Atrial Natriuretic Peptide Alleviates Cardiovascular and Metabolic Disorders in a Rat Endotoxemia Model: A Possible Role for Its Anti-inflammatory Properties

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Abstract

Background: Atrial natriuretic peptide (ANP) plays important roles in the regulation of cardiovascular and renal homeostasis. Furthermore, several studies have shown that ANP may have anti-inflammatory activities. We hypothesized that ANP may alleviate cardiovascular and/or metabolic disorders in rats with lipopolysaccharide (LPS)-induced endotoxemia.

Methods: In rats anesthetized with pentobarbital, LPS was injected and ANP was continuously infused at 0.15 μg/kg/min. Mean arterial pressure and pulse rate were monitored hourly, and arterial blood gases were analyzed before LPS injection and at 1, 4, and 6 hours after LPS injection. The expression in the rat left ventricle of mRNAs encoding nitric oxide synthase 2 and 3 (iNOS, eNOS), heme oxygenase 1 and 2 (HO-1, 2), tumor necrosis factor α (TNFα), and interleukin (IL)-1β was measured with the real-time reverse transcriptase-polymerase chain reaction.

Results: LPS increased the expression of TNFα, IL-1β, iNOS, and HO-1, which was inhibited by infusion of ANP. Furthermore, the LPS-induced decrease in mean arterial pressure was attenuated, and the acid-base imbalance caused by increased lactate production was improved 6 hours after the administration of ANP.

Conclusions: Our results suggest that continuous infusion of ANP counteracts the cardiovascular and metabolic disorders associated with endotoxemia, possibly via anti-inflammatory mechanisms.

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Key words: atrial natriuretic peptide, lipopolysaccharide, tumor necrosis factor-α, nitric oxide synthase 2, real-time reverse transcriptase-polymerase chain reaction

Introduction

Endotoxemia is a detrimental consequence of septic shock. Endotoxic or septic shock, and subsequent multiple organ failure, remains the main cause of morbidity and mortality in intensive care units. Endotoxic and septic shock can adversely affect cardiovascular function, by impairing...
myocardial contractility and decreasing vascular tone. A number of cytokines and mediators have been shown to be associated with impaired cardiac function in endotoxic and septic shock. Cytokines may play a key role in the decrease in heart contractility. In particular, tumor necrosis factor-α (TNFα) seems to be one of the main factors responsible for myocardial depression during endotoxemia. TNFα is an inflammatory cytokine that can induce cellular death via apoptosis and oncosis. In response to LPS, cardiomyocytes produce TNFα and provide an important local source of TNFα in the heart. The hemodynamic effects of TNFα include decreased myocardial contractile efficiency, reduced ejection fraction, hypotension, decreased systemic vascular resistance, and biventricular dilatation. The pathophysiological responses to interleukin (IL)-1β are similar to those to TNFα. It has also been reported that the combination of TNFα and IL-1β causes myocardial depression at concentrations 1/50th to 1/100th of the concentration of either cytokine alone.

Gaseous mediators, namely nitric oxide (NO) and carbon monoxide (CO), and their major endogenous regulators, such as nitric oxide synthase (NOS) and heme oxygenase (HO), play a pivotal role in endotoxemia. NO is an extensively studied molecule with vasorelaxation and oxidative stressor properties during endotoxic or septic shock. NO is produced by 3 distinct NOS enzymes: 2 are constitutively expressed in endothelial cells (eNOS) and in neuronal cells (nNOS), and 1 isozyme (iNOS) is inducible in a variety of cells (e.g., macrophages, hepatocytes, vascular smooth muscle cells, and cardiomyocytes) by stimuli that include lipopolysaccharide (LPS). iNOS is responsible for producing most of the NO that causes hypotension and oxidative stress during endotoxic shock, whereas eNOS has a minor role in the pathophysiology of endotoxemia.

