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Characterization of the hypothermic component of LPS-induced dual thermoregulatory response in rats

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Abstract

We have previously shown that *Escherichia coli* O111:B4 serotype lipopolysaccharide (LPS) produced a dual change in rectal temperature (T_b), in which hypothermia preceded fever at subthermoneutral ambient temperature (T_{amb}; 24–26 °C) in rats. In this study, the characteristics of the initial hypothermic response were evaluated. Hypothermia was significant when LPS (50 μg/kg, ip) was injected at thermoneutral T_{amb} (30 °C). There was no heat loss through tail skin during hypothermia. The open field activity of the rats did not change during this period. However, serum levels of tumor necrosis factor-α (TNF-α) elevated at the beginning of the hypothermia, whereas serum levels of interleukin (IL)-1β and interferon (IFN)-γ remained unchanged. A nonselective cyclooxygenase inhibitor (indomethacin, 5 mg/kg, sc) inhibited hypothermia and serum TNF-α elevation, which resulted in an acceleration of the subsequent pyrogenic response. Moreover, a nonselective inhibitor of nitric oxide synthase (nitro L-arginine methyl ester (L-NAME), 10 mg/kg, sc) not only abolished fever but also prolonged the initial hypothermic response. These data suggest that the hypothermic component of low dose LPS-induced dual response is a regulated decrease in T_b. The data also suggest that hypothermia and fever may occur independently as two different thermoregulatory strategies against immune challenge in rats.

Keywords: Acute phase response; Fever; Hypothermia; Tumor necrosis factor-α; Indomethacin; L-NAME; Rat

1. Introduction

Lipopolysaccharide (LPS) is a component of the outer cell wall of gram-negative bacteria. Systemic injection of LPS to experimental animals activates the immune system, leading to release of endogenous proinflammatory cytokines such as interleukin (IL)-1, IL-6 and tumor necrosis factor-α (TNF-α) (Elmquist et al., 1997; Kluger, 1991; Rothwell, 1997). These polypeptide mediators initiate a multisystem response, including immune, endocrine, behavioral and metabolic components, known as acute phase response, a nonspecific defence reaction of the organism to noxious stimuli such as inflammation or infection (Hart, 1988; Kushner and Rzewnicki, 1997; Yirmiya et al., 1994; DeRijk et al., 1991). Systemic LPS administration is widely used for the experimental modelling of the acute phase response.

From a thermoregulatory point of view, systemic administration of LPS causes fever in humans and in some of the laboratory animals such as rabbit, cat, dog and guinea pig (Kluger, 1991). However, rodents may also respond to LPS by hypothermia with or without fever (Filkins and Di Luzio, 1968; Ueno et al., 1982; Wang et al., 1997; Kozak, 1997; Paul et al., 1999). Several reasons have been put forward to account for this species-specific thermoregulatory response in rodents. In particular, it has been widely accepted that environmental conditions, the dose of LPS injected and the animal strain used are quite critical parameters for determination of thermoregulatory response pattern in rodents (Kluger, 1991; Long et al., 1991; Romanovsky et al., 1997).

It has been previously suggested that hypothermia could be a secondary response, which may possibly be related with the cardiovascular toxic effect of LPS (Feldberg and Saxena, 1975). Nevertheless, more recent experimental evidence revealed that LPS-induced hypothermia does not develop simply by peripheral heat loss. Rather, some thermoregulatory mechanisms are operational during hypo-

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thermic state. Thus, it has been shown that reduced thermogenesis, cold-seeking behavior and a decrease in the threshold of body temperature for metabolic heat production may account for the mediation of hypothermia (DeRijk et al., 1994; Romanovsky et al., 1996). Despite this regulated genesis, it should be noted that hypothermia has been regarded as a symptom of LPS-induced shock due to high doses of LPS used (0.5 mg/kg or above) in those experiments. Meanwhile, these studies have indicated a possible adaptive value of hypothermia against the LPS challenge in rodents (Romanovsky and Szekely, 1998).

