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### Pre-existing inflammatory state compromises heat tolerance in rats exposed to heat stress

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**Lim, Chin Leong, Gary Wilson, Lindsay Brown, Jeff S. Coombes, and Laurel T. Mackinnon.** Preexisting inflammatory state compromises heat tolerance in rats exposed to heat stress. *Am J Physiol Regul Integr Comp Physiol* 292: R186–R194, 2007. First published September 21, 2006; doi:10.1152/ajpregu.00921.2005.—This study investigated the roles of endotoxemia and heat-induced tissue damage in the pathology of heat stroke. In groups of eight, male Wistar rats were treated with heat exposure only (HE), or heat exposure with turpentine (T+HE), dexamethasone (D+HE), and turpentine and dexamethasone combined (TD+HE). The rats remained sedated for 2 h after receiving the respective treatments, followed by heat exposure until the core temperature ( $T_c$ ) was 42°C for 15 min; control rats received turpentine (T), dexamethasone (D), and turpentine and dexamethasone (TD) without heat stress. Blood samples were collected before treatment (*baseline I*), after 2 h of passive rest (*baseline II*), at  $T_c$  40°C (T40), and 15 min after achieving  $T_c$  42°C (T42). No rats died in the nonheat-stressed groups. Survival rate was lowest in the TD+HE rats (37.5%), followed by the HE (62.5%), T+HE (75%), and D+HE (100%) rats ( $P < 0.05$ ). The duration of survival at T42°C was shortest in the TD+HE rats ( $9.9 \pm 6.2$  min) ( $P < 0.01$ ), followed by the T+HE ( $11.3 \pm 6.1$  min) and the HE ( $12.2 \pm 4$  min) ( $P < 0.05$ ) rats. The increase in plasma IL-6 concentrations was highest in the T+HE (352%) and HE (178%) rats ( $P < 0.05$ ). D+HE treatment suppressed the increases in plasma aspartate transaminase, alanine aminotransferase, and IL-6 and LPS concentrations during severe heat stress. Heat stroke can be triggered by endotoxemia or heat-induced tissue damage, and preexisting inflammation compromises heat tolerance, whereas blocking endotoxemia increases heat tolerance.

inflammation; turpentine; dexamethasone

HEAT TOLERANCE REFERS TO THE ability to tolerate a high level of heat stress without lethality. During severe heat stress, heat intolerance (lethality) is due primarily to circulatory and thermoregulatory collapse (6, 14), which are also the underlying mechanisms of heat stroke (6). Heat stroke is a condition that comprises a multitude of biological responses during heat stress, which can become lethal if not properly managed. Although a universal definition of heat stroke is lacking at present, there is a general consensus that heat stroke is a hyperthermic condition that is associated with systemic inflammation, multiorgan dysfunction, and encephalopathy (6, 24,

43, 50). This definition of heat stroke is consistent with the clinical presentation of heat stroke patients, which includes a core temperature ( $T_c$ )  $>40^\circ\text{C}$ , with systemic inflammation, central nervous system (CNS) dysfunction (e.g., coma, delirium, convulsion), disseminated intravascular coagulation, and multiorgan failure; hot and dry skin and cardiovascular deterioration may be observed at times (12, 25, 35, 50).

The current model suggests that heat stroke is triggered by circulatory and thermoregulatory collapse during hyperthermia and driven by endotoxemia (6, 24, 43). Endotoxemia refers to an elevated level of gram-negative bacterial endotoxin, known as LPS, in the circulation (43). The current model of heat stroke was based on the observation that patients with heat stroke or sepsis present with similar clinical symptoms, such as septicemia, organ failure, hemorrhage, and systemic inflammation (23, 43). In addition, animals are protected from the lethality of heat stroke through the therapeutic methods for treating sepsis (17, 19) such as the inhibition of endotoxemia through gastric lavage (9), antibiotics (20), and anti-LPS antibodies (18). Although the current model of heat stroke can explain the clinical symptoms in heat stroke patients, this model cannot explain the inconsistency of heat in triggering heat stroke, which has led to the dual pathway model of heat stroke (35).

The dual pathway model of heat stroke suggests that heat stroke is triggered by two separate but connected pathways (35). One of the pathways is triggered by endotoxemia. When endotoxemia can be prevented or tolerated and heat exposure is prolonged, the other pathway of heat stroke can be triggered by the direct thermal effect of heat on tissue cells, causing tissue damage. Heat-induced tissue damage is likely to occur at  $T_c > 42^\circ\text{C}$ , where proteins begin to denature (8, 11). These two pathways of heat stroke are likely to exist in a continuum, with some degree of overlap in their activation (35).

Heat stress compromises the integrity of the epithelial tight junction of the gut mucosa and causes LPS to leak into the portal circulation (46). The liver is able to neutralize and remove the leaked LPS up to a point, but excessive LPS leakage overwhelms the liver, causing LPS to leak into the central circulation (15, 22). A second line of anti-LPS mechanisms comprising macrophages, anti-LPS antibodies, and high-density lipoproteins scavenge and neutralize circulating

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LPS (15, 22). Endotoxemia develops when the second line of anti-LPS mechanism fails to curb the increase in the concentration of LPS in the circulation. Endotoxemia can activate a cascade of host-defense responses, including the secretion of proinflammatory cytokines, such as IL-1 $\beta$  and TNF- $\alpha$ , which may trigger systemic inflammation (6, 15). When exaggerated, systemic inflammation can cause the clinical symptoms of heat stroke, such as systemic coagulation, hemorrhage, cellular necrosis and death, and multiple organ failure (41, 50).

The inflammatory response plays an important role in the pathology of heat stroke. Human heat stroke victims have elevated concentrations of IL-1 $\beta$  and TNF- $\alpha$  in the blood (4, 7). The secretion of these proinflammatory cytokines induces the production of IL-6, an inflammatory-responsive cytokine (4, 7). The role of IL-6 during heat stroke is significant because the concentration of IL-6 is positively associated with the severity of the heat stroke (4, 6, 24, 43). In animal studies, the concentrations of IL-1 $\beta$  in the brain and serum are elevated during heat stroke in rats (13, 38, 39) and rabbits (37) that develop arterial and cerebral hypotension and cardiovascular instability. However, the role of IL-1 $\beta$  during heat stroke was investigated in isolation in these studies, without considering the influences of other factors, such as TNF- $\alpha$ , IL-6, and LPS. The effects of a preexisting inflammation on the pathology of heat stroke have also not been investigated before. This study hypothesized that preexisting inflammation induces a greater magnitude of inflammatory response during heat exposure and contributes to systemic inflammation reported in heat stroke patients (4, 7).

