Effect of heat stress on LPS-induced febrile response in d-galactosamine-sensitized rats

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Dokladny, Karol, Anna Kozak, Maciej Wachulec, Erik S. Wallen, Margaret G. Menache, Wieslaw Kozak, Matthew J. Kluger, and Pope L. Moseley. Effect of heat stress on LPS-induced febrile response in d-galactosamine-sensitized rats. Am J Physiol Regulatory Integrative Comp Physiol 280: R338–R344, 2001.—We have previously reported that heat conditioning augments lipopolysaccharide (LPS)-induced fever in rats, which is accompanied by an accumulation of heat shock protein (HSP) in the liver and the reduction of the plasma level of tumor necrosis factor (TNF-α) (Kluger MJ, Rudolph K, Soszynski D, Conn CA, Leon LR, Kozak W, Wallen ES, and Moseley PL. Am J Physiol Regulatory Integrative Comp Physiol 273: R588–R683, 1997). In the present study we have tested whether inhibition of protein synthesis in the liver can reduce the effect of this heat conditioning on the LPS-induced febrile response in the rat. d-galactosamine (d-gal) was used to selectively inhibit liver protein synthesis. d-gal (50 μg/kg) or PBS as control was administered intraperitoneally 1 h before heat stress. LPS (50 μg/kg ip) was injected 24 h post-heat exposure. Treatment with d-gal blunted the febrile response to LPS. Moreover, heat-conditioned rats treated first with d-gal and subsequently with LPS demonstrated a profound fall in core temperature 10–18 h post-LPS. A significant increase of serum TNF-α accompanied this effect of d-gal on fever. Heat-conditioned animals receiving d-gal showed an inhibition in inducible HSP-70 in the liver. These data support the role of hepatic function in modulating the febrile response to LPS.

Several studies have demonstrated that HSPs regulate cytokine production in peripheral blood monocytes. Intracellular HSP accumulation is associated with a decrease in synthesis of tumor necrosis factor-α (TNF-α) and interleukin (IL)-1β (6, 32). Impaired HSP production causes enhanced TNF-induced cytotoxicity in cells (27), whereas overexpression of HSP-70 is associated with a decrease of TNF-induced cytotoxicity (13). We have shown that heat stress sufficient to induce liver HSP in the rat resulted in significant augmentation of LPS-induced fever compared with non-heat-stressed animals (15). This amplifying effect of heat conditioning on fever was accompanied by a suppression of LPS-induced plasma TNF-α and no change in LPS-induced elevation of plasma IL-6. Based on these data, we postulated that induction of HSPs in the liver may be associated with the heat-conditioning-induced alterations of the response to LPS injection in rats.

Whereas heat conditioning is protective, pretreatment with d-galactosamine (d-gal) increases sensitivity to subsequent LPS (2, 10). d-gal inhibits protein synthesis primarily in the liver (20, 35). Based on these data, we used d-gal to test the hypothesis that inhibition of protein synthesis in the liver would alter the effect of heat exposure on induction of liver HSPs and the subsequent LPS-induced fever in rats. The first aim of this study was to examine the ability of d-gal to block the cellular accumulation of HSP-70 in the liver compared with other organs. The second aim was to determine whether d-gal blocked the effect of heat conditioning on LPS-induced fever and cytokine production. We report that d-gal inhibited the febrile response to LPS both in heat-stressed and nonstressed rats, selectively blocked the expression of inducible type of HSPs in the liver, and increased the concentration of circulating TNF-α.

MATERIALS AND METHODS

Experimental animals. Male Sprague-Dawley rats weighing 200–220 g on arrival were obtained from Harlan (Indiana).
napolis, IN). Rats were housed one per cage in a room maintained at 24–25°C with 12:12-h light-dark cycle and were provided with ad libitum tap water and laboratory rodent chow (Teklad rodent diet W8604). All experimental procedures were approved by the Lovelace Respiratory Research Institute Institutional Animal Care Committee.

