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PREVIOUS HEAT SHOCK TREATMENT ATTENUATES LIPOPOLYSACCHARIDE-INDUCED HYPORESPONSIVENESS OF PLATELETS IN RATS

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ABSTRACT—Several studies demonstrated that previous heat shock treatment caused expression of heat shock proteins (HSPs) and reduced organ dysfunction and mortality in experimentally induced severe sepsis. However, the protective mechanism on platelet function remains unclear. The aim of this study was to investigate the effect of heat shock treatment on platelet aggregation *ex vivo* in endotoxin-induced rats with sepsis. Rats of the heated group were heated by whole-body hyperthermia 18 h before lipopolysaccharide (LPS) injection. Blood samples were obtained from the carotid artery 90 min after LPS injection. Platelet aggregation ability was measured by aggregometer. Results revealed that platelet aggregation *ex vivo* was significantly inhibited in LPS-induced rats in a manner of dose dependence. Previous heat shock treatment caused overexpression of HSPs and significantly attenuated the LPS-induced platelet hyporesponsiveness. This attenuation disappeared in accordance with absence of HSP72 at 7 days after heat shock treatment. Aggregation of normal platelets was also inhibited by incubating with plasma obtained from endotoxemic rats but not from preheated endotoxemic rats. Furthermore, no significant hyporesponsiveness was found in endotoxemic platelets in addition to preheated endotoxemic plasma. The addition of H_2O_2 scavenger catalase diminished the platelet hyporesponsiveness significantly only in nonheated endotoxemic rats. Moreover, the plasma nitrite and nitrate levels were significantly attenuated in preheated endotoxemic rats. These results revealed that previous heat shock treatment might attenuate LPS-induced hyporesponsiveness of platelets by changing the plasma components possibly through altering H_2O_2 and nitric oxide concentrations.

KEYWORDS—Heat shock, lipopolysaccharide, sepsis, platelet aggregation, catalase, hydrogen peroxide

INTRODUCTION

The presence of gram-negative bacteria or bacterial products in the circulation initiates a series of inflammatory responses from septic syndrome to multiple organ dysfunction. Although the advancement of anti-inflammatory agents and life-saving equipment has been achieved, the mortality of sepsis remains unchanged at about 30% (1). The most potent inducer in the cascade is lipopolysaccharide (LPS), the major component of endotoxin, released from the lysis of gram-negative bacteria. During endotoxemia, cardiac dysfunction, progressive hypotension, coagulopathies, and organ perfusion defects are common sequences that lead to multiple organ failure. In animal studies, coagulopathy has been reported to be the most critical manifestation after endotoxemia, in which platelet and fibrin deposit in blood vessels and finally, disseminated intravascular coagulation (DIC) occurs. DIC leads to thrombotic and hemorrhagic events, which are the main causes of death in sepsis (2–5). Therapeutically, various blood components, including the donor platelets and coagulation factors, have been administered to prevent or arrest the hemorrhagic episodes (3–5). However, the effect is not promising, and it raises the hypothesis that hyporesponsiveness of platelets might be present in the circulatory circumstance of endotoxemia. Therefore, it is essential to further understand the response of platelets during a severe infection when searching for a better therapeutic intervention to the pathologic condition.

Since 1962, a self-protective phenomenon, a so-called heat shock response (HSR), in almost all living cells and organisms was investigated intensively worldwide. The response was first observed in *Drosophila* embryos by Ritosa et al. (6). Sudden heat stress (termed as hyperthermia for a short time, or heat shock) can induce the expression of a set of proteins in living cells referred to as stress or heat shock proteins, HSPs). They are synthesized constitutively or inducibly under normal and stressed conditions. HSP overexpression allows the cells to tolerate further stresses that might otherwise be lethal (7). Several reports demonstrated that external heating or administration of sodium arsenite result in expression of HSPs and significantly reduce end-organ injury and mortality rate in endotoxemia by LPS or cecal ligation and puncture (8–15). The protective mechanism is still not conclusive. Particularly, the potential role of the HSR or the HSPs overexpression on the platelet function during endotoxemia is seldom mentioned.

The present study was undertaken to examine the platelet responsiveness *ex vivo* in LPS-treated rats and to understand whether heat shock could modulate the effect to the reduction

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in mortality of experimental sepsis. The possible mechanism will be discussed.

MATERIALS AND METHODS

Materials

ADP, pentobarbital, and *Escherichia coli*-derived LPS were obtained from Sigma (St. Louis, MO).