According to a current hypothesis, hypothermia is initiated by TNF-α, which is possibly released from peripheral macrophages (Kozak, 1997). Certain cyclooxygenase or lipoxygenase metabolites of arachidonic acid and neuropeptide arginine vasopressin have been proposed as possible central mediators of this response (Paul et al., 1999; Ueno et al., 1982; DeRijk and Berkenbosch, 1994). It has also been shown that TNF-α may have an endogenous cryogenic role for the modulation of febrile rise of body temperature (Long et al., 1990, 1992; Leon et al., 1997). Thus, it has been proposed that TNF-α-mediated hypothermia may have an antipyretic role, which prevents the increase in body temperature to harmful levels.

In our previous experiments, we have observed that the serotype of bacteria from which LPS was isolated is also critical in determining the pattern of the thermoregulatory response in rats (Doğan et al., 2000). For example, *Escherichia coli* O55:B5 LPS causes only pyrogenic response, whereas *E. coli* O111:B4 LPS is either pyrogenic or hypothermic depending on the dose administered. Furthermore, even much lower dose of LPS (50 μg/kg) could cause hypothermia, which was subsequently followed by fever. This observation raised a possibility that hypothermia does not necessarily develop as a symptom of shock.

Thus, in this study, we evaluated some characteristics of the hypothermic component of dual thermoregulatory response pattern in rats to compare the characteristics of hypothermia observed due to high dose of LPS injection. Furthermore, we intended to inhibit selectively each component of the dual response by pharmacological interventions in order to test the hypothesis that hypothermia develops for the modulation of subsequent pyrogenic response.

2. Materials and methods

2.1. Animals

Adult male Wistar albino rats weighing 220–300 g were used. They were maintained in an air-conditioned room with an ambient temperature (T_{amb}) 21 ± 3 °C and a 12-h light/dark cycle (light on at 7:00 AM), and fed ad libitum by standard rat chow and tap water. Rats were housed in groups of three to five in polyethylene cages. Each animal was used only once.

2.2. Body (rectal) temperature (T_b) measurements

Measurements of T_b were made in lightly restrained (handheld) conscious animals at 20-min intervals. A plastic coated thermocouple, connected to an electrically operated thermometer (Ellab, Denmark), was inserted 4 cm into the rectum and kept 30–45 s before readings were taken. All animals were adapted regularly to rectal probe for several days before the experiments in order to minimize the stress response induced by measurement procedure.

2.3. Heat Loss Index (HLI)

To evaluate heat loss through the tail skin, the tail skin temperature (T_{skin}) was measured with a thermistor (Type H3 probe, Ellab) attached to the ventral surface of the tail at the border of its proximal and middle thirds. The measurements were made at every 20 min for 30–45 s simultaneously with T_b recordings. T_{skin} values were then compared with T_{amb} and T_b and were expressed as HLI according to the formula:

$$\mathrm{HLI} = (T_{skin} - T_{amb})/(T_b - T_{amb})$$

The HLI may range from 0 to 1, indicating the maximal heat conservation (due to skin vasoconstriction) or maximal heat loss (due to skin vasodilatation), respectively (Romanovsky et al., 1997).

2.4. Behavioral analysis

The open field activity of the rats was evaluated in a 1 × 1 m Plexiglas box during a 6-min period using a real-time video image analyser (Videomex-V, Columbus Instruments, OH, USA). The open field activity parameters analysed were the distance traveled, the time spent with ambulatory movement, the time spent with stereotypic movement and the time spent in resting position.

2.5. Cytokine assay

The blood samples for cytokine determination were taken by intracardiac puncture under light diethylether anaesthesia. The samples were kept at 4 °C for 1 h, centrifuged (10 000 rpm, 5 min), and then the sera were separated and stored at −70 °C. The cytokine levels were determined by using enzyme-linked immunosorbent assay (ELISA). Rat-specific ELISA kits for TNF-α, IL-1β and interferon (IFN)-γ were purchased from Biosource (Camarillo, CA, USA). The detection limits of the assays were 4, 3 and 13 pg/ml for TNF-α, IL-1β and IFN-γ, respectively.