Measurement of body temperature. Core body temperature (±0.1°C) was monitored using a Datacom 3 biotelemetry system (Mini-Mitter, Sunriver, OR) (15). Seven days before the experiments, the animals were anesthetized with halothane for intra-abdominal implantation of the Mini-Mitter transmitters (15). Body temperature was recorded at 5-min intervals, beginning at least 24 h before experimental procedures and continuing until the animals were killed. After each experiment, the transmitters were recalibrated to ensure that recorded temperatures were accurate.

Reagents. Purified LPS (Escherichia coli endotoxin 0111: B4, L2630, Sigma, St. Louis, MO) was dissolved in sterile pyrogen-free saline and injected intraperitoneally at a dose of 50 μg/kg. This dose causes robust, but not maximal, fever in control rats (15). d-gal (G0500, Sigma) was dissolved in sterile pyrogen-free PBS and injected intraperitoneally at a dose of 500 mg/kg.

Tissue preparation and Western blot analysis. At 24 h after heat or sham exposure (experimental protocol 1) or 4 h after LPS injection (experimental protocol 2), tissue samples from the liver, kidney, and brain were quickly removed and frozen in liquid nitrogen and then stored at −80°C for subsequent analysis. Samples were homogenized with a Potter-Elvehjem tissue grinder in an equal volume of ice-cold NaHCO3 (10 mM) for 30 s, subjected to three freeze-thaw cycles, then centrifuged to remove undissolved material. Protein was quantitated using the Bradford method. Fifteen micrograms of the animals was stored at 20°C until assayed. IL-6 concentration was determined using an ELISA kit (Bio-Rad Laboratories, Hercules, CA).

Measurement of IL-6. IL-6 plasma levels were measured 4 h after intraperitoneal injection of LPS (50 μg/kg) or saline (experimental protocol 2). Blood was withdrawn into syringes by heart puncture. After centrifugation, recovered plasma was stored at −20°C until assayed. IL-6 concentration was determined using an ELISA kit (R&D Systems, Minneapolis, MN).

Experimental protocol 1. All experiments were begun between 0800 and 0830 to minimize circadian variation. Rats were removed from their cages (25°C ambient temperature) and placed in new cages in a climatic chamber (44°C ambient temperature) for heat conditioning. Before exposure to the warm ambient temperature, the average body temperature of the animals was −37°C. When the body temperature reached 42°C, animals were removed from the warm chamber and returned to their home cages. Control rats were paired with the heat-stressed rats and treated similarly: they were removed from their home cages and placed into new ones in the 25°C room and returned to their home cages when the heat-treated rats were returned to their cages. One hour before the heat or sham exposure, all rats were injected with either d-gal or PBS. Changes in HSP level in the liver, kidney, and brain were measured.

Experimental protocol 2. Rats were treated with d-gal or PBS and exposed to heat (see Experimental protocol 1). At 24 h after heat or sham exposure, animals were injected with either PBS or saline. To check effects of LPS on rat body temperature, recordings were continued for 24 h. Changes in HSP level in the liver, kidney, and brain were measured.

Data analysis. Separate statistical analyses were performed for the temperature data from experimental protocols 1 and 2 and for the cytokine data. For all analyses, the data were analyzed using ANOVA. Depending on experimental protocol, the ANOVA had two or three factors with interactions. The possible factors were: heat stress (yes or no), d-gal treatment (yes or no), and LPS treatment (yes or no). If the overall model F for the ANOVA was statistically significant (P < 0.05), then the factor and interaction term F tests were examined for statistical significance. If any interaction terms were observed to be statistically significant and there were more than two comparison groups, subtesting was performed using t-tests. If a factor was statistically significant, there was no need for further subtesting as each factor had only two possible comparison groups. In that case, the factor F

Fig. 1. Effect of d-galactosamine (d-gal) on changes in body temperature in heat-stressed rats. d-gal (500 mg/kg ip) or PBS was injected intraperitoneally 1 h before heat or sham-heat exposure (♦ in the time axis). During nighttime, heat-stressed (HS) rats (injected with PBS or d-gal) had higher body temperature compared with sham-heat-exposed animals. The solid line just above axis represents the hours of darkness in the room. Values are means ± SE 1-h averages; n, numbers of rats in each group. cont, Control.
test and the subtest t-test would have the same \( P \) values, making the subtesting redundant.