Experimental animals and handling

In conducting the research described in this study, the authors adhered to the guidelines of the National Institutes of Health for the use of the experimental animals, and all experiments were approved by the Committee for the Use of Experimental Animal of Kaohsiung Medical University. Male Sprague-Dawley rats (350–400 g) were purchased from the National Experimental Animal Center (Nan-Kang, Taipei, Taiwan). The Sprague-Dawley rats were anesthetized by intraperitoneal injection of pentobarbital (25 mg/kg). Heat shock treatment was similar to that previously described (15). In brief, rats were heated gently with an electric heating pad to 41°C to 42°C (rectal temperature) for 15 to 20 min. Rectal temperature was kept at 37°C until consciousness was clearly regained. Rats in the nonheated group were also anesthetized, but no heating procedure was performed. Endotoxemic rats were anesthetized by pentobarbital (25 mg/kg, i.p.) and achieved by femoral venous injection of lipopolysaccharide (2 and 5 mg/kg) 18 h after heat shock treatment. The sham group was treated by injection of the same volume of normal saline. Blood samples were drawn from the carotid artery under anesthesia 90 min after LPS injection. All rats were divided into five subgroups: control rats without heat shock and LPS treatment (n = 16); heat-pretreated rats without LPS treatment (n = 16); nonheated, endotoxemic rats (n = 18); preheated, endotoxemic rats (1st day after heat shock; n = 14); and endotoxemic rats 7th day after heat shock treatment (n = 6).

Hematological parameters

Erythrocyte count (red blood cells [RBC]), leukocyte count (white blood cells [WBC]), platelet count, hematocrit, and hemoglobin concentration (Hb) from arterial blood were determined by an electronic counters (Sysmex microcellcounter F-800; Toa Medical Electronics, Kobe, Japan).

Platelet aggregation ex vivo

Blood samples were drawn from the carotid artery and collected into a tube containing sodium citrate (3.8%; 1 v for 9 v of blood) 90 min after LPS injection. Platelet-rich plasma (PRP) was prepared by centrifugation at 1000 rpm for 10 min at room temperature. Platelet-poor plasma (PPP) was prepared by centrifugation at 3000 rpm for 5 min. There were no significant differences of WBC ($0–0.1 \times 10^3$ /μL) in PRP and PPP among rats of control, heat-pretreated, nonheated endotoxemic rats and preheated endotoxemic groups. Platelet aggregation of PRP was measured by Aggregometer (PACKS-4 platelet aggregation chromegenic kinetic system; Helena Laboratories, Beaumont, MT) at 37°C induced by 2.5 μM ADP. The tests were performed at 37°C in 250 μL of PRP in a siliconized cuvette with continuous stirring. The platelet count in the PRP was adjusted to 400,000/μL by dilution with PPP as needed. *Ex vivo* data of platelet aggregation are presented as the percentage of aggregation relative to PPP.

HSP analysis

The liver, kidney, and leukocytes were selected to be the target cells for identifying a successful HSR. The protein expression of HSP72 was detected by Western immunoblotting analysis. Total proteins were extracted and separated by 10% SDS-polyacrylamide gel electrophoresis, and the proteins were then transferred to a polyvinylidene difluoride nitrocellulose membrane with a semidry transblot apparatus for 1 h. The nitrocellulose membrane was soaked in 5% milk-t Tris-buffered saline blocking solution at room temperature for 1 h, and was then incubated with monoclonal anti-HSP72 antibody as the primary antibody. After washing twice, the membranes reacted with 5% milk-t Tris-buffered saline containing goat anti-mouse immunoglobulin G (from goat) as the secondary antibody. The target protein band was visualized by an enhanced chemiluminescence system (Amersham International, Buckinghamshire, UK) (15–16).

Assay of plasma nitrite and nitrate

Five-hundred microliters of heparinized blood (5 U/mL heparin) was centrifuged at 3000 rpm for 15 min at 4°C. The plasma was collected by recentrifugation at 14,000 rpm for 10 min to discard blood cells. The nitrite and nitrate were measured by an NOX Analyzer (ENO-20; Eicom Corporation, Kyoto, Japan).

Statistics

Ex vivo data of platelet aggregation are presented as the percentage of aggregation relative to PPP. Data are expressed as the mean ± SEM. Significance was determined by *t* test, and a *P* value less than 0.05 was considered as a significant difference.

