Effects of Bai-Hu decoction on fever induced by lipopolysaccharide

Li-Long Jia, Ran Li, Ji Ma, Ying Fan, Hai-Bo Li

School of Graduate, Liaoning University of Traditional Chinese Medicine, Shenyang, China
School of Basic Medical Sciences, Liaoning University of Traditional Chinese Medicine, Shenyang, China

Abstract
This study was designed to investigate the antifebrile effect and mechanisms of Bai-Hu decoction (BHD), a traditional Chinese medical (TCM) prescription. The rabbits used in this study received an intravenous injection of lipopolysaccharide (LPS) after being orally administered with BHD, ibuprofen, or saline, and their rectal temperatures were monitored by a copper–constantan thermocouple. Concentrations of interleukin-1 beta (IL-1β) and tumor necrosis factor alpha (TNF-α) in serum and hypothalamus were assayed using the commercially available rabbit IL-1β and TNF-α enzyme-linked immunosorbent assay kits following the manufacturer’s instructions. The BHD treatment group exhibited a significant fall in body temperature in both peaks compared with the LPS group (p < 0.05). BHD reduced the concentrations of IL-1β and TNF-α in serum, and of TNF-α in hypothalamus to control the febrile responses at 1 hour. Besides the levels of IL-1β in hypothalamus and serum, the concentration of TNF-α in hypothalamus was decreased remarkably in the BHD group than in the LPS group at 3 hours. The main findings, the partial mechanisms of BHD in reducing biphasic fever elicited by LPS, were that treatments with the crude extract of BHD could remarkably reduce the increased concentrations of IL-1β and TNF-α, not only in serum but also in hypothalamus. The results indicated that BHD would be a valuable candidate for further investigation as a traditional antifebrile and anti-inflammatory natural drug.

KEYWORDS
Bai-Hu decoction; Interleukin-1 beta; Lipopolysaccharide; Traditional Chinese medicine; Tumor necrosis factor alpha

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* Corresponding author. School of Basic Medical Sciences, Liaoning University of Traditional Chinese Medicine, 79 Chongshan East Road, Shenyang 110032, China.
E-mail address: lnihb@hotmail.com (H.-B. Li).

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Introduction

Since ancient times, fever has been recognized as a sign of illness and is still widely used as an indication of infection. Pyrogens can be classified into two categories: exogenous and endogenous. It is well known that an exogenous pyrogen elicits a febrile response by stimulating the production of endogenous pyrogen (EP). Some exogenous pyrogens such as bacterial endotoxin are believed to act directly on the putative hypothalamic thermoregulatory center, in addition to stimulating EP production. Some investigations conducted on the pathogenesis of fever have focused primarily on the characterization of EP. So far, cytokines, such as interleukin-1 (IL-1) and tumor necrosis factor (TNF), are the most likely candidates for EP.

Bai-Hu decoction (BHD) is a famous antifebrile Chinese herbal formula that consists of four herbal drugs: gypsum (mineral, calcium sulfate, CaSO₄·2H₂O), Anemarrhena asphodeloides Bunge (common Anemarrhena Rhizome), Radix Glycyrrhizae (Glycyrrhiza), and rice; it was described in Shang-Han-Lun, a classical piece of traditional Chinese medical (TCM) literature of the Han dynasty. The prescription has been clinically used in treating diseases with fever, such as influenza, epidemic hemorrhagic fever, epidemic encephalitis B, rheumatic fever, diabetes mellitus [3], and so on. However, the underlying mechanism of BHD is still unclear. In this study, we made a lipopolysaccharide (LPS)-induced fever model in rabbits to detect the concentrations of IL-1β and TNF-α after injecting them with LPS only and after they were administered with BHD or ibuprofen (IBF) separately. Through this research, we want to elucidate the mechanism of BHD in inhibiting a febrile response.

Materials and methods

Animals

The animals used in this study were male New Zealand white rabbits weighing 2.0–3.0 kg. They were housed in a room maintained at 21 ± 1°C with a 12-hour light–dark cycle. Food and water were available ad libitum. All the animals used in this experiment were cared for according to the ethical regulations on animal research of our university.

Reagents and preparation of herbal extract

LPS from Escherichia coli O127:B8 was purchased from Sigma Aldrich (Shanghai, China Mainland). Rabbit IL-1β and TNF-α enzyme-linked immunosorbent assay (ELISA) kits were purchased from Shanghai Lang-dun Biologic Technology Company. BHD is composed of gypsum (250 g), A. asphodeloides Bunge (94 g), Radix Glycyrrhizae (31 g), and rice (100 g), according to the ancient records. The first three herbs were purchased from the Tong Ren Tang Medicinal Materials Company of Shenyang. The herbs were immersed in distilled water (2000 mL) for at least 30 minutes, and boiled for 30 minutes until the volume of the material became 150 mL. The extract obtained was filtered and then stored at −20°C until use. IBF was made in the third plant of the Harbin Pharmaceutical Industry (Lot Number 080135, Harbin, China). It was solubilized in normal saline, and the final concentration was 0.01 g/mL. To determine a suitable dosage of BHD, the conversion ratio of dosage between people and animals was performed as described elsewhere [4]. BHD 22 g/kg (equals to 7 mL/kg) and IBF 0.01 g/kg were used as suitable dosages for the rabbits.