Intramuscular (im) injection of turpentine is an established experimental model for sterile induction of the inflammatory response (3, 34). Turpentine treatment causes fever after 4 h (34) and increases the concentrations of IL-6 in the brain and circulation more than 2 h postinjection (40), although the effects on circulating IL-1 $\beta$  and TNF- $\alpha$  are less consistent (34, 40). Turpentine has not been used as an experimental model to investigate the pathology of heat stroke. Instead, exogenous LPS injection has been used to induce the inflammatory response and sepsis (29, 42). Exogenous LPS treatment is not suitable for studying heat-induced endotoxemia because the exogenous LPS may confound the measurement of circulating LPS originating from the gut. Turpentine injection is sterile, and it induces the production of proinflammatory cytokines, which makes it a useful model for investigating the pathology of heat stroke. Local inflammation may increase the resting concentration of tissue and circulating proinflammatory cytokines, which can become excessively elevated during heat exposure.

In a study of heat-stressed primates, corticosteroid treatment protected the animals at  $T_c$  43.5°C with minimal changes in plasma LPS concentration, whereas 77% of placebo-treated primates died at the same  $T_c$  and exhibited a threefold increase in plasma LPS concentration (19). Although all of the primates died when  $T_c$  was increased to 44.4°C, plasma LPS concentration was lower than preheat-stressed concentration at death with corticosteroid treatment but increased by fivefold from resting concentration with placebo treatment (17). These results demonstrate that inhibiting endotoxemia protects the animals up to a certain level of heat stress, after which heat tolerance can deteriorate in the absence of endotoxemia. Although corticosteroid increases heat tolerance, the combined

effects of corticosteroid and turpentine on heat tolerance have not been studied. It is possible that the combination of turpentine and corticosteroid treatments may induce heat stroke through heat-induced tissue damage because endotoxemia is inhibited by corticosteroid.

The evidence presented suggests that elevated concentrations of circulating IL-1 $\beta$ , TNF- $\alpha$ , and IL-6, induced by preexisting inflammation, may play complementary roles with heat-induced endotoxemia in triggering systemic inflammation during heat stroke. At high  $T_c$ , heat stroke can also occur through tissue damage in the absence of endotoxemia (8, 19). Heat-induced tissue damage would increase the concentrations of alanine aminotransferase (ALT) and aspartate transaminase (AST), which are clinical biomarkers of tissue damage in human heat stroke patients (1, 5, 25, 50) and indicators of heat stroke in sedated rats (26, 27, 30, 31). This study investigated the effects of local inflammation and immune suppression on the pathways of heat stroke involving endotoxemia and thermal effects of heat. It was hypothesized that heat stroke occurs through pathways involving endotoxemia or heat-induced tissue damage, and that immune disturbance in the form of turpentine-induced local inflammation compromises heat tolerance, but that preventing endotoxemia during heat exposure increases heat tolerance.

## METHODS

Male Wistar rats (10 to 11 wk old,  $410.2 \pm 43.5$  g,  $n = 56$ ) were obtained from the Central Animal Breeding House of The University of Queensland. These rats were kept on a 12-h day (0700) and night (1900) cycle and were fed standard commercial chow and water ad libitum. The rats were kept in groups of four in plastic cages (40 cm  $\times$  65 cm  $\times$  22 cm) with a layer of soft woodchips, and the room environment was maintained at about 24°C and 40% relative humidity. All of the experiments were conducted in the first day hour of the light-dark cycle. The experimental protocols were approved by the Animal Experimentation Ethics Committee at The University of Queensland under the guidelines of the National Medical and Health Research Council of Australia. This conforms to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publication No. 85–23, revised 1996).

**Group 1: effects of heat stress on plasma LPS and cytokine concentrations.** Eight rats were sedated using ketamine (80 mg/kg) and xylazine (12 mg/kg ip) repeated with half of the initial dose every 60 to 90 min as required. After cannulation of the jugular vein with a sterile Silastic tube (Dow Corning, Auburn, MI) under anesthesia, a 22-gauge needle was attached for drug administration and removal of blood samples.  $T_c$  was measured by a rectal probe (series 4400, Yellow Springs Instrument, Yellow Springs, OH) inserted 8 to 10 cm past the anal sphincter and connected to a digital display (model 8502–12; Cole Palmer, Vernon Hills, IL) for continuous display.  $T_c$  was maintained at  $37 \pm 0.2^\circ\text{C}$  with an infrared heating lamp for 2 h of passive rest under sedation. The  $T_c$  was then raised gradually to 42°C with the infrared heating lamp positioned 40 cm above the rat and then maintained at about 42°C for 15 min [heat exposure (HE) group]. This level of heat stress is higher than the 50% level for heat stress mortality (41.5°C) in rodents (28) and has been shown to increase gut epithelial permeability (33) and cause tissue damage (8) in rats.

**Group 2: effects of intramuscular turpentine injection on heat tolerance and plasma LPS and cytokine concentrations.** These experiments compared rats treated with turpentine and heat exposure (T+HE) and turpentine only (T). Eight anesthetized rats were given 50  $\mu\text{l}$  turpentine im in the right hindlimb, followed by 2 h of passive rest under sedation and heat stress as in group 1. The duration of

passive treatment was based on a previous report of IL-6, IL-1 $\beta$ , and TNF- $\alpha$  production 2 h after turpentine injection (40). Each T+HE rat was paired with a T rat, which was sedated and given turpentine, but not exposed to heat.

**Group 3: effects of dexamethasone treatment on heat tolerance and plasma LPS and cytokine concentrations.** Two groups of eight rats were treated with 4 mg/kg of dexamethasone intravenously after sedation, followed by 2 h of passive rest. The dexamethasone (D-159, Lot 122K 1026; Sigma, St. Louis, MO) was dissolved in pyrogen-free saline at a concentration of 4 mg/ml. The appropriate volume of the solution, corresponding to a dosage of 4 mg/kg, was administered through the jugular vein of the rat. For example, a 400-g rat would receive 0.4 ml (1.6 mg) of the dexamethasone solution. This dosage of dexamethasone was shown to have neuroprotective effects in heat stroke rats (39). At the end of the passive treatment, one group of rats was subjected to heat exposure at T<sub>c</sub> 42°C for 15 min (D+HE). These rats were matched with another group that received the same treatment, but without heat exposure (D).

**Group 4: effects of turpentine and dexamethasone on heat tolerance and plasma LPS and cytokines concentrations.** Two groups of eight rats were given turpentine (50  $\mu$ l im) injection in the right hindlimb and dexamethasone (4 mg/kg iv) after sedation. One group of rats was exposed to the heat stress protocol described above (TD+HE), 2 h after the treatment. The TD+HE rats were matched with the second group of rats that received the same treatment, but without heat exposure (TD).