RESULTS

Hematological parameters

All parameters, including counts of RBC, WBC, platelets, and Hb, were measured. LPS treatment induced a decrease in WBC count, whereas heat pretreatment significantly attenuated the leukopenic response ($3.0 \pm 0.7 \times 10^3$/μL vs. $5.4 \pm 2.2 \times 10^3$/μL, n = 11, $P < 0.05$). There were no significant differences of RBC, Hb, and platelet counts among rats of control, heat-pretreated, nonheated, endotoxemic and preheated, endotoxemic groups.

Platelet responsiveness ex vivo to ADP in LPS-treated rats without or with previous heat shock treatment

As shown in Figure 1, heat shock pretreatment did not affect the aggregation ability of platelets in response to ADP, 69% ± 10% (n = 16) of control rats versus 65% ± 12% (n = 16) of the heat-pretreated rats. The aggregation response was dose dependently inhibited to 26% ± 12% (LPS, 2 mg/kg, n = 14) and 18% ± 4% (LPS, 5 mg/kg, n = 4) in nonheated, endotoxemic rats, respectively. However, with previous heat shock treatment, the platelet hyporesponsiveness revealed in endotoxemic rats was significantly diminished to 53% ± 10% (LPS, 2 mg/kg, n = 14, $P < 0.01$) and 44% ± 12% (LPS, 5 mg/kg, n = 4, $P < 0.01$) in preheated rats. In addition, the attenuation effect disappeared 7 days after heat shock treatment (n = 6), in coincidence with the absence of HSP72 (Fig. 2).

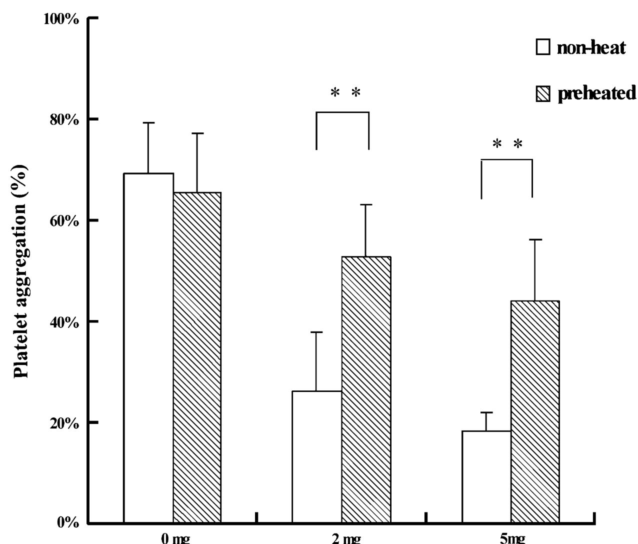

FIG. 1. **Effect of previous heat shock treatment on the *ex vivo* platelet hyporesponsiveness of endotoxemic rats induced by 2 mg/kg LPS (n = 14) and 5 mg/kg LPS (n = 4).** Heat shock treatment was performed 18 h before LPS administration. Platelet-rich plasma was triggered by ADP and the aggregation ability was measured by aggregometer. $^{**}P < 0.01$ by *t* test.

Role of plasma on the influence of platelet hyporesponsiveness in LPS-treated rats without or with previous heat shock treatment

To further define the candidate factors modulating the platelet hyporesponsiveness in endotoxemic rats by previous heat shock treatment, cross-reactions of plasma and platelets from opposite groups were evaluated separately. Plasma (100 μL) obtained from PPP of control, nonheated, endotoxemic and preheated, endotoxemic rats were reacted with PRP (250 μL) separated from normal rats for 5 min. Similar to the findings from the above experiment, nonheated, endotoxemic plasma inhibited the response to 32% ± 7% (n = 6) compared with 62% ± 5% of control plasma ($P < 0.05$). As expected, plasma from preheated, endotoxemic rats only showed a very mild inhibitory response to ADP (Fig. 3). Plasma from nonheat-treated and heat-pretreated without LPS treatment could alone prevent platelet hypoaggregation (figure not shown). Furthermore, cross-interactions of preheated endotoxemic plasma with nonheated endotoxemic platelets or nonheated endotoxemic plasma with preheated endotoxemic platelets confirmed the modulating factors seem in the plasma. It indicated that no matter where the platelets come from, their response decreased in the presence of nonheated endotoxemic plasma (Fig. 4).