CO is a potentially life-threatening gas. Recently, however, CO has been shown to have anti-inflammatory and vasorelaxation properties and to be essential for life. HO is the rate-limiting enzyme in the catabolism of heme, a process that promotes the formation of equimolar amounts of the bile pigment biliverdin, free iron, and CO2. Together with HO, which has anti-inflammatory and anti-oxidative functions, the CO/HO system is recognized to have vasodilatory and cytoprotection properties. HO has 2 distinct isozymes. HO-1 is highly inducible by heme and other nonheme substances, including LPS. In contrast, HO-2 is expressed in a constitutive manner and acts as a heme-binding molecule in normal cells.

The cardiovascular hormone atrial natriuretic peptide (ANP) is secreted by the cardiac atria in response to an increased plasma volume. ANP binds to the guanylate cyclase-coupled natriuretic peptide receptor (NPR)-A and NPR-C, which lacks guanylate cyclase function. ANP exerts a hypotensive effect through its natriuretic, diuretic, and vasodilating actions and thus mediates cardiovascular and renal homeostasis. Indeed, carperitide (alpha-human ANP) has been approved for the treatment of acute heart failure in Japan. Interestingly, ANP has been reported to possess additional functions beyond blood pressure regulation. For example, ANP is also expressed in various immune organs and can influence immune cells by attenuating their inflammatory response. Indeed, studies performed in vitro have revealed anti-inflammatory activities of ANP. ANP can enhance phagocytic activity and the production of reactive oxygen species of phagocytes. ANP also reduces the production of proinflammatory mediators by inhibiting iNOS and cyclooxygenase-2 (COX-2) and decreasing TNFα synthesis. ANP has also been reported to counteract TNFα-induced endothelial permeability and the adhesion and attraction of inflammatory cells.

Therefore, the aim of the present study was to determine whether continuous infusion of ANP affects the gene expression of proinflammatory cytokines (TNFα and IL-1β) and the major endogenous enzymes regulating the gaseous mediators (NOS and HO) in the LPS-induced endotoxic rat left ventricle and to determine whether ANP reverses cardiovascular disorders induced by LPS.
Materials and Methods

Animal Preparation
This study was approved by the Animal Experimental Ethical Review Committee of Nippon Medical School. Eight- or 9-week-old male Wistar rats (Saitama Experimental Animals, Inc., Saitama; body weight, 270–330 g) were anesthetized with pentobarbital sodium (65 mg/kg i.p., followed by 25 mg/kg/h i.v.). A tracheotomy was performed with a 14-G Teflon catheter to facilitate spontaneous respiration. Rectal temperature was maintained at 37°C using a heat lamp. The tail vein was then cannulated with a 24-G Teflon catheter for intravenous infusion of drugs, and the left femoral artery was cannulated with a 24-G Teflon catheter and connected to a pressure transducer (TP-300T; Nihon Kohden Corp., Tokyo) to measure mean arterial pressure (MAP) and pulse rate (PR). Blood samples were obtained through the femoral artery.

Preliminary Dose-finding Study
To evaluate the hypotensive effect of ANP, we performed a dose-finding study and administered various doses of ANP to rats with LPS-induced endotoxemia. The rats were given LPS derived from Escherichia coli 055: B5 (10 mg/kg i.v. dissolved in 0.4 mL of saline 5 minutes before injection) as a slow bolus injection over 5 minutes. The rats were then randomized into 3 groups (4 rats per group) and received infusions of ANP (carperitide; Daiichi Sankyo, Tokyo) at a rate of 1 mL/hr at doses of 0.15, 0.5, or 1.5 µg/kg/min. The infusion rate was controlled with an electronic pump (STC-525; Terumo Corp., Tokyo). MAP and PR were recorded hourly.