2.6. Compounds and drugs

LPS of *E. coli* serotype O111:B4 (lot 3922-25-0, Difco) was used. LPS was aliquoted as a stock solution of 2 mg/ml under aseptic conditions, and kept at −20 °C until use. LPS was further diluted with sterile apyrogenic saline solution. The injection volume was kept constant as 1 ml for 100 g of each rat. Indomethacin, a nonselective cyclooxygenase inhibitor (Merck, Darmstad, Germany), was dissolved in ethanol at a concentration of 20 mg/ml. The dilutions of indomethacin stock were made by saline. The highest ethanol concentration injected was 2.4%. For the highest dose of indomethacin (15 mg/kg), a suspension was prepared from stock solution by Tween 20 (0.025%). Nitro L-arginine methyl ester (L-NAME; Sigma), a nonselective nitric oxide synthase inhibitor, was dissolved in saline.

2.7. Experimental protocols

The experiments were started at 9:00 AM. Seven or eight control T_b values were recorded before the injections of LPS (50 μg/kg, ip) and/or drugs. All injections were made between 11:20 and 11:40 AM. Thereafter, T_b was recorded for 7 h at 20-min intervals and expressed as a difference from the mean of the last three control T_b (basal T_b) values of each rat (ΔT). All experiments were carried out in an air-conditioned room, maintained at a subthermoneutral T_{amb} of 24–26 °C, except during the experiments for evaluation the effect of LPS on T_b at thermoneutral T_{amb}, i.e. 30 °C.

In order to evaluate the characteristics of the hypothermic response, the following experimental protocols were performed.

(1) The effect of LPS on T_b was tested at 24 or 30 °C ambient temperatures. The tail skin temperatures of some rats were measured concomitantly with T_b after LPS or saline injections at 24 °C, and HLI was calculated.

(2) The open field activity of the rats was recorded at the initial period of the hypothermic response which corresponded to 80–100 min after LPS injection. The open field activity of the control rats was evaluated at the indicated time interval after saline injection.

(3) Cytokine determination: Blood samples of LPS- or LPS + drug-injected rats (other then used for T_b recordings) were taken at the initial period of the hypothermia. Control samples were collected at the intervals corresponding chronologically to the initial phase of the hypothermic response (80–100 min after injection). Cytokine assay was made for TNF-α, IL-1β and IFN-γ in the same sample.

(4) LPS injection was made together with one of the following compounds: indomethacin (0.5, 5 and 15 mg/kg, sc) or L-NAME (10 mg/kg, sc). Drug injection was made simultaneously with LPS injection.

The local Ethical Committee of Medical Faculty of Ankara University has approved all the procedures.

2.8. Statistical analysis

ΔT values were expressed as mean ± standard error of the mean. Multiple comparisons of the fever curves were made