The temperature measurements were made at 5-min intervals. For the purpose of the figures, the data from experimental protocol 2 were collapsed into 1-h averages, and the data from experimental protocol 2 were collapsed into 20-min averages. For the purposes of the statistical analyses, the data were averaged as follows. There were three time intervals of interest in experimental protocol 1: before heat stress (0600–0800); post-heat stress during the afternoon when the animals might be expected to be less active (1200–1700); and during the night (1800–0600). The temperatures were averaged for each individual animal corresponding to each of these three periods. The two-factor with interaction ANOVAs were performed separately for each time period.

There were five time intervals of interest in experimental protocol 2: before LPS (0900–1040), the first fever peak (1300–1420), the second fever peak (1520–1720), the night (1900–0500), and the following morning (0520–0900). For the two fever peaks, the maximum temperature was used rather than the average; however, for the other three time points, the averages were calculated for each animal. The three-factor with interactions ANOVAs were performed separately for each of the five periods.

RESULTS

Effect of D-gal and heat stress on changes in body temperature. Before heat (44°C) or sham heat (25°C) exposure, animals were injected with D-gal or PBS. Heat stress caused an immediate increase in body temperature (Fig. 1). When the body temperature reached 42°C, animals were removed to their home cages (25°C) and a decrease in body temperature was observed. Figure 1 shows 1-h temperature averages. The average body temperatures were 40.11 and 40.59°C for D-gal and PBS-treated rats, respectively (\( P \neq 0.05 \)). Shortly after heat stress (1300–1700), PBS-treated animals had significantly higher temperatures compared with three other groups (PBS-control, D-gal-control, D-gal-heat stress; \( P < 0.01 \)). During the dark period (1800–0600), body temperatures of the heat-conditioned animals pretreated with either PBS or D-gal were higher than sham-heated rats (\( P < 0.01 \)). D-gal had no effect on changes in body temperature during the dark period.

Sham-heat-stressed animals developed a moderate, transient rise in body temperature from being put into new cages and then returned to their home ones. The normal circadian rhythm in body temperature was observed both in D-gal- and PBS-treated animals.

Effect of D-gal and heat stress on LPS-induced fever. Rats were injected with D-gal or PBS 1 h before exposure to heat or sham heat. Twenty-four hours later animals were subjected to 50 mg/kg ip of LPS or saline. The patterns of body temperature changes after LPS and saline injection are shown in Fig. 2 and Fig. 3, respectively. Biphasic fever was recorded after LPS injection in both heat-stressed and sham-heat-stressed animals that had been injected (1 h before heat conditioning) with PBS. However, D-gal-treated rats with or without heat conditioning did not develop the second phase of fever to 50 mg/kg LPS (\( P < 0.01 \)) compared with control (PBS treated) animals exposed to heat or sham heat, respectively.

![Fig. 2. Effect of heat stress on lipopolysaccharide (LPS)-induced fever in D-gal-sensitized rats. Animals were exposed to D-gal (500 mg/kg ip) or PBS and heat stress or sham heat stress 24 h before intraperitoneal injection of 50 \( \mu \)g/kg of LPS (\( P \) in the time axis). D-gal treatment regardless of heat conditioning blocked the second phase of fever. Moreover, a profound drop in body temperature was observed in D-gal-sensitized rats exposed to heat and LPS. The solid line just above the axis represents the hours of darkness in the room. I and II, the first and the second peak of fever, respectively. Values are means ± SE 20-min averages; \( n \), numbers of rats in each group.](http://ajpregu.physiology.org/)

![Fig. 3. Effect of heat stress on changes in body temperature after saline (control) injection in D-gal-sensitized rats. Animals were exposed to D-gal (500 mg/kg ip) or PBS and heat stress or sham heat stress 24 h before intraperitoneal injection of saline (\( P \) in the time axis). The solid line just above the axis represents the hours of darkness in the room. Values are means ± SE 20-min averages; \( n \), numbers of rats in each group.](http://ajpregu.physiology.org/)
On the first day after heat or sham heat exposure (Fig. 1), during the dark period d-gal did not affect the body temperature in any experimental group. Its effect compared with PBS ($P < 0.05$), regardless of heat stress or LPS appeared during nighttime (on the next day) in all experimental groups (shown in Figs. 2 and 3). Furthermore, starting at 2100 to 0500 (at 10–18 h after LPS injection) in d-gal-heat stress-LPS-treated animals (Fig. 2), a profound drop in body temperature was observed compared with all other experimental groups (PBS-control-LPS, PBS-heat stress-LPS, d-gal-control-LPS, $P < 0.01$). After the night period (0600–0900), no statistically significant changes between groups were detected.