**Blood sampling.** For all groups, blood samples (1 ml, replaced by pyrogen-free saline solution) were taken at *baseline I* (after cannulation). The respective treatments (turpentine, dexamethasone, or saline) were administered following the collection of baseline I blood sample. A second blood sample (*baseline II*) was drawn after the passive treatment, just before heat exposure began. The third blood sample was taken when T<sub>c</sub> reached 40°C (T40), and the final blood sample was taken after the T<sub>c</sub> had been maintained at 42°C for 15 min (T42) or immediately upon death if the rat did not survive the heat stress.

To maintain consistency in blood collection, each rat that was not exposed to heat was matched with a rat that was subjected to heat exposure within each group. The blood sampling time points at the T40 and T42 intervals in the nonheated rats were matched with the paired heat-stressed rat. When a heat-stressed rat died before the end of the experiment, a blood sample for the matched nonheated rat was collected at the same time point (death in the heat-stressed rat), but the nonheated rat was allowed to continue with the experiment for another 15 min so that differences in mortality between heated and nonheated rats could be documented. Animals that survived the heat exposure and nonheated rats were euthanized by injecting 100  $\mu$ l of pentobarbital into the jugular vein.

**Measurements.** Blood samples were collected in sterile EDTA tubes and centrifuged at 400 g for 10 min. The plasma was extracted and stored at -80°C in separate pyrogen-free microtubes for analysis of ALT, AST, IL-1 $\beta$ , TNF- $\alpha$ , IL-6, and LPS. Pyrogen-free pipette tips were used to transfer plasma samples, which were stored in pyrogen-free microtubes. AST and ALT were analyzed using a robotic assay system (Cobas Mira; Thermo Electron, Melbourne, Australia) with commercially available kits from the same company.

**Cytokine analysis.** The cytokine assay was performed using a multiplex assay kit (Lincyto-80-03; Lyncoplex, St. Charles, MO) according to the instructions of the manufacturer. Assay standards of 4.88, 19.53, 78.13, 312.5, and 1,250 pg/ml were created with the stock provided by the kit, and the assay buffer served as the background (0 concentration). The assay kit also came with control solutions for "high" and "low" concentrations. The antibody-immobilized beads for each cytokine were mixed with the bead diluent (supplied) after being sonicated for 30 s and vortexed for 1 min. The assay buffer (200  $\mu$ l) was added into each well of the 96-well microtiter plate (supplied), and the contents in the wells were removed by a vacuum pump after incubating for 10 min on a shaker. Twenty-five microliters of the

assay buffer (background), standards, "high" and "low" control solutions and undiluted plasma samples were then added into the designated wells. Another 25  $\mu$ l of the assay buffer were added into each sample well, and 25  $\mu$ l of the serum matrix solution (supplied) were added into the background, standard, and control wells. The cocktail of antibody-immobilized beads (25  $\mu$ l) was then added into each well. The plate was sealed, covered with aluminum foil, and incubated on a shaker in a cold room ( $\approx$ 4°C) overnight. The next morning, the plate was brought to room temperature, and the solution in the wells was removed by a vacuum pump. The plate was then washed three times with the wash buffer (supplied).

The detection antibody cocktail (50  $\mu$ l, supplied) was added into each well after the third wash, and the plate was sealed, covered with aluminum foil and left to incubate for 2 h on a shaker. At the end of the incubation, 50  $\mu$ l of streptavidin-phycoerythrin was added into each well, and the plate was again sealed, covered with aluminum foil, and incubated for another 30 min on a shaker. At the end of the incubation, the content in the wells was removed by a vacuum pump, and the plate was washed three times with the wash buffer. Following the wash, 100  $\mu$ l of sheath fluid was added into each well, and the plate was covered with aluminum foil and agitated on the shaker for another 10 min. The reactions of the antibody-coated beads to the cytokines in the wells were then read with the Luminex 100 machine (Luminex, Austin, TX). The concentrations of the cytokines were calculated by matching the optical density of the samples against a regression equation established with the standard curve. The assay was performed in duplicate for the background, standards, controls, and samples.

**LPS analysis.** LPS analysis was performed using the chromogenic limulus amoebocyte lysate (LAL) assay kit (Charles River Laboratories, Wilmington, MA) according to the instructions of the manufacturer. The standard concentrations of 0.075, 0.15, and 0.3 endotoxin units (EU)/ml were created with the reconstituted control standard endotoxin stock provided by the kit, and the LAL reagent water (LRW) served as the background for the standards (0 concentration). Plasma samples of the rats were thawed and diluted five times with sterile saline solution. The samples were vortexed for 1 min and placed in a water bath at 75°C for 10 min. After heating, the samples were vortexed and 50  $\mu$ l of the standards and plasma samples were added into the designated wells of a 96-well pyrogen-free microplate in duplicate. The LAL reagent was then mixed with the substrate-buffer solution at a ratio of 1 LAL reagent: 2 substrate buffer solution. Once mixed, 150  $\mu$ l of this solution were added into each well with an eight-channel pipette. There was also a negative control for each sample, which received 150  $\mu$ l of LRW instead of the LAL reagent-substrate buffer solution mix. Reactions to the reagents were observed after about 5 min. The reaction of the samples was then read with a microplate reader (Titertek Multiskan, MCC 340 MK II, Flow Laboratories, Lugano, Switzerland) at 580 nm at 1- to 2-min intervals, until the highest concentration in the standards reached a plateau (about 25 min for all plates). Plasma concentration of LPS was determined by matching the optical density of the samples against a regression equation established with the standard curve, after subtracting the negative control values from the corresponding sample wells. Sterile procedures were used in this assay. All instruments and accessories that came into contact with the sample or reagents were pyrogen-free.

**Statistics.** The results are presented as means  $\pm$  SD. The differences between heat- and nonheat-exposed rats were analyzed using a 3 (groups)  $\times$  4 (time points) ANOVA. If a significant main (group or time) or interaction (group  $\times$  time) effect was found, the data were further analyzed for pairwise difference at each time point with an independent *t*-test. Differences between heat-stressed groups were compared using a 4 (groups)  $\times$  4 (time points) ANOVA. The four heat-exposed groups were HE, T+HE, D+HE, and TD+HE. Post hoc analysis for pairwise difference at each time point was performed using Tukey's test when a significant main (group or time) or



interaction (group  $\times$  time) effect was found in the ANOVA. The categorical data for survival rate (survived vs. died) were analyzed using the  $\chi^2$ -test statistics. The  $\chi^2$ -test statistics compared only the survival rate of the groups that were exposed to heat stress. The data for duration of survival in the 15 min where  $T_c$  was  $42^\circ\text{C}$  were analyzed nonparametrically with the Mann-Whitney  $U$ -test because the data were not normally distributed.  $P < 0.05$  was considered significant.