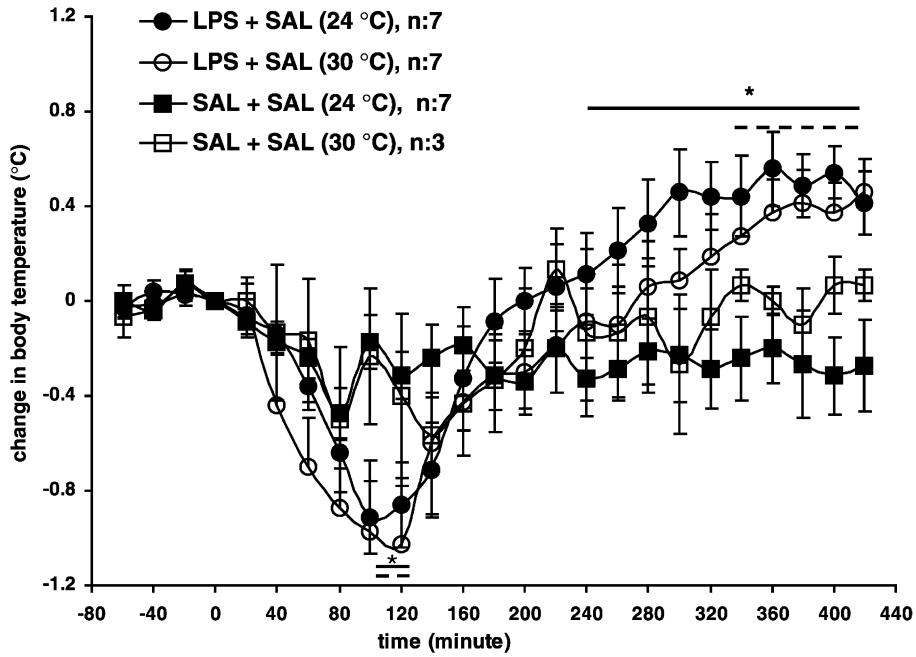

Fig. 1. Changes on the T_b following the intraperitoneal injection of the 50 μg/kg dose of *E. coli* O111:B4 LPS at 24 and 30 °C T_{amb}. All injections were made at the time "zero". Each point represents the mean ± S.E.M. of the specified observations. *: $P < .05$ compared to the SAL + SAL-injected group; ———: LPS + SAL (24 °C); – – – –: LPS + SAL (30 °C).

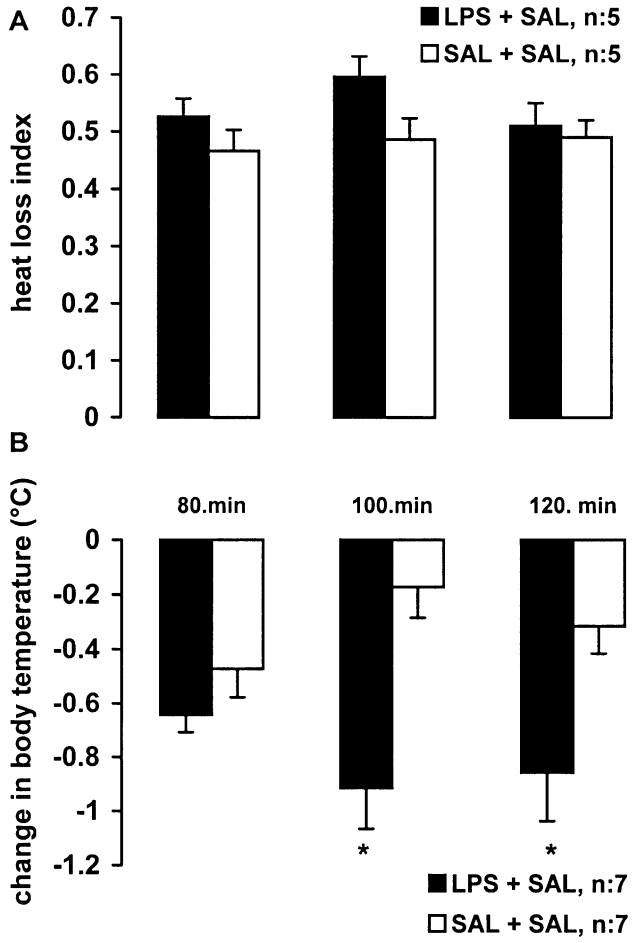

Fig. 2. Changes on the HLI (A) and the T_b (B) of saline- or LPS (50 μg/kg, ip)-injected rats at the initial phase (80–120 min) of the hypothermic response.

by analysis of variance (one-way ANOVA) with Newman–Keuls post hoc test. Comparisons of two independent groups were made by Student's *t* test. The Mann–Whitney *U* test and the Fisher–Pitman randomisation test were used for nonparametrical statistical analysis of the serum cytokine levels. The α value was corrected by using the Bonferroni procedure for multiple comparisons. Significance was noted when $P < .05$.

3. Results

3.1. T_b of control rats at subthermoneutral and thermoneutral T_{amb}

The saline-injected rats served as the control. Basal T_b of the control rats were 37.3 ± 0.1 °C at subthermoneutral T_{amb} and 37.4 ± 0.1 °C at thermoneutral T_{amb}. The basal T_b's of the other treatment groups were not significantly different than the basal T_b of control rats. Saline injection did not produce any change on T_b at both T_{amb}'s during 420 min of observation (Fig. 1).