The normal circadian rhythm in body temperature was observed in animals treated with saline 24 h after exposure to d-gal and heat or sham heat (Fig. 3).

Effect of d-gal and heat stress on changes in inducible and constitutive HSP in accumulation. One hour after d-gal or PBS injection, animals were exposed to heat. Twenty-four hours later tissues (liver, kidney, and brain) were harvested, and HSP-70 and HSC-70 were measured. In liver tissue d-gal suppressed the inducible type of HSP-70 (Fig. 4, top), with no effect on the constitutive type of HSC-70 (Fig. 4, bottom), synthesis that normally occurs even in unstressed cells. In the brain and kidney, however, accumulation of both HSP-70 and HSC-70 were unchanged (data not shown).

In the second protocol, animals were also heat stressed 1 h after d-gal or PBS injection. Twenty-four hours later they were injected with LPS (50 $\mu$g/kg). After 4 h, tissues (liver, kidney, and brain) were harvested, and expression of HSP-70 (Fig. 5, top) and HSC-70 (Fig. 5, bottom) were measured. Administration of d-gal in heat-stressed animals subjected to LPS resulted in markedly decreased inducible HSP-70 accumulation in liver tissue, with no effect on induction of HSP-70 in the kidney or brain. Moreover, d-gal did not affect constitutive HSC-70 in any of these tissues.

Cytokine production. One hour before heat stress or sham heat stress, rats were injected with d-gal or PBS. Twenty-four hours after heat or sham exposure, the rats were given LPS or saline. At 1 h and 4 h after injection, blood from the heart was harvested, and serum TNF-α (1 h) and plasma IL-6 (4 h) were measured. Plasma IL-6 concentration (Fig. 6) was significantly elevated ($P < 0.01$) in LPS-treated animals that had been subjected to PBS and sham heat stress, compared with the three other groups of animals also treated with LPS. IL-6 was absent in the plasma of sham-LPS-treated (saline treated) animals 24 h after heat exposure or sham heat exposure, with or without d-gal. Similarly, in the sham-LPS-injected (saline injected) animals serum TNF-α was not detected (Fig. 7). On the other hand, TNF-α concentration increased in LPS-treated rats that were subjected to d-gal and heat stress, relative to the three other LPS-treated groups. A trend toward an increase of the TNF-α level was also observed in LPS-treated nonheated (25°C) animals compared with their heat-stressed (44°C) counterparts. But these differences were not statistically significant ($P = 0.2$).

DISCUSSION

The present study demonstrates that d-gal given shortly before heat conditioning dramatically alters...
the response to LPS in rats. D-gal treatment before heat conditioning not only blocks the augmented febrile response to LPS, but actually results in a drop in body temperature. We also demonstrate that D-gal effectively blocks the accumulation of inducible HSP-70 in the liver, but not in other organs we studied, in response to heat. Finally, we show that D-gal coupled with heat actually augments the TNF-α response to LPS. These data are consistent with our earlier reports (15, 30), demonstrating a protective effect of heat conditioning on subsequent LPS stress and extend the model to support the hypothesis that inhibition of hepatic HSP function before heat conditioning and LPS exposure changes the heat conditioning from a protective to an additive stress.

D-gal is thought to alter hepatic function by inhibiting hepatic mRNA synthesis through depleting uridine phosphates and uridine diphosphate sugars (14). This results in an inability of the cells to produce important components of the cell membrane, leading to cell damage. The Kupffer cells are thought to play a major role in the response to inflammatory agents, mainly to those entering the circulation from the gut, which, in turn, promotes the expression of TNF-α (33). LPS-induced TNF-α may be responsible for pathogenesis of liver injury and increase in mortality in D-gal-sensitized mice (2, 21, 31). Moreover, anti-TNF-α inhibits the lethal activity of killed gram-negative and gram-positive bacteria in D-gal-sensitized mice (9) and rhesus monkeys (8). This cytokine is considered a key factor inducing hepatocellular apoptosis after exposure to LPS in D-gal-treated mice (1, 34).