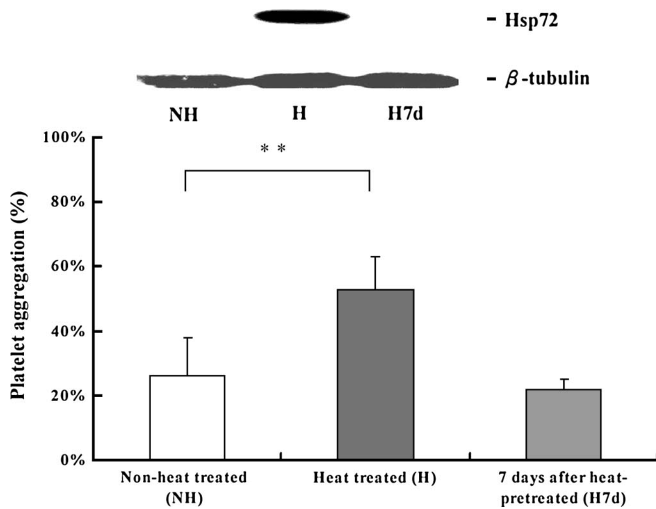

FIG. 2. **Correlation of HSP72 synthesis and the *ex vivo* hyporesponsiveness of platelets induced by intravenous endotoxin treatment.** The measurements were performed 90 min after LPS (2 mg/kg) injection. Top panel, Representative result of immunochemical study of HSP72 in the liver obtained from different group of rats. NH, Nonheated endotoxemic rats (n = 14); H, endotoxemic rats 18 h after heat shock treatment (n = 14); H7d, endotoxemic rats 7 days after heat treatment (n = 6). β-Tubulin was selected as the internal control. Bottom panel, Quantitative analysis of platelets aggregation activity measured as described in Figure 1. $^{**}P < 0.01$ by t test.

Effects of catalase on the* ex vivo *platelet hyporesponsiveness to ADP in endotoxemia rats

To investigate whether the hyporesponsiveness of platelets to ADP was from H_2O_2, human erythrocyte catalase, an H_2O_2 scavenger, was premixed with the different PRP at 37°C for 3 min. As shown in Figure 5, the addition of catalase (100 U/mL) enhanced the aggregation ability in all samples with various intensity. Samples from LPS-induced endotoxemia showed a significant increment (26% ± 12% vs. 58% ± 8%, n = 6, $P < 0.01$) after the addition of catalase compared with that from the preheated, endotoxemic group (53% ± 10% vs. 55% ± 4%, n = 6). The results indicated that previous heat shock treatment may have attenuated endotoxemic platelet hyporesponsiveness due to H_2O_2 content.

Nitric oxide (NO) release in LPS-induced endotoxemia without or with previous heat shock treatment

NO production was chosen as the other potential factor that might influence the phenomenon mentioned above. Nitrate and nitrite were measured in the plasma from different groups of rats by an NOX Analyzer. As shown in Figure 6, the plasma nitrite and nitrate level increased 90% ± 14% (n = 6) in nonheated, endotoxemic rats 90 min after LPS challenge, whereas only 16 ± 20% (n = 6) could be detected in the preheated, endotoxemic group ($P < 0.05$). The plasma nitrite and nitrate level in endotoxemic plasma was significantly decreased by previous heat shock treatment.

DISCUSSION

HSR induces synthesis of a family of proteins named HSP, most quantitatively the HSP72 in mammalian cells. The induction of HSPs expression is crucial for the survival of cells and, ultimately, organisms under stress conditions. The possible

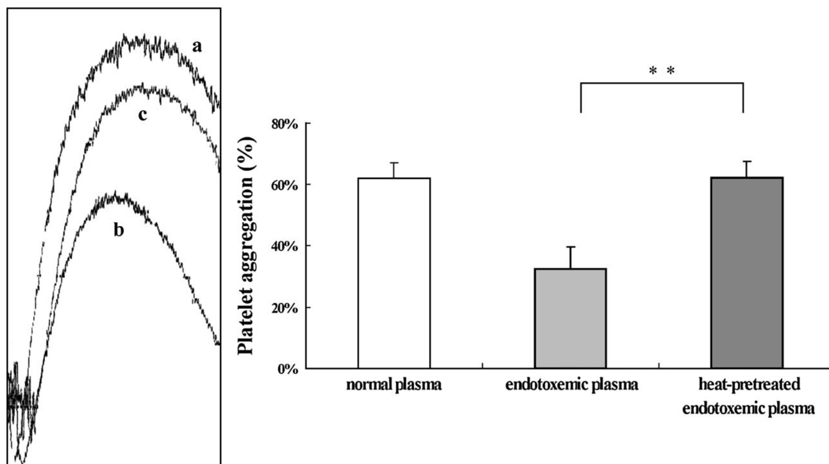

FIG. 3. **Effect of endotoxemic plasma on the normal platelets aggregation activity.** Left panel, Representative real-time aggregation curves obtained from aggregometer. (a) Normal platelets in normal plasma; (b) normal platelets in endotoxemic plasma; (c) normal platelets in preheated endotoxemic plasma. Right panel, Quantitative analysis of platelets aggregation activity measured as described in Figure 1 (n = 6 for each group). $^{**}P < 0.01$ by t test.