3.2. Effects of LPS on T_b at subthermoneutral T_{amb}

The injection of *E. coli* O111: B4 LPS (50 μg/kg, ip) caused a dual change in T_b of rats kept at 24–26 °C [Fig. 1; $F(1,292) = 19.2$, $P < .0001$]. The T_b began to fall 80 min after the injection and reached its minimal value (ΔT: − 0.9 ± 0.2 °C) by 20 min. The hypothermia subsided in 40 min. The T_b started to rise 240 min after the injection of the LPS and reached a peak value (ΔT: 0.6 ± 0.2 °C) in about 120 min and remained elevated until the end of the experiment (Fig. 1).

3.3. Effects of LPS on T_b at thermoneutral T_{amb}

Similarly, LPS produced a dual change in T_b at 30 °C. The minimal value and the duration of the hypothermic response were not significantly different than those observed

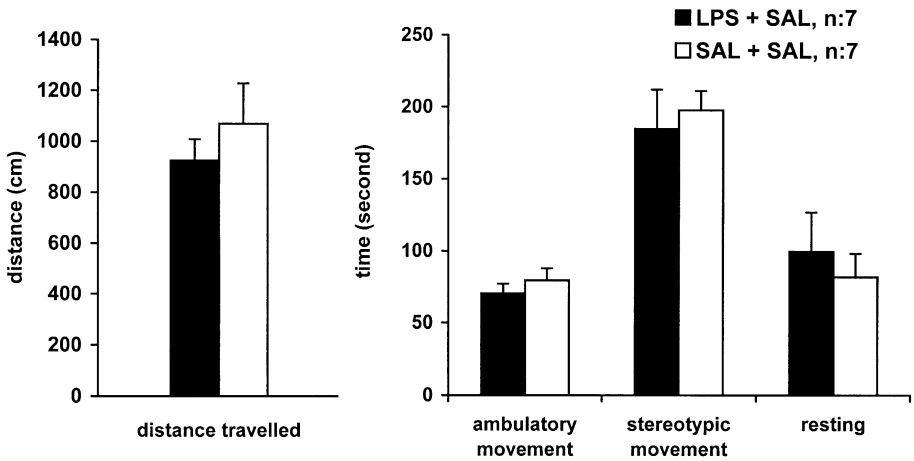

Fig. 3. The open field activity of saline or LPS (50 μg/kg, ip)-injected rats at the initial phase (80–120 min) of the hypothermic response.

Table 1
Changes in serum cytokine levels at the initial phase of the *E. coli* O111:B4 serotype LPS-induced hypothermia and the effect of indomethacin (5 mg/kg, sc) or L-NAME (10 mg/kg, sc) treatment

Treatment		*n*	TNF-α median (pg/ml)	*n*	IL-1β median (pg/ml)	*n*	IFN-γ median (pg/ml)
A	saline	12	114 [9]	8	172 [6]	8	224 [7]
B	LPS	16	2000 [2]*	6	33 [2]	6	– [6]
C	LPS + indomethacin	9	886 [2]*,§		ND		ND
D	LPS + L-NAME	7	2217 [3]*		ND		ND

The number of samples that were below detection limits are indicated in brackets.
ND: not determined.
* $P < .05$ compared to saline.
§ $P < .05$ compared to LPS.

at subthermoneutral T_{amb}. Meanwhile, fever began later at 30 °C (Fig. 1).

3.4. Effects of LPS on heat loss

Despite a significant decrease in T_b during 80–120 min after LPS injection (Fig. 2B), HLI did not differ significantly between LPS-treated and control rats at subthermoneutral T_{amb} (Fig. 2A).

3.5. Effects of LPS on the open field activity

As shown in Fig. 3, LPS injection did not alter the distance traveled, the time spent in ambulatory movement, the time spent in stereotypic movement or the time spent in a resting position at the initial phase of the hypothermic response.

3.6. Effects of LPS on proinflammatory cytokine levels in serum

There was a significant increase in the serum TNF-α levels at the initial phase of the hypothermia, whereas serum IL-1β and IFN-γ levels were found to be unchanged (Table 1A and B). Briefly, TNF-α was detectable in 3 of the 12 control samples, ranging from 6 to 454 pg/ml (median: 114 pg/ml). In LPS-treated animals, elevated TNF-α levels were found in 14 of the 16 samples (range: 591–4174 pg/ml; median: 2000 pg/ml).