Measurement of changes in body temperature

Briefly, rectal temperatures of the rabbits were monitored by a copper–constantan thermocouple for 6 hours after an intravenous injection of LPS, and the rectal temperature change (ΔT) was calculated by subtracting the temperature before the injection from the temperature at each time point. Before the formal experiment, body temperatures of the rabbits were monitored for 2 days, and the basic temperatures were noted.

On the day of conducting the body-temperature experiment, the animals were minimally restrained in conventional rabbit stocks, at an ambient temperature of 21 ± 1°C between 09:00 and 16:00 hours. Throughout the experiment, the rectal temperature was measured every 20 minutes with a copper–constantan thermocouple. The rectal temperature in each animal was allowed to stabilize for at least 90 minutes before any injections were administered.

Drug treatment

All animals (n = 72) were randomly divided into three parts during the experiment, and the rabbits (n = 24) of each experiment were separated into four groups: control (n = 6, orally administered with 0.9% saline solution only); LPS (n = 6), received an intravenous injection of LPS only, 200 ng/kg; BHD + LPS (n = 6), received an intravenous injection of LPS 30 minutes after being orally administered with BHD 7 mL/kg); and IBF + LPS (n = 6), received an intravenous injection of LPS 30 minutes after being orally administered with IBF 1 mL/kg). The rectal temperature in each animal was allowed to stabilize for at least 90 minutes before any injections were given. Intravenous injections were made into the marginal ear vein. In the first series of experiments, we observed the body temperature for 6 hours only to prove the antifebrile effect of BHD. In the second and third series of experiments, we observed the body temperature for 1 and 3 hours, respectively.

Preparation of blood sample and hypothalamus tissue

Blood samples were collected into tubes from the carotid artery of rabbits and centrifuged (2500 r/min) at 4°C for 10 minutes after being deposited for 2 hours. Then, blood serum was stored at −80°C for IL-1β and TNF-α activity assays. After sampling the blood, the rabbits were killed by venous air embolism, and their brains were removed immediately and placed on ice. The hypothalamus was dissected between the anterior margin of the optic chiasma and that of the mamillary bodies, and the lateral sulci, to a depth of 3 mm. The hypothalamus tissue homogenate was prepared by mixing 1 mL homogenate solution (pH 7.2–7.4, containing 1 mL 1% aprotinin per
100 mL 0.2% PBS) with 100 mg hypothalamus tissue. After deposition for 5 minutes, the supernatants were collected from the hypothalamus tissue homogenate by centrifugation (3000 r/min) at 4°C for 10 minutes. The supernatants were used to detect the concentrations of IL-1β and TNF-α following the manufacturer’s instructions.

**Enzyme-linked immunosorbent assay**

The concentrations of IL-1β and TNF-α in serum and hypothalamus were assayed using the commercially available rabbit IL-1β and TNF-α ELISA kits (R&D Systems, Inc., Minneapolis, MN, USA, Lot Number 0811252:F2019; Lot Number 0811252:F2041), following the manufacturer’s instructions. Plasma samples and working standards were added (100 μL per well) in duplicate and incubated at 37°C for 2 hours on a rotator. After washing the plates, biotinylated antibodies were added (100 μL per well), and the plates were incubated for 1 hour at 37°C on a rotator. The plates were incubated for 0.5 hour with streptavidin–horseradish peroxidase at a dilution of 1:20,000, and the antibodies were detected with 3,3',5,5'-tetramethylbenzidine dissolved in dimethyl sulfoxide to a concentration of 1% in a solution containing 0.1 M citric acid, 0.1 M sodium acetate (pH 6), and 0.016% H2O2 for 30 minutes. The reaction was stopped by the addition of 1.5 M H2SO4. The plates were read using wavelengths (450 nm) on a microplate reader (BIO-RAD iMark, made in Japan in 2010, serial no. 12843), and total cytokine concentrations were calculated using the standard curve prepared from recombinant cytokines. The lower limit of detection for the cytokines based on the standard curves ranged from 10 to 16 pg/mL.