## RESULTS

**Survival rate, duration of survival and heating rate.** All of the rats that were not exposed to heat survived. Dexamethasone alone protected the animals from lethal heat stress, whereas turpentine alone or in combination with dexamethasone compromised heat tolerance and survival time under heat stress. Survival rate was 100% in the D+HE, 75% in the T+HE, 62.5% in the HE and 37.5% in the TD+HE groups ( $P < 0.05$ ) (Fig. 1). The duration of survival at T42 was the shortest in the TD+HE rats ( $9.9 \pm 6.2$  min), followed by the T+HE ( $11.3 \pm 6.1$  min) and HE ( $12.2 \pm 4.0$  min) rats. These durations of survival differed significantly between the D+HE and HE ( $P < 0.05$ ) and between the D+HE and TD+HE ( $P < 0.01$ ) groups (Fig. 2). The mean duration taken for  $T_c$  to increase from resting to  $42^\circ\text{C}$  was  $21.1 \pm 4.3$  min for HE rats,  $21.6 \pm 4.3$  min for T+HE rats,  $21.7 \pm 4.6$  min for D+HE rats, and  $21.2 \pm 5.0$  min for TD+HE rats. These heating durations were not significantly different. The  $T_c$  profiles of one rat that died and one rat that survived during heat exposure in each of the heat-stressed group are depicted in Fig. 3.

**ALT and AST.** Heat stress induced a significant increase in plasma concentrations of ALT and AST in the HE ( $P < 0.001$ ), T+HE ( $P < 0.001$ ) and TD+HE ( $P < 0.001$ ) rats, but not in the D+HE rats (Table 1). The concentrations of plasma ALT ( $P < 0.05$ ) and AST ( $P < 0.001$ ) also differed significantly between the heat-stressed groups at T42. In the D+HE rats, plasma concentrations of ALT at T42 were significantly lower than the TD+HE rats ( $P < 0.05$ ), and plasma concentrations of AST were significantly lower than the T+HE ( $P < 0.05$ ) and TD+HE ( $P < 0.01$ ) rats.

**Plasma IL-1 $\beta$ , TNF- $\alpha$ , and IL-6.** Plasma IL-1 $\beta$  concentrations increased significantly in all of the heat-stressed groups

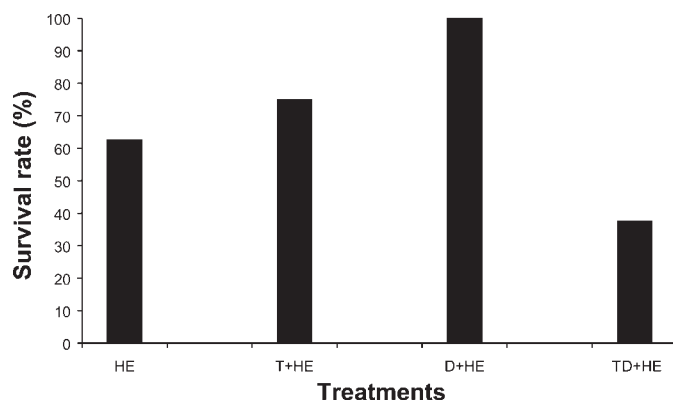


Fig. 1. Survival rate of rats treated with heat exposure only (HE), turpentine combined with heat stress (T+HE), dexamethasone combined with heat stress (D+HE) and a combination of turpentine, dexamethasone, and heat stress (TD+HE). The data were analyzed in categorical scale (died vs. survived) using  $\chi^2$  statistics. The survival rates among the heat-stressed rats are significantly different ( $P < 0.05$ ).

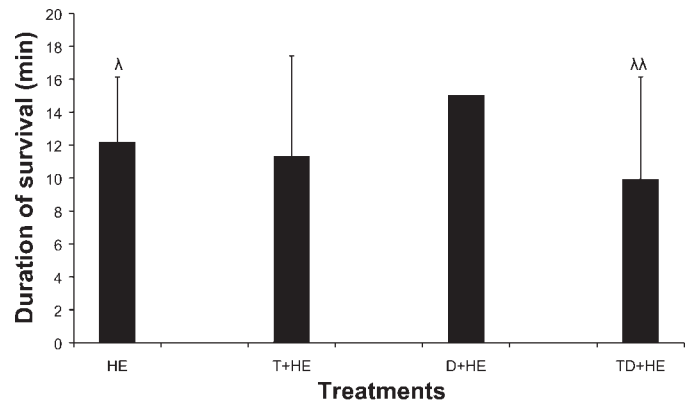


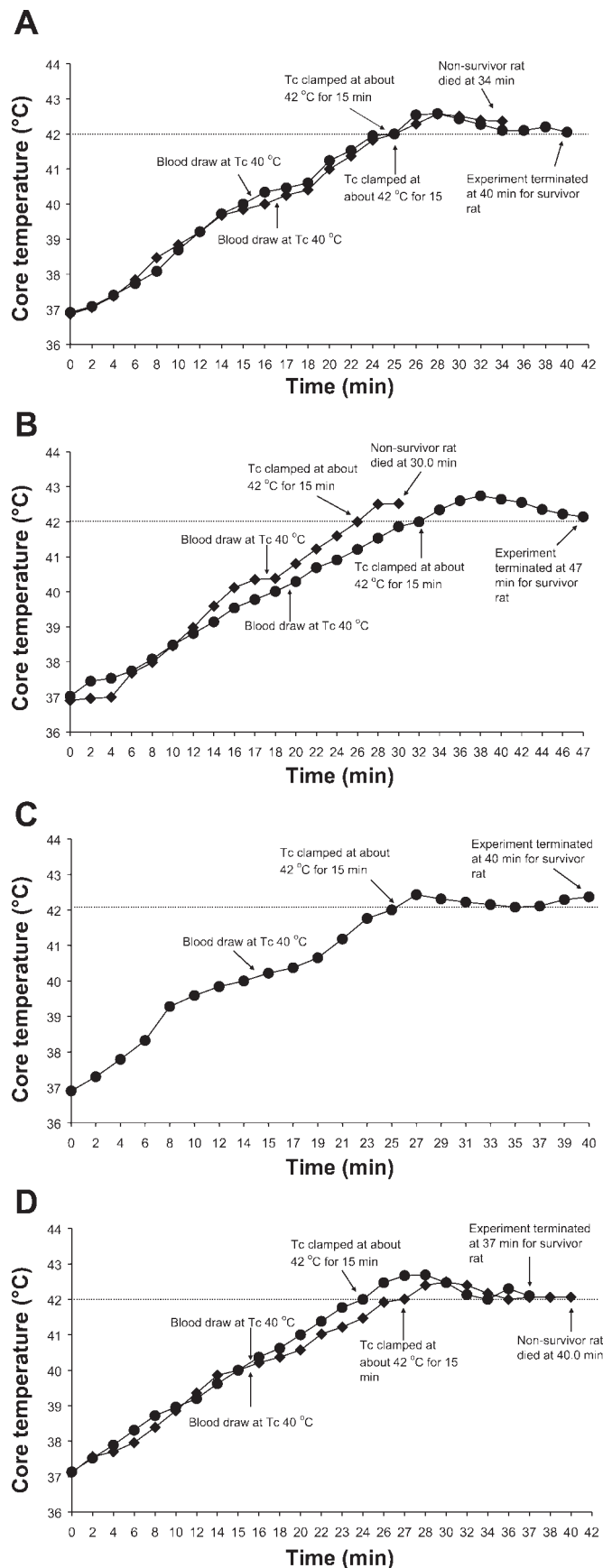
Fig. 2. Mean (SD) of duration of survival of rats treated with HE, T+HE, D+HE, and TD+HE. There is no SD in the D+HE group because all the rats in that group survived the 15 min of heat stress at  $T_c \approx 42^\circ\text{C}$ .  $\lambda$  ( $P < 0.05$ ) and  $\lambda\lambda$  ( $P < 0.01$ ) represents significant difference in duration of survival from the D+HE group.