3.7. Effect of indomethacin on LPS-induced changes in T_b and serum TNF-α levels

Indomethacin (0.5 and 5 mg/kg, sc) completely abolished the hypothermic response to LPS and accelerated the

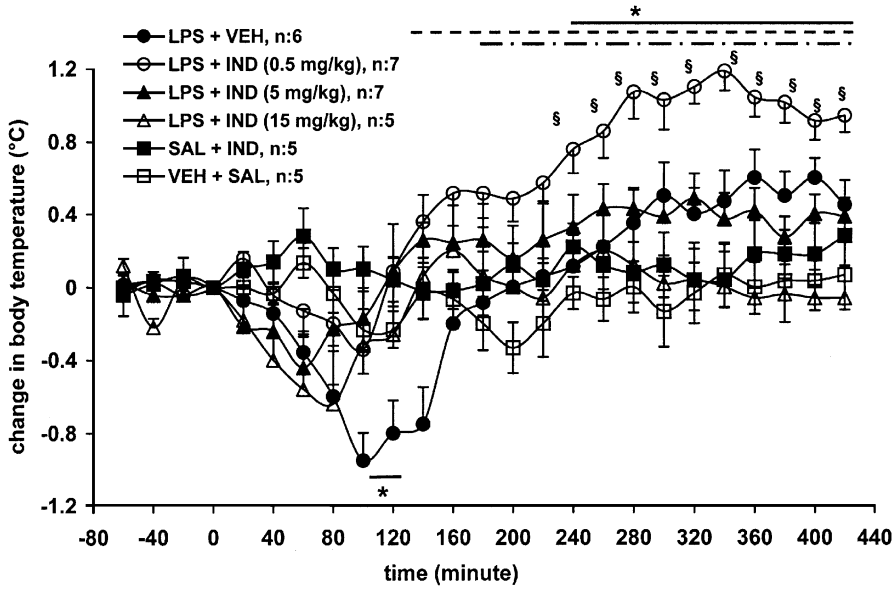

Fig. 4. The effects of subcutaneously injected indomethacin (IND) at doses of 0.5, 5 and 15 mg/kg on the *E. coli* O111:B4 LPS (50 μg/kg, ip)-induced T_b changes. The basal T_b values for indomethacin-injected groups were 37.0 ± 0.1, 37.1 ± 0.1 and 37.4 ± 0.2 °C, respectively. All injections were made at the time "zero". Each point represents the mean ± S.E.M. of the specified observations. (VEH: vehicle; ethanol 2.4%.) *: $P < .05$ compared to the VEH + SAL-injected group. §: $P < .05$ compared to the LPS + VEH-injected group. ——: LPS + VEH group; – – –: LPS + IND 0.5 mg/kg group; — · —: LPS + IND 5 mg/kg group.