We demonstrate that D-gal inhibits the production of inducible HSP in the liver of animals that had been exposed to heat, whereas synthesis of the inducible type of HSP-70 in the kidney and brain was unaltered (data not shown). Levels of constitutive HSC-70 were not affected by D-gal either in heat-stressed or in sham-exposed animals in the examined organs. Morikawa et al. (25) have found that D-gal or LPS injections alone were not able to significantly alter the synthesis of HSC-70 and HSP-70 in mice. However, simultaneous treatment with D-gal and LPS led to overexpression of the inducible type of HSP-70 and suppression of constitutive HSC-70 in the liver. In our experiments we used D-gal (1 h before heat stress) to prevent the synthesis of HSPs in the liver.

We have previously shown (15) a heat stress-associated decrease in the synthesis of HSPs by D-gal-sensitized rats. Animals were injected with D-gal 1 h before heat stress. Twenty-four hours later animals were given an intraperitoneal injection of LPS or saline (control). Heat stress markedly increased serum TNF-α in D-gal-sensitized rats after intraperitoneal LPS injection. Values are means ± SE. Numbers in parentheses indicate samples sizes. **Significantly different from control (P < 0.001).

Finally, we propose that the reduced febrile response to LPS in animals pretreated with heat stress and D-gal can result from blunted production of IL-6 (Fig. 2). During endotoxemia (5, 16, 28), stroke (24), exposure to organophosphate pesticides (12), and terminal liver failure (11), a fall in body temperature is often observed. TNF is thought to be involved in endotoxin-induced decrease in body temperature in rats (4) and in mice (17). Moreover, impaired HSP production enhances TNF-induced cytotoxicity in cells (27). From
these data we speculate that loss of the HSP-70 response in the liver and subsequent increased concentration of TNF-α in d-gal-sensitized rats might be key factors responsible for the long-lasting reduction in body temperature observed in Fig. 2. Based on in vitro studies (6, 32), it is possible that these are linked phenomena, given the role of HSP production in blocking LPS-associated TNF transcription.

In summary, in the present study we demonstrate that the febrile response in d-gal-sensitized and heat-conditioned rats was dramatically changed, whereas heat-conditioned animals in the absence of d-gal showed increased febrile response to LPS. d-gal exposure combined with heat conditioning not only resulted in reduction of fever, but also in a long-lasting drop in body temperature. We also show that d-gal effectively blocks liver HSP accumulation in heat-exposed animals. Finally, we demonstrate augmented concentration of TNF to LPS in d-gal-sensitized rats with heat exposure. In conclusion, these data are consistent with our earlier study demonstrating a protective effect of heat conditioning on subsequent LPS stress and extend the model to support the hypothesis that inhibition of hepatocyte function changes the heat conditioning from protective to additive stress.

Perspectives

Expression of HSP, which is one of the most basic and highly conserved mechanisms of protection, affects many physiological functions on cells and organisms. Our recent studies have shown that heat conditioning, accompanied by an accumulation of HSP-70, augments LPS-induced fever in rats. In our present study, we have shown that the treatment with d-gal blunted the febrile response to LPS. Moreover, heat-conditioned animals treated first with d-gal and then exposed to LPS demonstrated a profound fall in body temperature. Although the exact mechanism responsible for the effect remains to be determined, we speculate that overexpression of TNF may be one possible explanation. Further studies will be needed to determine the role of proteins synthesized in liver on the development of fever or anaparexia (fall in body temperature). Moreover, we hope such further study will help us figure out whether the LPS-induced drop in body temperature during liver disfunction that we observed here might have had physiological and protective effects or whether it was only a sign of disease. Answering that question may contribute to our understanding of the role of fever and anaparexia in hepatic disease and find some practical applications.

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