( $P < 0.001$ , HE, T+HE, and TD+HE, and  $P < 0.01$ , D+HE) (Fig. 4). However, IL-1 $\beta$  concentrations at T42, were significantly higher in the heat-stressed than in the control rats in T+HE ( $P < 0.05$ ) and TD+HE groups ( $P < 0.05$ ), but not in the D+HE group. Within the HE group, plasma IL-1 $\beta$  at T42 was significantly higher than at *baseline I* ( $P < 0.01$ ), *Baseline II* ( $P < 0.05$ ), and T40 ( $P < 0.05$ ). The concentrations of plasma IL-1 $\beta$  were also significantly different between the heat-stressed groups ( $P < 0.05$ ). IL-1 $\beta$  concentration was significantly higher in the HE than in the D+HE rats at T42 ( $P < 0.05$ ).

Plasma TNF- $\alpha$  concentrations did not change significantly within each group, but TNF- $\alpha$  concentrations at T42 differed significantly between the heat-stressed and control rats in the T+HE ( $P < 0.05$ ) and TD+HE ( $P < 0.05$ ) groups (Fig. 5). The concentrations of plasma TNF- $\alpha$  also differed significantly among the heat-stressed groups ( $P < 0.05$ ), but there was no significant pairwise difference between these groups.

Heat stress induced a significant increase in plasma IL-6 concentrations in each heat-exposed group ( $P < 0.001$ ) (Fig. 6), and IL-6 concentrations were significantly higher at T42 in the heat-stressed than in the control rats ( $P < 0.01$ , T+HE and D+HE and  $P < 0.001$ , TD+HE). In the HE group, IL-6 concentration at T42 was significantly higher than at *baseline I* ( $P < 0.05$ ) and T40 ( $P < 0.05$ ). Plasma IL-6 concentrations differed significantly among the heat-stressed rats ( $P < 0.05$ ). Plasma IL-6 concentrations in the D+HE rats were significantly lower than in the T+HE ( $P < 0.05$ ) and TD+HE ( $P < 0.05$ ) rats at T42.

**Plasma LPS.** Heat stress induced a significant increase in plasma concentrations of LPS in the HE ( $P < 0.01$ ) and T+HE rats ( $P < 0.05$ ), but LPS concentrations decreased significantly in the TD+HE ( $P < 0.05$ ) and D+HE ( $P < 0.05$ ) rats (Fig. 7). At T42, plasma LPS concentrations were significantly higher in the T+HE than in the T group ( $P < 0.05$ ) but not between D+HE and TD+HE and the respective control groups. In the HE group, plasma LPS concentration at T42 was significantly higher than at *baseline I* ( $P < 0.05$ ) and *II* ( $P < 0.05$ ) and T40 ( $P < 0.01$ ). Plasma LPS concentrations were significantly lower in the D+HE than in the T+HE rats ( $P < 0.001$ ). Plasma LPS concentrations were also significantly different



among the heat-stressed groups ( $P < 0.05$ ). The concentration of plasma LPS was significantly higher in the T+HE than in the D+HE rats ( $P < 0.05$ ).

## DISCUSSION

This study investigated the hypotheses that turpentine-induced local inflammation compromises heat tolerance and that preventing endotoxemia during heat exposure increases heat tolerance. D+HE protected the rats from lethal heat stress, but T+HE alone and when combined with dexamethasone, TD+HE, decreased survival rate, and TD+HE decreased the duration of survival at T42. Heat exposure caused endotoxemia and tissue damage in the T+HE and HE rats but only caused tissue damage in the TD+HE rats. Turpentine treatment enhanced the inflammatory response in the presence of heat-induced endotoxemia. Dexamethasone could have protected the rats by inhibiting endotoxemia during lethal heat exposure. Our results suggest that heat stroke can be induced by either the endotoxemia or tissue damage pathways, with both pathways being driven by systemic inflammation.

Heat stroke is defined as a hyperthermic condition that is associated with systemic inflammation, multiorgan dysfunction, and encephalopathy (6). Two of the authors have reviewed and reported on the clinical and forensic presentations of heat stroke in patients, who are usually presented with hyperthermia ( $T_c > 40^\circ\text{C}$ ), systemic inflammation, CNS dysfunction (e.g., coma and delirium), disseminated intravascular coagulation and multiorgan failure (35). Hot and dry skin and cardiovascular deterioration are observed inconsistently (35). For example, only 32.6% of civilian (48) and 35% of military (49) heat stroke patients are hypotensive. The concentrations of inflammatory cytokines are increased and endotoxemia is present in heat stroke patients (4, 7, 24, 43). In animals, some experiments used the achievement of a high  $T_c$  as the criterion for inducing heat stroke. For example, the criterion  $T_c$  for inducing heat stroke are  $>43^\circ\text{C}$  in dogs (10, 53, 54) and  $\geq 43.5^\circ\text{C}$  in primates (18, 19). Heat stroke rabbits (9) and primates (18, 19) have elevated concentrations of circulating LPS and the inhibition of endotoxemia protected the animals from the lethal effects of heat (9, 18, 19). In unconscious rats, an increase in the concentrations of circulating ALT and AST (27) and the occurrence of hypotension (36, 39) under severe heat stress have been used, separately, as indicators of heat stroke.