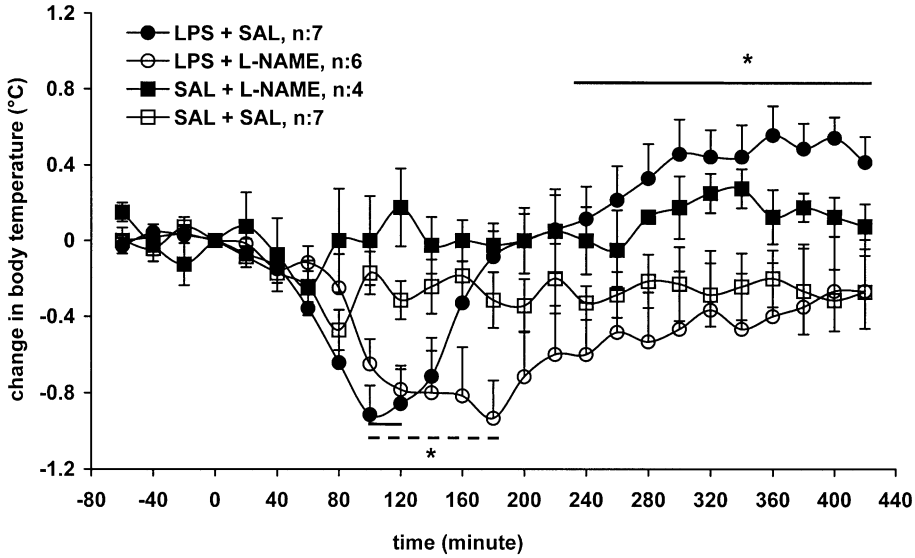

Fig. 5. The effect of L-NAME (10 mg/kg, sc) pretreatment on the *E. coli* O111:B4 LPS (50 μg/kg, ip)-induced T_b changes. The T_b of the L-NAME-injected group was 37.3 ± 0.2 °C. All injections were made at the time "zero". Each point represents the mean ± S.E.M. of the specified observations. *: $P < .05$ compared to the SAL + SAL-injected group. ——: LPS + SAL; – – –: LPS + L-NAME.

development of fever [Fig. 4; $F(1,244) = 10.1$, $P < .0001$]. Furthermore, the peak value of the pyrogenic response (LPS + indomethacin 0.5 mg/kg group) was significantly higher than the peak value obtained in LPS-treated group (ΔT: 1.2 ± 0.1 °C vs. 0.6 ± 0.2 °C, respectively). Meanwhile, the highest dose of indomethacin (15 mg/kg, sc) completely abolished both hypothermia and fever. Indomethacin itself did not cause any change in T_b (Fig. 4).

Indomethacin (5 mg/kg, sc) reduced the LPS-induced elevation in TNF-α levels (median: 886 pg/ml, range: 104–1535 pg/ml); however, TNF-α values were still significantly higher than control (Table 1C).

3.8. Effects of L-NAME on LPS-induced changes in T_b and serum TNF-α levels

L-NAME treatment (10 mg/kg, sc) completely inhibited fever and prolonged the initial hypothermia for 60 min (Fig. 5). L-NAME injection alone had no effect in T_b.

L-NAME did not cause any inhibition on LPS-induced TNF-α elevation (median: 2217 pg/ml, range: 168–5956 pg/ml; Table 1D).

4. Discussion

In this study, it was shown that low dose of *E. coli* O111:B4 LPS injection (50 μg/kg, ip) causes a dual T_b response at subthermoneutral and thermoneutral T_{amb}, in which hypothermia preceded fever in rats. A selective elevation on serum TNF-α levels accompanied by hypothermia. On the other hand, there was neither heat loss through skin vasculature nor locomotor activity depression during hypothermia. A selective inhibition of one component of the dual response resulted in a facilitation of the other one. Thus, it may be suggested that hypothermic component of the dual response is not related by LPS-induced shock, and, in this case, hypothermia and fever may develop independently from each other.

It has been shown that the effects of LPS on body temperature in rodents have a heterogeneous nature, in which hypothermia and fever can be seen either separately or subsequently (Kluger, 1991; Kozak, 1997). The dose and the serotype of LPS injected and the environmental conditions such as T_{amb} of the laboratory are among the critical variables determining the thermoregulatory response pattern of LPS in rats (Romanovsky et al., 1997; Doğan et al., 2000). Recent experimental evidence has revealed that LPS-induced hypothermia is a regulated decrease in body temperature in which several thermoregulatory mechanisms are involved in the mediation of the response. However, the characteristics of the hypothermia has been generally evaluated following the injection of high dose of LPS (0.5 mg/kg and above) of which manifestations of LPS-induced shock were significant (Romanovsky et al., 1996; DeRijk et al., 1994). Thus, it has been generally accepted that hypothermia is a thermoregulatory symptom of LPS-induced shock at subthermoneutral T_{amb}, because high dose of LPS injection does not produce hypothermia at thermoneutral T_{amb} (DeRijk et al., 1994; Romanovsky and Szekely, 1998).