Experimental Procedure
Rats were assigned to 1 of 4 groups (9 rats per group) and treated with saline alone (control group), LPS alone, LPS plus ANP, or ANP alone. The control group received a single dose of saline (0.4 mL), which was followed by infusion of saline at 1 mL/hr. The LPS group received 10 mg/kg LPS in 0.4 mL saline followed by saline infusion as before. The LPS/ANP group received LPS followed by infusion of ANP in saline at a rate of 0.15 µg/kg/min (1 mL/hr). The ANP group received saline as in the control group, followed by ANP infusion as in the LPS/ANP group. As in the dose-finding study, the rate of infusion was controlled with an electronic pump. MAP and PR were recorded hourly. At 6 hours after LPS or saline injection, the rats were killed and the left ventricle of the heart was removed after a brief in situ perfusion with normal saline. The tissues were then immediately frozen in liquid nitrogen. We measured the expression of mRNAs encoding iNOS, eNOS, HO-1, HO-2, TNFα, and IL-1β using real-time quantitative polymerase chain reaction (PCR) analysis.

Real-time Reverse Transcriptase-PCR
Total RNA was extracted from the left ventricles using the Trizol method followed by isogen-chloroform extraction and isopropanol precipitation2. Before reverse transcription, any residual genomic DNA was removed with DNase I (Takara Shuzo, Otsu). A spectrophotometer was used to determine the amount of isolated mRNA.

The mRNA was then reverse-transcribed with a High Capacity cDNA Reverse Transcription kit (Applied Biosystems Japan, Tokyo) and PCR Express (Thermo Fisher Scientific, Waltham, MA, USA). Reverse transcription was performed in a final volume of 100 µL comprising 10 µg of eluted mRNA, 10 µL of 10× reverse transcription buffer, 4 µL of deoxyribonucleoside triphosphate, 10 µL of 10× random primers, 5 µL of MultiScribe Reverse Transcriptase, 5 µL of RNase inhibitor, and 16 µL of nuclease-free H2O.

The resulting solution was incubated in a thermal cycler at 25°C for 25 minutes, followed by 37°C for 120 minutes and 85°C for 5 seconds. The cDNA obtained was aliquoted and stored at −20°C until PCR reactions were performed.

We used Taqman® Gene Expression Assays (Applied Biosystems) for PCR primers using the primers summarized in Table 1.

One microliter of cDNA was used for quantitative PCR in 20 µL reaction volumes containing 10 µL of Taqman® Universal Master Mix (Applied
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Table 1 Primers used for reverse transcriptase—PCR (Applied Biosystems Japan)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Code</th>
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<tbody>
<tr>
<td>Nitric oxide synthase 2</td>
<td>Rn00561646_ml</td>
</tr>
<tr>
<td>Nitric oxide synthase 3</td>
<td>Rn02132634_ml</td>
</tr>
<tr>
<td>Heme oxygenase 1</td>
<td>Rn00561387_ml</td>
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<tr>
<td>Heme oxygenase 2</td>
<td>Rn00577761_ml</td>
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<tr>
<td>Tumor necrosis factor-α</td>
<td>Rn99999017_ml</td>
</tr>
<tr>
<td>Interleukin-β</td>
<td>Rn00580432_ml</td>
</tr>
<tr>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>Rn99999916_ml</td>
</tr>
</tbody>
</table>

Biosystems). 1 μL of each primer, and 8 μL of deionized water. The PCR conditions were as follows: 50°C for 2 minutes and 95°C for 10 minutes, followed by 40 cycles of amplification for 15 seconds at 95°C and 1 minute at 60°C. The Taqman® probe labeled with 6-carboxyfluorescein was cleaved during amplification, generating a fluorescent signal. Experimental samples and calibration curve samples were run in triplicate. Unknown values were automatically interpolated from the standard curve. We also used primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (TaqMan® Gene Expression Assays; Applied Biosystems) to correct for total nucleic acid content. The assay used an instrument capable of measuring fluorescence in real time (ABI PRISM 5700 Sequence Detector; Applied Biosystems). The results of the real-time PCR data are represented as cycle threshold (Ct) values, where Ct represents a unitless value defined as the fractional cycle number at which the sample fluorescence signal passes a fixed threshold above baseline. Values that were markedly different value for the triplicate samples, which was considered due to inaccurate operation, were omitted. Relative amounts of all mRNAs were calculated with the comparative Ct method (Applied Biosystems, Foster City, CA, USA)16.