In the present study, the  $T_c$  of the rats was increased to  $42^\circ\text{C}$  for 15 min. This level of heat stress had induced tissue injury in heat stroke rats (27) and caused damages to rat liver (8) and intestinal (33) tissues. Heat stroke-related mortality under this level of heat stress is 70% to 90% in rats (26). Blood pressure was not measured in the present study, but the concentrations of AST and ALT were significantly increased in all the heat-stressed rats, except for the D+HE rats. An earlier study showed in rats that the increase in AST and ALT concentrations under severe heat stress is due to heat stroke-induced tissue injury (27). The absence of blood pressure data in the

Fig. 3. Core temperature ( $T_c$ ) profiles of a rat that died (◆) and a rat that survived (●) in the HE (A), T+HE (B), D+HE (C), and TD+HE (D) groups during the 15 min when  $T_c$  was maintained at  $\approx 42^\circ\text{C}$ . All of the rats survived the heat stress in the D+HE group.

Table 1. Effects of heat stress on plasma concentrations of ALT and AST

Groups	ALT				AST			
	Baseline I	Baseline II	T40	T42	Baseline I	Baseline II	T40	T42
HE	42.7 (9.0)	48.2 (15.9)	49.1 (15.3)	73.3 (22.6) <sup>a</sup>	88.4 (25.6)	104.7 (47.7)	131.7 (45.9)	162.9 (48.1) <sup>a</sup>
T	39.0 (10.6)	44.1 (12.0)	46.7 (9.2)	49.7 (13.6)	83.9 (30.6)	106.2 (32.2)	111.1 (27.7)	113.6 (24.5)
T+HE	42.1 (14.8)	48.0 (13.8)	53.0 (16.0)	79.8 (24.3) <sup>a</sup>	77.5 (23.2)	131.6 (43.8)	155.0 (53.1)	200.5 (63.0) <sup>a,b,c,d</sup>
D	39.9 (7.1)	40.2 (11.6)	40.5 (9.9)	40.7 (11.5)	77.1 (7.9)	82.71 (12.1)	88.73 (18.1)	89.7 (14.7)
D+HE	41.0 (7.9)	43.7 (9.0)	44.3 (9.1)	50.1 (20.8)	86.5 (17.9)	89.9 (37.0)	98.4 (27.6)	102.4 (35.6)
TD	42.9 (8.1)	49.3 (16.9)	50.0 (17.0)	63.2 (30.0)	77.2 (17.6)	121.3 (44.0)	120.8 (43.5)	130.1 (42.3)
TD+HE	42.3 (7.8)	61.4 (18.4)	63.8 (21.3)	100 (42.2) <sup>a,d</sup>	87.3 (32.5)	132.0 (43.8)	168.1 (69.1)	252.8 (105.5) <sup>a,b,c</sup>

Values are expressed as means  $\pm$  SD (in parentheses) concentrations of plasma alanine aminotransferase (ALT) and aspartate transaminase (AST) (U/l) in rats treated with heat exposure only (HE), turpentine only (T), T combined with heat stress (T+HE), dexamethasone only (D), D combined with heat stress (D+HE), T and D combined without heat stress (TD), and TD with heat stress (TD+HE). T40, core temperature 40°C; T45, core temperature 45°C. <sup>a</sup>Significant changes in ALT and AST concentrations in each group ( $P < 0.001$ ). <sup>b</sup> $P < 0.05$  and <sup>c</sup> $P < 0.01$ , significant difference at the specific time point between the heat-stressed and control rats within each treatment group. For comparisons between the heat-stressed groups, <sup>d</sup> $P < 0.05$  and <sup>e</sup> $P < 0.001$  represent a significant difference from the D+HE rats at the specific time point.

rats should not suggest that heat stroke was not induced in the present study. Although heat stroke is a syndrome that induces a variety of biological responses (6, 35), including hypotension, it is unlikely that all of these responses occur at the same time at the onset of heat stroke. This postulation could explain the finding that only 32.6% of civilian (48) and 35% of military (49) heat stroke patients were hypotensive. Some researchers have used the decrease in mean arterial pressure to indicate the onset of heat stroke in rats (36, 39). Such a model of defining heat stroke assumes that hypotension is the first observable biological mechanism to respond to heat stroke. To the best knowledge of the authors, such an assumption has yet to be supported and the sequence of biological responses during a heat stroke is also not determined. However, there is a good agreement on the type of biological responses during heat stroke, which have been described earlier (hyperthermia, CNS dysfunction, systemic inflammation, etc.). The observations of these responses under heat stress condition are suggestive of

heat stroke. CNS dysfunction could not be determined in our study because the rats were unconscious. However, the heat-stressed rats were hyperthermic (42°C), and the concentrations of ALT and AST are indicative of tissue damage in the HE, T+HE, and TD+HE rats. At T42, endotoxemia was present in the HE and T+HE rats, but plasma LPS levels were lower than at baseline in rats treated with dexamethasone (D+HE and TD+HE). In addition, the concentrations of IL-1 $\beta$  and IL-6 were increased significantly in all the heat-stressed rats. These responses in T<sub>c</sub>, liver enzymes, plasma LPS, and inflammatory cytokines are consistent with the definition of hyperthermia, systemic inflammation, and multiorgan failure in heat stroke (6). These results support the suggestion that heat stroke was induced in the present study.

Our results suggest that there are two mechanisms of heat stroke, and these are driven by endotoxemia in the HE and T+HE rats and tissue damage in the TD+HE rats. The high concentrations (0.81 to 0.88 EU/ml), and the magnitude of increase (88% to 100%) in plasma LPS, coupled with the relatively lower magnitude of increase in the concentrations of plasma ALT and AST in the HE (73% ALT and 84% AST) and T+HE (79% ALT and 158% AST) rats suggest that heat stroke

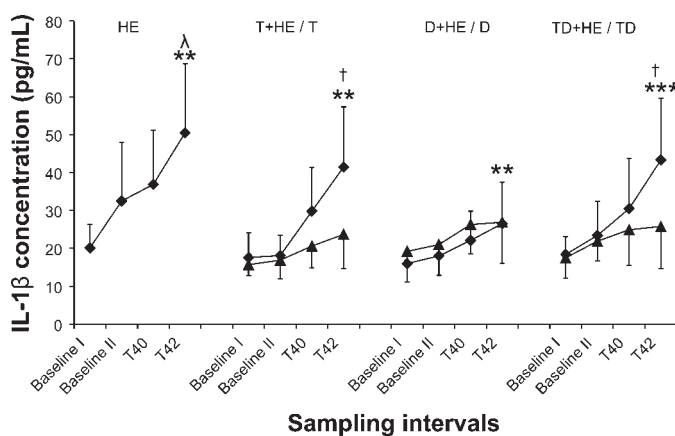


Fig. 4. Mean (SD) of plasma IL-1 $\beta$  concentration in rats treated with HE, turpentine only (T), T+HE, dexamethasone only (D), D combined with heat stress (D+HE), turpentine and dexamethasone combined without heat stress (TD), and TD+HE. T40, T<sub>c</sub> 40°C; T45, T<sub>c</sub> 45°C. Within each group, the heat-stressed rats are represented by solid diamonds (◆), and the nonheat-stressed rats are represented by solid triangles (▲). \*\* $P < 0.01$ , and \*\*\* $P < 0.001$ , significant change in IL-1 $\beta$  concentrations within each group. † $P < 0.05$  significant difference at the specific time point between the heat-stressed and control rats within each treatment group. For comparisons between the heat-stressed groups, † $P < 0.05$  represents a significant difference from the D+HE rats at the specific time point.