We have recently reported that a considerable lower dose of LPS (50 μg/kg, ip) causes a hypothermic response, which was subsequently followed by fever in rats (Doğan et al., 2000). This observation raised the possibility that hypothermia may also develop as a specific thermoregulatory res-

ponse without LPS-induced shock in rats. In this study, we showed that hypothermia was still significant at thermoneutral T_{amb}. Furthermore, there was no heat loss through skin vasculature, which could eliminate the possible shock-inducing effect of LPS (Romanovsky et al., 1996). There was also no inhibition on open field activity of rats, which has eliminated the central depressant activity of LPS on that dose (Yirmiya et al., 1994). All these data may be taken as evidence indicating that LPS-induced hypothermia at low dose is a specific thermoregulatory change and may not be a component of LPS-induced shock.

It has been hypothesised that LPS-induced hypothermic response is initiated by TNF-α elevation in peripheral blood circulation. A considerable body of experimental findings is in favour with this hypothesis. LPS causes significant increase in plasma TNF-α level before the development of hypothermia in mice (Paul et al., 1999). It has been reported that inhibition of TNF-α bioactivity by several ways attenuates LPS-induced hypothermia in mice and rats (DeRijk and Berkenbosch, 1994; Leon et al., 1997; Kozak et al., 1995; Tollner et al., 2000). Thus, our finding that a selective elevation of serum TNF-α levels occurs at the initial phase of hypothermia is in line with these observations.

As a proinflammatory cytokine, TNF-α has been suggested as an endogenous pyrogen for the initiation of the pyrogenic response (Kluger, 1991). However, recent data have indicated that TNF-α may have also an endogenous cryogenic role. It has been reported that TNF-α inhibits LPS-induced fever, whereas TNF-α antiserum potentiates the response (Long et al., 1990, 1992). Furthermore, LPS produces an exacerbated febrile response without any hypothermia in TNF-α receptor double-knockout mice (Leon et al., 1997). All these findings have suggested that hypothermia may have a role for the modulation of pyrogenic response in rodents. In order to test this possibility, we inhibited each component of the dual response by pharmacological treatments. Selective inhibition of fever by L-NAME treatment resulted in a prolongation of the initial hypothermic response. This finding confirms the previous observation by Scammell et al. (1996). If the hypothermia developed for the modulation of the pyrogenic response, then inhibition of fever would reduce the hypothermia. However, that was not the case. On the other hand, selective inhibition of hypothermia by indomethacin treatment accelerated and potentiated the subsequent pyrogenic response. In this case, however, the initiation of the fever did not overlap with the initiation of the hypothermia. This may implicate that hypothermic signal occurs before than the pyrogenic signal. Actually, the elevation in serum TNF-α levels was selective at the initial phase of the hypothermia, as there was no elevation on the levels of other proinflammatory pyrogenic cytokines. Taken together, it is reasonable to suggest that the development of hypothermia may not be dependent to the genesis of fever. It seems that hypothermia is an independent thermoregulatory event rather than a cryogenic response for the modulation of subsequent fever. The changes in body temperature due to low dose of LPS treatment in rats presumably reflects the net effect of opposing hypothermic and pyrogenic mechanisms.

From a pharmacological point of view, indomethacin has been previously shown to potentiate LPS-induced hypothermia and TNF-α elevation in mice (Kozak et al., 1994, 1997). However, it should be noted again that the dose of LPS used in those studies is 2.5 mg/kg, which is about 50 times higher than the dose used in this study. It is apparent that the effectiveness of indomethacin is on opposite direction during LPS-induced shock state. Meanwhile, our study clearly showed that indomethacin preferentially inhibits hypothermia and TNF-α elevation in a dose range that did not inhibit fever in rats. Thus, inhibitory effect of indomethacin on elevated TNF-α levels may contribute to the antihypothermic activity of the compound. The classical antipyretic effect of indomethacin was observed as the dose increased, which is presumably more directly related with the cyclooxygenase enzyme inhibitory activity of the drug (DeRijk et al., 1994).

In conclusion, low dose of *E. coli* O111:B4 LPS injection produces a dual thermoregulatory response in rats in which hypothermia precedes fever. Both components of the response seems to represent two different and independent adaptive thermoregulatory strategies against exogenous immune challenge in rats.

Acknowledgments

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