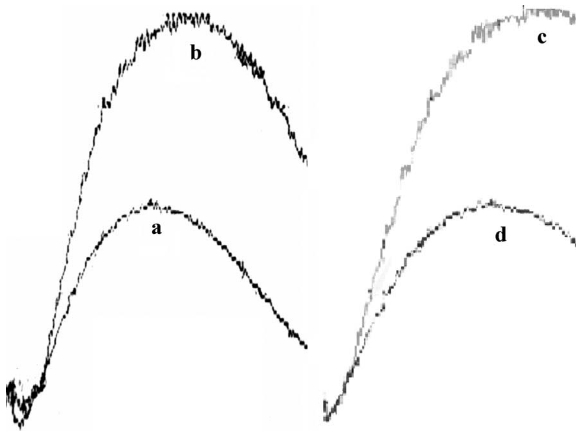

Fig. 4. **Crossed interaction of platelets and plasma from nonheated or preheated endotoxemic rats.** Platelets from LPS (2 mg/kg)-injected rats were kept in plasma of LPS-injected rat (a) or mixed with plasma from preheated endotoxemic animals (b); platelets from preheated endotoxemic animals were kept in plasma of preheated endotoxemic animals (c) or mixed with plasma from LPS-injected rat (d). The results indicated the modulators influencing the decrement effect of platelet hyporesponsiveness are in the plasma.

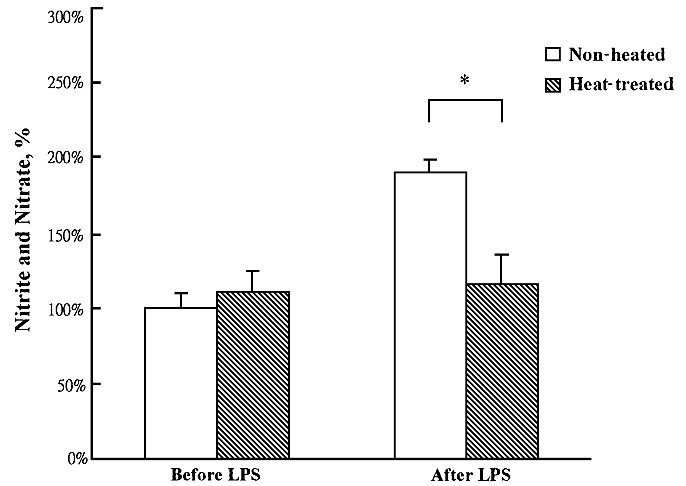

Fig. 6. **Synthesis of nitric oxide influenced by LPS (2 mg/kg) in nonheated or preheated endotoxemic rats.** Data were expressed as relative percentage compared with those of nonheated normal rats. C, control rats (n = 5); H, heat-pretreated rats (n = 5); L, nonheated endotoxemic rats (n = 5); HL, preheated endotoxemic rats (n = 5). $^*P < 0.05$ by *t* test.

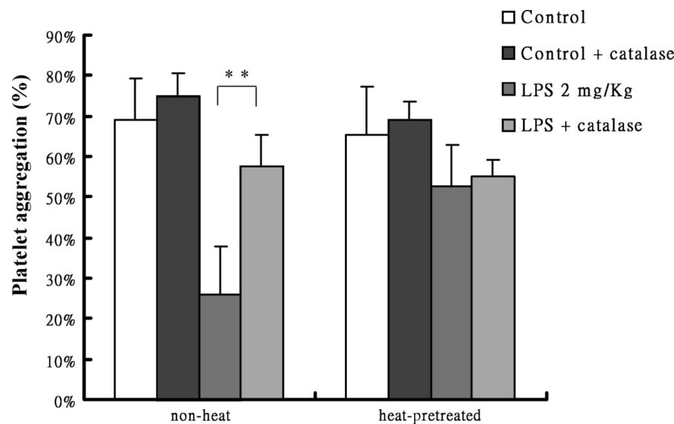

Fig. 5. **Effect of catalase (hydrogen peroxide scavenger) on the reservation of platelet hyporesponsiveness induced by LPS (2 mg/kg) treatment.** Catalase was added into the samples 3 min before ADP triggering. $^{**}P < 0.01$ by *t* test. The results indicated the previous heat shock treatment may attenuate endotoxemic platelet hyporesponsiveness through H_2O_2 content.