Statistical Analysis

All values are expressed as mean ± standard deviation (SD). Non-repeated-measures analysis of variance (ANOVA) and Bonferroni’s multiple comparison tests were performed to determine differences in MAP, PR, and blood gas data between the groups. All gene expression data were analyzed with the Kruskal-Wallis H-test, followed by the Mann-Whitney U-test with Bonferroni correction when significant differences were obtained. The significance level was set at p<0.05.

Results

Figure 1 shows the changes in MAP and PR in the preliminary dose-finding study. Continuous infusion of ANP decreased MAP in a dose-dependent manner. In all groups, the maximum change in MAP occurred within 1 hour of ANP infusion. In the group given ANP at 15 μg/kg/min, MAP decreased rapidly, and none of the rats survived for 1 hour (data not shown). In the group given ANP at 0.5 μg/kg/min, the mortality rate during the 6-hour recording period was 75%. Therefore, on the basis of the results of this preliminary study, we infused ANP at 0.15 μg/kg/min in the following study.

Table 2 shows the blood gas levels in each group before (0 hours) and at 1, 4, and 6 hours after LPS or saline injection. Compared with the values in the control group, after 6 hours the pH, HCO₃⁻, and BE in the LPS group decreased significantly (p<0.05), and the Lac increased (p<0.05). These changes in
Lac, BE, and HCO₃⁻ were attenuated in the LPS/ANP group at 6 hours.

**Figure 2** shows the changes in MAP and PR for up to 6 hours after LPS or saline injection. In the LPS/ANP group, the LPS-induced decrease in MAP was attenuated at 6 hours, indicating that ANP may alleviate metabolic acidosis during endotoxemia by maintaining blood pressure.

There were no significant differences between the ANP and control group in any of the variables, indicating that ANP does not affect blood gas analyses or blood pressure in normal conditions.

The expression of TNFα, IL-1β, iNOS, and HO-1 were greater in the LPS group than in the control group (**Fig. 3**). However, these effects of LPS were inhibited in the LPS/ANP group, indicating that ANP may directly inhibit the expression of cytokines and gaseous mediators in the endotoxic rat left ventricle. The expression of eNOS was lower in the LPS and LPS/ANP groups than in the control group but did not differ significantly between the LPS and LPS/ANP groups. There were no significant differences in HO-2 expression between any groups.

**Discussion**

In the present study, we have shown that continuous infusion of ANP alleviates the decrease in blood pressure and metabolic acidosis in a rat
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Table 2  pH, arterial oxygen tension (PaO₂), arterial carbon dioxide tension (PaCO₂), bicarbonate ion concentration ([HCO₃⁻]), lactate concentrations (Lac) and base excess value (BE) before (0 hour) and 1, 4, and 6 hours after the injection of LPS (or saline)

<table>
<thead>
<tr>
<th></th>
<th>pH</th>
<th>PaO₂ (mmHg)</th>
<th>PaCO₂ (mmHg)</th>
<th>HCO₃⁻ (mmol/L)</th>
<th>Lac (mmol/L)</th>
<th>BE (mmol/L)</th>
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<tbody>
<tr>
<td></td>
<td>0 hour</td>
<td>1 hour</td>
<td>4 hours</td>
<td>6 hours</td>
<td>0 hour</td>
<td>1 hour</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CON</td>
<td>7.33 ± 0.06</td>
<td>7.40 ± 0.05</td>
<td>7.45 ± 0.05</td>
<td>7.46 ± 0.04</td>
<td>65.9 ± 10.23</td>
<td>75.0 ± 7.06</td>
</tr>
<tr>
<td>LPS</td>
<td>7.43 ± 0.05</td>
<td>7.41 ± 0.02</td>
<td>7.46 ± 0.03</td>
<td>7.33 ± 0.11*</td>
<td>75.2 ± 7.96</td>
<td>77.8 ± 11.57</td>
</tr>
<tr>
<td>LPS/ANP</td>
<td>7.36 ± 0.05</td>
<td>7.40 ± 0.04</td>
<td>7.48 ± 0.03</td>
<td>7.45 ± 0.08</td>
<td>72.5 ± 12.42</td>
<td>77.5 ± 12.16</td>
</tr>
<tr>
<td>ANP</td>
<td>7.38 ± 0.07</td>
<td>7.42 ± 0.06</td>
<td>7.45 ± 0.04</td>
<td>7.44 ± 0.07</td>
<td>69.9 ± 10.09</td>
<td>74.8 ± 10.14</td>
</tr>
</tbody>
</table>