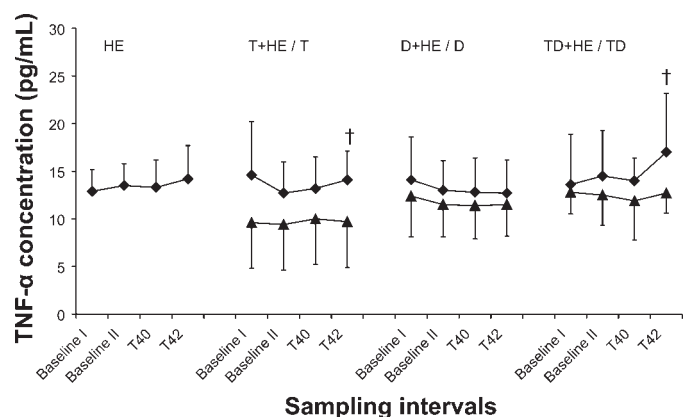


Fig. 5. Mean (SD) of plasma TNF- $\alpha$  concentration in rats treated with HE, T, T+HE, D, D+HE, TD, and TD+HE. Within each group, the heat-stressed rats are represented by solid diamonds (◆), and the nonheat-stressed rats are represented by solid triangles (▲). †Significant difference at the specific time point between the heat-stressed and control rats within each treatment group;  $P < 0.05$ .

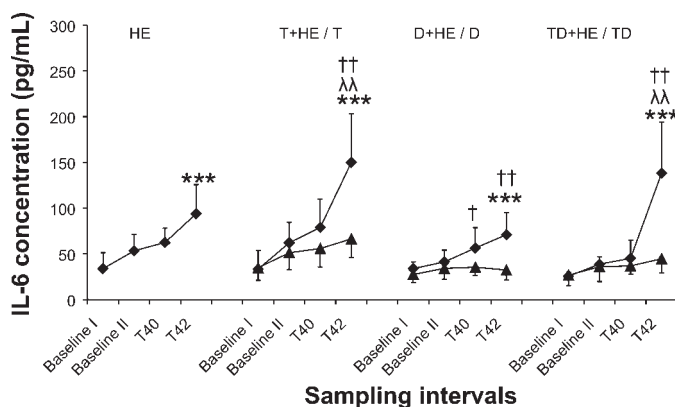


Fig. 6. Mean (SD) of plasma IL-6 concentration in rats treated with HE, T, T+HE, D, D+HE, TD, TD+HE. Within each experiment, the heat-stressed rats are represented by solid diamonds (◆), and the nonheat-stressed rats are represented by black triangles (▲). \*\*\* $P < 0.001$ , significant change in IL-6 concentrations within each group. † $P < 0.05$  and †† $P < 0.01$ , significant difference at the specific time point between the heat-stressed and control rats within each treatment group. For comparisons between the heat-stressed groups,  $\lambda\lambda$  ( $P < 0.01$ ) represents a significant difference from the D+HE rats at the specific time point.

was triggered primarily by endotoxemia in these rats (32). In contrast, the near-resting plasma LPS concentrations and the higher increase in plasma ALT (138%) and AST (190%) concentrations suggest that heat stroke occurred because of heat-induced tissue damage in the TD+HE rats. Heat-induced tissue damage can occur at  $T_c > 42^\circ\text{C}$  in rats and humans (8, 11, 27). These results support the notion that heat stroke can be triggered by endotoxemia and heat-induced tissue damage, and suggest that heat-induced tissue damage can trigger heat stroke in the absence of endotoxemia. However, the role of tissue injury in endotoxemia-driven heat stroke is less clear presently because the increase in ALT and AST concentrations in the HE and T+HE rats could be induced by endotoxemia alone or in combination with the effects of tissue damage.

Our results suggest that turpentine treatment compromised heat tolerance by enhancing the inflammatory response and tissue damage at a given level of heat stress. The concentrations of plasma IL-6 in turpentine-treated rats (T+HE and TD+HE) were significantly higher than in the D+HE rats. IL-6 is an inflammatory responsive cytokine that is stimulated by IL-1 $\beta$  and TNF- $\alpha$  (47). Under heat stress, the concentrations of circulating IL-6 are positively associated with the severity of systemic inflammation and heat stroke (4, 7, 43). The higher IL-6 concentrations in the T+HE and TD+HE rats reflect the more severe heat stroke and inflammatory response in turpentine-treated rats, which are also supported by the higher ALT and AST concentrations, and the shorter duration of survival at T42. In contrast, the increases in the concentrations of plasma LPS, TNF- $\alpha$ , and IL-1 $\beta$  were not significantly different between the HE and T+HE rats, indicating that the elevated LPS, IL-1 $\beta$ , and TNF- $\alpha$  concentrations in the T+HE rats resulted from heat stress and not the turpentine treatment. These results suggest that turpentine treatment may contribute to the inflammatory response during severe heat stress by exaggerating IL-6 response to an increase in IL-1 $\beta$  and TNF- $\alpha$  concentrations resulting from heat-induced endotoxemia. This suggestion is consistent with the observation of a positive

association between IL-6 concentration and the severity of systemic inflammation and heat stroke (4, 7, 43).

Dexamethasone treatment protected the rats from the lethality of heat stroke, possibly by preventing endotoxemia, tissue damage, and the inflammatory response, and this is shown by the responses of plasma LPS, IL-1 $\beta$ , IL-6, AST, and ALT in the D+HE rats. Plasma LPS concentrations increased significantly in the HE and T+HE rats but decreased below resting concentrations in the D+HE rats during the experiment, and AST and ALT concentrations increased significantly in all of the heat-stressed rats, except in the D+HE rats. Plasma IL-1 $\beta$  concentrations increased significantly in all the heat-stressed groups, including D+HE, but comparisons with the respective control groups showed that the increases in IL-1 $\beta$  concentrations were due to the heat stress in the HE, T+HE, and TD+HE rats, but not in the D+HE rats. Plasma IL-6 concentrations increased significantly in all the heat-stressed rats, including the D+HE rats, and these increases were due to the heat exposure in the experiment. However, plasma IL-6 concentrations in the T+HE and TD+HE rats were much higher than in the D+HE rats, whereas IL-6 concentrations were not significantly different between the HE and the D+HE rats. These results suggest that the anti-inflammatory effect of dexamethasone was effective in suppressing, but not in preventing, the increase in IL-6 during severe heat stress. In contrast, turpentine had an additive effect on IL-6 response during severe heat stress. These results support the suggestion that dexamethasone treatment alone protected the rats from heat stress by preventing endotoxemia and tissue damage and suppressing the increase in inflammatory cytokines. The protection against severe heat stress by dexamethasone has been reported before. For example, primates treated with corticosteroid survived  $T_c > 43.5^\circ\text{C}$ , with minimal changes in the concentration of plasma LPS, whereas mortality was  $> 77\%$  and plasma LPS concentration increased by three to fivefold in placebo-treated animals (17, 19). The researchers speculated that corticosteroid treatment protected the primates from the lethality of severe heat stress by maintaining the integrity of the