The ELISA for rabbit IL-1β detects both precursor and mature IL-1β, but not TNF-α. Similarly, the ELISA for rabbit TNF-α does not cross-react with that for rabbit IL-1β. The protocol of the ELISA for rabbit IL-1β is similar to that for rabbit TNF-α. The sensitivities of the assays were as follows: IL-1β, 10 pg/mL, and TNF-α, 16 pg/mL. Given the high concentrations of cytokines at the site of inflammation compared with those in the circulation, some samples required dilutions.

**Statistical analysis**

All results were confirmed in at least three separate experiments. Data were expressed as mean ± standard deviation (SD). A one-way ANOVA followed by LSD-t test was used for comparing the differences between groups. These comparisons were made at a two-sided α level of 0.05. A p value of <0.05 was considered significant. Statistical analysis was performed using the SPSS software version 13.0.

**Results**

**Effects of BHD on body temperature during LPS-induced fever in rabbits**

In the first series of experiments, we injected LPS intravenously 30 minutes after an oral administration with BHD or IBF, and the changes in the body temperature of the rabbits were recorded, as given in Fig. 1. Fig. 1 depicts changes in the rectal temperature in the four different groups. An injection of LPS (200 ng/kg) induced a biphasic fever, in which the first peak occurred at 1 hour and the second peak at 3 hours after the injection. In contrast, the BHD treatment group exhibited a significant fall in body temperature in both peaks (p < 0.05), and treatment with IBF also attenuated both peaks of the fever (p < 0.05). There were no significant differences between the BHD and IBF treatment groups in the first peak (p > 0.05); however, there were significant differences between these groups in the second peak (p < 0.05). Control animals that had been administered only saline failed to exhibit any change in body temperature.

**Concentrations of IL-1β and TNF-α in both serum and hypothalamus 1 hour after an intravenous injection of LPS in the four different groups**

With an aim of discussing the mechanism of BHD in reducing the first peak of the body temperature in LPS-induced fever, the concentrations of IL-1β and TNF-α in both serum and hypothalamus were measured at 1 hour (Table 1); the results showed that all the levels of IL-1β and TNF-α exhibited significant differences between the control and LPS groups (p < 0.05). Both the BHD and IBF treatment groups exhibited a significant fall in the concentrations of IL-1β and TNF-α in serum compared with the groups treated with LPS only (p < 0.05) at 1 hour. There was no reduction in the concentration of IL-1β due to BHD in hypothalamus compared with the LPS group (p > 0.05); however, there was a reduction in the concentration of IL-1β due to IBF in hypothalamus compared with the LPS group (p < 0.05). On
the other hand, the concentration of TNF-α in hypothalamus of the BHD group exhibited a remarkable decrease compared with that of the LPS group (p < 0.05), whereas the IBF group failed to decrease the level of TNF-α in hypothalamus compared with the LPS group (p > 0.05).

### Concentrations of IL-1β and TNF-α in both serum and hypothalamus 3 hours after an intravenous injection of LPS in the four different groups

Blood samples and hypothalamic samples were collected at 3 hours after an injection of LPS, and the concentrations of IL-1β and TNF-α were assayed (Table 2). The concentrations of IL-1β in both serum and hypothalamus of the BHD and IBF groups were significantly decreased compared with those in the LPS group (p < 0.05). There was no significant difference in the concentration of TNF-α in serum between the LPS and control groups (p > 0.05), but there was a significant difference in the concentration of TNF-α in hypothalamus between these two groups (p < 0.05); the BHD group represented remarkable control on the rising concentration of TNF-α in hypothalamus at 3 hours compared with the LPS group (p < 0.05). However, there was no remarkable difference in the concentration of TNF-α in hypothalamus between the IBF and LPS groups (p > 0.05). From our experiment, we found that IL-1β in serum and IL-1β and TNF-α in hypothalamus played significant roles in the second peak of LPS-induced fever.

### Discussion

Fever is a regulated rise in body temperature and one of the most common responses to infection, injury, or trauma. Administration of bacterial endotoxin LPS is widely used as a laboratory model of fever. Some endogenously produced proteins are considered responsible for the induction of fever by altering the “set point” for body temperature regulation. IL-1 is considered an EP during LPS-induced fever. Intraperitoneal, intravenous, intracerebroventricular, and intrahypothalamic injections of recombinant IL-1 induce fever in various species [5]. The proinflammatory cytokine IL-1 is a pivotal mediator of local and systemic responses to infection and inflammation, of which fever is the most widely studied, experimentally and clinically [5, 6]. The rabbit TNF injection also elicited biphasic fever in rabbits, the second phase of which was found to be mediated by similar EP, and endogenous TNF played an important role in eliciting a febrile response to endotoxin [7].