Values are means ± SD for 9 rats per group. * p<0.05 vs. the control group; †p<0.05 vs. the LPS group; CON=control group; LPS=lipopolysaccharide group; ANP=carperitide group; LPS/ANP=carperitide and lipopolysaccharide group.

endotoxemia model. Nevertheless, it is important to determine whether continuous infusion of ANP in a rat endotoxemia model is associated with any adverse effects. Cardiovascular changes in endotoxic shock or septic shock included impaired myocardial contractility and a decrease in vascular tone, resulting in hypotension and hypoperfusion. In our preliminary study, the mortality of rats treated with
MAP

![Graph showing changes in mean arterial pressure (MAP) and pulse rate (PR) over time](image)

Fig. 2 Changes in mean arterial pressure (MAP) and pulse rate (PR) after injection of LPS or saline. Results are means ± SD for 9 rats per group. *p<0.05 vs. the control group; †p<0.05 vs. the control group; CON=control group; LPS=lipopolysaccharide group; ANP=carperitide group; LPS/ANP=carperitide and lipopolysaccharide group.

0.5 or 1.5 μg/kg/min ANP after LPS was higher than that of rats treated with LPS alone. This result suggests that the vasodilatory effect of high doses of ANP may aggravate the effects of endotoxemia. This possibility is consistent with the previously reported effects of ANP in acute heart failure therapy, and the effect of ANP on blood pressure may be a limiting factor. Indeed, hypotension was the most frequently reported adverse event during treatment and usually occurred within 3 hours of the start of ANP infusion, although hypotension was overcome by dose reductions or discontinuation of ANP infusion. Clearly, it is necessary to titrate the dose of ANP according to changes in blood pressure, but appropriate doses of ANP can be effective in endotoxemia.

The main finding of the present study is that the expression of TNFα, IL-1β, iNOS, and HO-1 in the left ventricles of rats with LPS-induced endotoxemia is decreased in the rats treated with both LPS and ANP. The cardiovascular changes in endotoxic shock or septic shock or both have usually been attributed to the activity of various mediators in the inflammatory cascade, including cytokines (particularly TNFα and IL-1β) and secondary mediators, such as NO, via iNOS. High levels of TNFα and NO produced by activated macrophages may injure the host cells and contribute to the pathogenesis of several inflammatory diseases, such as endotoxic and septic shock. Previous in vitro studies have shown that ANP inhibits the production of TNFα and iNOS in macrophages.
The ability of ANP to inhibit the induction of TNFα and iNOS supports the anti-inflammatory activities of ANP. Furthermore, Ladetzk-Baehs et al. have reported that the administration of ANP decreases TNFα expression in the spleen and liver, prevents increases in the serum TNFα concentration in mice with LPS-induced endotoxemia, and ultimately improves the survival rate of these mice. Together with the previous findings, our present results suggest that the administration of ANP protects the left ventricle during endotoxemia by inhibiting the expression of TNFα, IL-1β, and iNOS.

HO-1 appears to play vital roles in various biological processes, including inflammation, oxidative stress, cell survival, and cell proliferation. Surprisingly, we found that the expression of HO-1 was inhibited in rats treated with both LPS and ANP. Previous studies have shown that ANP induces the endothelial expression of HO-1 at the transcriptional level via cyclic guanosine monophosphate (cGMP) and mitogen-activated protein kinase (MAPK) pathways. Additionally, the expression of HO-1 is upregulated in a variety of cell types and is involved in the resolution of inflammation, which has been reported in both in vitro and in vivo studies. Therefore, the expression of HO-1 and its regulation by ANP may be an important factor in the anti-inflammatory effects of ANP.

