endotoxins can induce significant upregulation of inflammatory mediators. Our data revealed that the concentrations of chemokines, cytokines (e.g., TNF-α, IL-1β, and IL-6), NO/iNOS mRNA, and PGE_2/COX-2 mRNA were significantly higher in murine macrophages that were treated with endotoxin than in macrophages treated with PBS. Data from this study also demonstrated that vasopressin at a dosage of 10 pg/mL posed no significant effect on modulating the endotoxin-induced upregulation of inflammatory mediators: our data revealed that the concentrations of inflammatory mediators in macrophages treated with endotoxin plus 10 pg/mL of vasopressin and the concentrations of inflammatory mediators in macrophages treated with endotoxin alone were comparable. By contrast, our data demonstrated that vasopressin at dosages of 100 pg/mL and 1000 pg/mL could significantly inhibit the endotoxin-induced upregulation of inflammatory mediators: the concentrations of inflammatory mediators were significantly lower in macrophages treated with endotoxin plus 100 pg/mL or endotoxin plus 1000 pg/mL of vasopressin than in macrophages treated with endotoxin alone. These data confirmed our hypothesis, and provide direct evidence that vasopressin in a dose-dependent manner can inhibit the endotoxin-induced upregulation of inflammatory mediators in activated murine macrophages.

Our data confirmed the potent anti-inflammatory effects of vasopressin. However, the underlying mechanisms remain unstudied.

The expression of inflammatory mediators is tightly regulated by the upstream transcriptional factors nuclear factor-κB (NF-κB) and mitogen-activated protein kinases (MAPKs) [23,24]. The cellular recognition of endotoxin—including the binding of endotoxin to inflammatory cells and subsequent toll-like receptor 4 (TLR-4) activation—is essential in mediating the endotoxin-induced activation of NF-κB and MAPKs [25–27]. Judging from the crucial roles of the aforementioned signaling pathways in regulating the expression of inflammatory mediators, we speculate that vasopressin may act through inhibiting endotoxin binding and thereby inhibiting the subsequent activation of TLR-4, NF-κB, and/or MAPKs. In this way, vasopressin inhibits the endotoxin-induced upregulation of inflammatory mediators. Previous data that NF-κB activation in septic mice can be mitigated by vasopressin [28] seems to support our speculation. More studies are needed before further conclusions can be reached.

As mentioned previously, vasopressin has several important physiological functions, including vasoconstriction and antidiuresis [8,9]. To exert vasoconstriction, vasopressin needs to interact with V1 receptors located on vascular smooth muscle cells [29]. To exert its antidiuretic effect, vasopressin needs to interact with the V2 receptors located on the cells of the distal convoluted tubules and collecting ducts [29]. Previous data nevertheless has indicated that sepsis can downregulate the V1 receptors [30]. Previous data also reveals that the effects of vasopressin on mitigating sepsis-induced pulmonary inflammation could be blocked by antagonizing the V2 receptors [18]. These data indicate the involvement of the V1 and V2 receptors in sepsis. Judging from these data, we further speculate that the anti-inflammatory effects of vasopressin observed in this study may very likely involve the V1 and/or V2 receptors.

In summary, our data confirmed that vasopressin in a dose-dependent manner inhibits the endotoxin-induced upregulation of inflammatory mediators in activated murine macrophages.

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