A biphasic fever model was made in rabbits using a high dosage of LPS (200 ng/kg) that had been performed by others [3]. The first phase of biphasic fever appeared about 1 hour and the second phase about 3 hours after the administration of LPS, which were similar to the findings of the investigation reported earlier [8]. The main findings of this study were that treatments with the crude extract of BHD remarkably reduced the biphasic fever induced by LPS. IBF, a specific cyclooxygenase inhibitor, not only obviously reduced the first phase of biphasic fever, but also affected

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<thead>
<tr>
<th>Test group</th>
<th>Serum</th>
<th>Hypothalamus</th>
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<tbody>
<tr>
<td></td>
<td>IL-1β</td>
<td>TNF-α</td>
</tr>
<tr>
<td>LPS</td>
<td>22.58 ± 0.72</td>
<td>81.29 ± 1.53</td>
</tr>
<tr>
<td>Control</td>
<td>19.20 ± 0.20</td>
<td>76.55 ± 3.88</td>
</tr>
<tr>
<td>IBF + LPS</td>
<td>19.65 ± 0.36</td>
<td>97.62 ± 1.39</td>
</tr>
<tr>
<td>BHD + LPS</td>
<td>20.74 ± 0.18</td>
<td>85.95 ± 6.44</td>
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Concentrations of IL-1β and TNF-α in serum and hypothalamus 3 hours after the injection of LPS in the different groups were detected. Data are expressed as mean ± SD (n = 6). Compared with the LPS group, *p < 0.05; **p > 0.05.

BHD = Bai-Hu decoction; IBF = ibuprofen; IL-1β = interleukin-1 beta; LPS = lipopolysaccharide; SD = standard deviation; TNF-α = tumor necrosis factor alpha.
the latter fever peak. This result that the IBF could lead to a significant fall in body temperature in both fever peaks was consistent with the investigation reported earlier [9].

Results of the present study showed that IL-1β and TNF-α were increased in serum and hypothalamus during biphasic fever induced by an intravenous injection of LPS (200 ng/kg). This observation is consistent with previous reports that LPS increases IL-1β mRNA levels in hypothalamus after a systemic injection [10] and that endogenous TNF plays an important role in eliciting a febrile response to endotoxin [7]. In addition, at 3 hours, there were no differences in the TNF-α level in serum between the LPS and control groups. Therefore, it was possible that the early peak of fever was induced by the indirect action of IL-1β and TNF-α produced in serum by an intravenous injection of LPS, and the latter fever peak that appeared at 3 hours was mediated by LPS-induced endogenous IL-1β in serum and endogenous TNF-α in hypothalamus. A previous study had shown that endogenous TNF activity was detected in 1-hour blood in an endotoxin dose-dependent manner that was coincident with the early fever peak but was not detected in 2.5-hour blood [7]. BHD reduced the concentrations of IL-1β and TNF-α in serum, and that of TNF-α in hypothalamus to control the febrile responses at 1 hour; the levels of IL-1β in hypothalamus and serum and that of TNF-α in hypothalamus decreased remarkably in the BHD group compared to that in the LPS group at 3 hours. However, IBF failed to reduce the rising concentrations of TNF-α in both serum and hypothalamus in the latter fever peak, and the reason might be the dissimilar anti-inflammation mechanism between IBF and BHD. Compared with IBF, according to TCM selective treatment based on the differential diagnosis, the TCM prescription BHD has some advantages in reducing fever induced by various diseases. Both gypsum and A. asphodeloides Bunge have been found to exert antifebrile and anti-inflammatory activities in this complex formula, according to the investigations reported earlier. Ca²⁺ is the main chemical composition of gypsum. The dissolution rate of Ca²⁺ is much higher when gypsum is used in combination with A. asphodeloides Bunge than when used singly [11]. The possible mechanism of gypsum’s antifebrile effect may be that it reverses the changes in the firing rate of pyrogen-treated, warm- and cold-sensitive neurons in the preoptic anterior hypothalamus region [12]. The main chemical constituents of A. asphodeloides Bunge are mangiferin and neomangiferin, which exert anti-fever effects. Different processing methods and different compatibility proportions of A. asphodeloides Bunge and Gypsum can change the concentration of mangiferin and neomangiferin, according to modern pharmacology research findings [13, 14]. Through studies conducted on the antifebrile effect and mechanisms of BHD and the single herbs of BHD, our results further proved that the synergistic effect of herbs plays a crucial role in treating fever compared to their cumulative effect. Meanwhile, the complex formula BHD may increase the dissolution rate of the effective contents.

Conclusion

In conclusion, the effects of TCM prescriptions are multi-target and complex; however, at least in part, the present study showed that the prescription could obviously reduce the increased concentrations of IL-1β and TNF-α, not only in serum but also in hypothalamus, which are the mechanisms of the prescription in reducing fever induced by LPS. All these results indicated that BHD could serve as a valuable candidate for further investigation and as a traditional antifebrile and anti-inflammatory natural drug.

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