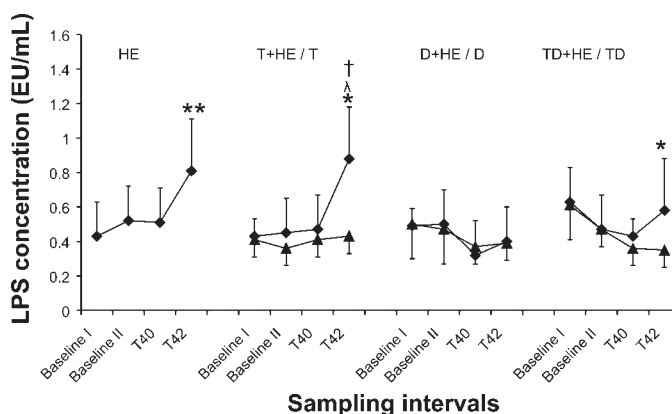


Fig. 7. Mean (SD) of plasma LPS in rats treated with HE, T, T+HE, D, D+HE, TD, and TD+HE. Within each experiment, the heat-stressed rats are represented by solid diamonds (◆), and the nonheat-stressed rats are represented by black triangles (▲). \* $P < 0.05$  and \*\* $P < 0.01$ , significant change in LPS concentrations within each group. †Significant difference at the specific time point between the heat-stressed and control rats within each treatment group,  $P < 0.05$ . For comparisons between the heat-stressed groups, significant difference from the D+HE rats at the specific time point,  $\lambda\lambda P < 0.05$ .



gut-mucosa tissue and by inhibiting gut-related LPS translocation (17, 19).

Dexamethasone treatment increased the survival time of rats during heat stroke by protecting cerebral functions through the suppression of IL-1 $\beta$  concentration in the brain (39). IL-1 $\beta$  causes instability in cardiovascular (13, 38) and cerebral (13, 39) functions during heat stroke in rats (13, 38) and rabbits (37), and inhibition of IL-1 $\beta$  protects the animals from these adverse effects (36–39). In the present study, heat stress induced the production of IL-1 $\beta$  in all of the heat-stressed rats but not in the D+HE rats, and IL-6 response was significantly lower in the D+HE rats than in the HE and T+HE rats. The links between corticosteroid treatment, suppression of IL-1 $\beta$ , and thermal protection were investigated before without taking other factors such as IL-6 and endotoxemia into consideration. Our results suggest that the lower responses of IL-1 $\beta$  and IL-6 in the D+HE rats were due to the inhibition of endotoxemia by dexamethasone treatment and that the suppression of both IL-1 $\beta$  and IL-6 protected the rats from the lethality of heat stroke, possibly by maintaining cerebral and cardiovascular stability.

Although dexamethasone treatment protected the rats from lethal heat stress, the survival rate was the lowest when dexamethasone treatment was combined with turpentine (TD+HE). Dexamethasone treatment inhibited endotoxemia in the TD+HE rats, but these rats had much higher concentrations of plasma IL-1 $\beta$  (65%) and IL-6 (100%) than the D+HE rats. In addition, ALT and AST concentrations were more than twice as high in the TD+HE rats than in the D+HE rats. The contrasting effects of dexamethasone when acting alone and when coupled with turpentine may be explained by its role in the acute phase response (APR). The APR involves the secretion of acute phase proteins from the liver into the circulation in response to proinflammatory cytokines; these proteins promote the repair of tissue subscript injury (6). Acute-phase proteins such as C-reactive protein,  $\alpha_2$ -macroglobulin, and mannan-binding lectin are released and their concentrations increase markedly during inflammation (2); these proteins are also involved in the opsonization of LPS through a cascade of complement activation. However, an exaggeration of the APR contributes centrally to the pathology of heat stroke by triggering the systemic inflammatory response (6). Dexamethasone acts synergistically with catecholamines and proinflammatory cytokines to trigger the APR during an infection (21) and acts independently to induce C-reactive protein production (53). Dexamethasone does not have an independent effect on  $\alpha_2$ -macroglobulin, but  $\alpha_2$ -macroglobulin production is greatest when dexamethasone is combined with turpentine (2). This suggests that the combination of dexamethasone and turpentine may cause an overshoot of the APR and activation of the systemic inflammatory response. This suggestion is supported by the markedly elevated concentrations of IL-1 $\beta$  and IL-6 in the TD+HE rats in the present study. It is possible that heat-induced tissue damaged in the TD+HE rats activated the APR and that the combination of corticosteroid and turpentine caused an exaggerated APR, resulting in the greatly elevated concentrations of proinflammatory cytokines and liver enzymes. The exaggerated APR and inflammatory response would have caused further tissue damage and may have acted in a positive feedback loop to further exaggerate the APR. The elevated concentrations of proinflammatory cytokines and liver

enzymes in the TD+HE rats are consistent with this conclusion.

In conclusion, the results of this study support the proposed dual pathway model of heat stroke, which advocates that heat stroke can be triggered by two separate pathways. One pathway is triggered by heat-induced endotoxemia and the other pathway by heat-induced tissue damage, with both pathways being driven by systemic inflammation. Local inflammation may compromise heat tolerance by exaggerating the proinflammatory cytokine response in the presence of endotoxemia, and endotoxemia-related heat stroke can be prevented by suppressing LPS translocation at the gut mucosa. Dexamethasone alone may protect against endotoxemia-related heat stroke by inhibiting endotoxemia, but may also compromise heat tolerance when combined with turpentine, possibly by contributing to an overdriven APR response. Besides controlling heat strain, the prevention of heat stroke should also consider the state of the immune system and how it may influence proinflammatory cytokine production and anti-LPS mechanisms during exercise and heat stress. Athletes undergoing intense training may have a higher risk of heat stroke because of exercise-induced immune suppression (45), musculoskeletal injuries (51), subclinical infection (44), and gastrointestinal disturbances, which promote the secretion of proinflammatory cytokines in the blood (52) and suppress anti-LPS response during exercise and heat stress (15, 22).

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#### DISCLOSURES

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