In summary, the results of this study suggest that ANP can protect the left ventricle during endotoxemia by inhibiting the expression of TNFα, IL-1β, and iNOS. Furthermore, the upregulation of HO-1 may contribute to the anti-inflammatory effects of ANP. These findings provide new insights into the mechanisms of ANP's protective effects and may lead to the development of new therapeutic strategies for various inflammatory conditions.
monophosphate. However, whether the induction of HO-1 within a physiological range, as induced by ANP, is cytoprotective remains controversial. In previous studies LPS-induced HO-1 expression was significantly decreased after the administration of iNOS inhibitors, indicating that HO-1 expression is mediated, at least in part, by a NO/NOS-dependent mechanism. If the relationship between HO-1 and NO/NOS is functional, our results suggest that the expression of HO-1 is inhibited by a NO/NOS-dependent mechanism, which is decreased by ANP. Of course, ANP itself might inhibit HO-1 expression. Further studies are needed to investigate whether this relationship is functional in the ANP infusion model.

The expression eNOS is moderate, but eNOS is essential for life. Its expression is increased by stimuli such as shear stress and hydrogen peroxide and is decreased by LPS and TNFα. However, the magnitude of these responses differs between organs and cells and according to the time-point of LPS-induced endotoxemia. The expression eNOS typically increases within 1 hour after stimulation with LPS but gradually decreases thereafter, and the pivotal role then shifts to iNOS. Of note, LPS has been reported to decrease the expression of eNOS in the lung, aorta, and heart and to increase its expression in the liver. In the present study, the expression of eNOS in the left ventricle was decreased 6 hours after LPS injection and was similar in rats treated with LPS and in rats treated with LPS and ANP. Therefore, the results of our study were consistent with those of the previous studies showing time- and organ-specific changes in eNOS expression in a rat endotoxemia model. However, the expression of eNOS should be further investigated to better understand the effect of ANP during the early and later stages.

Traditional therapies for endotoxemia and septic shock include eradication of infection with antibiotics, removal of the infective focus through surgical procedures, and intensive care to manage cardiovascular abnormalities and organ failure. Several researchers have used anti-TNFα agents, cyclooxygenase inhibitors, and NOS inhibitors, for example, in an attempt to modulate myocardial dysfunction in endotoxemia and septic shock as an adjunct to traditional therapy. Unfortunately, most of these strategies have failed to increase survival rates and, in some cases, have increased mortality rates. A previous study has revealed that a multimodal therapeutic approach targeting different pathophysiologic pathways of endotoxic and septic shock may have additive or synergistic effects. We speculate that comprehensive inhibition of TNFα, IL-1β, and iNOS expression constitutes a potential therapeutic use of ANP for endotoxic shock.

The present study had several limitations. First, we measured gene expression but not protein expression. Quantitative reverse transcriptase-PCR data represent only a snapshot of the quantity of a given transcript in a tissue. Furthermore, the expression of genes for proinflammatory cytokines and mediators may not necessarily induce inflammatory actions. Further protein expression studies are needed to better understand the potential use of ANP as an anti-inflammatory drug. Second, we only assessed gene expression in the left ventricle. An important manifestation of cardiovascular dysfunction is myocardial depression; however, the systemic inflammatory response to infection ultimately leads to organ dysfunction, including myocardial dysfunction in endotoxic and septic shock. Therefore, further studies are needed to elucidate the dynamic state by measuring gene expression in several organs.

In summary, this study showed that ANP attenuates the expression of TNFα, IL-1β, and iNOS in the left ventricle of rats with LPS-induced endotoxemia and improved metabolic acidosis. We suggest that ANP, a cardiovascular hormone, has anti-inflammatory properties and warrants further investigation from a therapeutic perspective in endotoxic and septic shock.

References

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