mechanisms underlying the protective effect include their function as molecular chaperones by refolding and renaturing the damaged proteins. HSPs may also stabilize or modulate the assembly of the cytoskeleton and provide protection for their constituent proteins inside the cell (17). Even in the increasing number of articles, the influence of HSPs on platelets in sepsis is seldom mentioned. In this study, we demonstrated that intravenous injection of LPS caused platelet hyporesponsiveness *ex vivo* to ADP and that the effect could be attenuated by previous heat shock treatment. Furthermore, the protective effect disappeared 7 days after heat shock treatment, coincident with the absence of HSPs. It was clear that the attenuation of platelet hyporesponsiveness was highly correlated with the effect of previous HSR. In addition, the factors participating in the phenomenon seemed not to be the HSPs or platelet itself, but might be present in the plasma, which synthesis or/and release were influenced by heat shock.

Generally, the administration of LPS is characterized by activation of the humoral cascade system, which might subsequently influence the platelet aggregation ability directly or indirectly. Activation of coagulation cascade during the inflammation is well known to be the major trigger of DIC. However, hyporesponsiveness of platelets during infection has been also reported in several articles (18–21). Recently, Yaguchi et al. (18) found that patients with sepsis had consistently decreased platelet aggregation compared with controls, regardless of the platelet count, thrombin generation, or overt DIC status. The severity of sepsis correlated to the platelet aggregation defect. Cicala et al. (19) additionally demonstrated that when platelet aggregation was evaluated on PRP obtained from rats 4 h after endotoxin administration, platelet response to ADP and collagen was significantly reduced. In the present study, we also revealed that the platelet hyporesponsiveness effect definitely occurred as early as 1.5 h after LPS administration, whereas the platelet count was unchanged. However, the massive aggregations of platelets and the appearance of massive microhemorrhages were found by 1 h from the *in vivo* microscopic observation of mesenteries of LPS-treated rabbit (2). Accordingly, it was suggested the fact of endotoxin on platelet function display a continuous and progressive phenomenon that timing factor should be considered in this hyporesponsive effect and needed to be further investigated.

It has been reported that the antiplatelet activity of LPS may be involved in two pathways: conformational changes in platelet membrane and activation of the NO/cyclic GMP formation. Recently, other molecular mechanisms have been evaluated. For instance, the phosphoinositide breakdown and the intracellular Ca^{++} mobilization in human platelets stimulated by collagen were inhibited by LPS dose dependently. Sheu et al. (20, 21) further showed that protein kinase C activation was markedly inhibited by LPS. Thromboxane A2 formation was also inhibited by endotoxin administration (20, 21). In our study, we found the

platelet itself seems not influence the effect significantly, based on the results that normal aggregation activity could be obtained in the LPS-administered platelet suspended in normal plasma. Similar results have also been reported by Cicala et al. (19) demonstrating that during endotoxemia, platelets are functionally unaltered and that platelet hyporesponsiveness is only observed in the presence of plasma by using an *ex vivo* platelet aggregation test in rats. Therefore, the alteration of plasma components might play a more potential role in the hyporesponsive effect of platelet in endotoxemia.

Many humoral changes happen in endotoxemic animals, including the most potent factors influencing the platelet aggregation such as platelet-activating factor or thrombin. However, both of them were unlikely the key factors because the LPS-induced platelet hyporesponsiveness could not be modulated *in vivo* or in *ex vivo* experiments when animal pretreated platelet-activating factor antagonist or thrombin inhibitor (19). One of the other potent factors that must be considered is NO. During gram-negative shock, LPS triggers the release of cytokines, which, in turn, induce inducible NO synthase (iNOS), leading to high output of NO and subsequently inhibiting human platelet adhesion to vascular endothelium, which may facilitate hemorrhage. HSR has been reported to inhibit expression of NF-κB and inhibit expression of iNOS, leading to inhibition of overproduction of NO (22). Our results also show that previous heat shock treatment attenuated platelet hyporesponsive effect caused by LPS, with significant decreases in the plasma nitrite and nitrate levels. However, Cicala et al. (19) have reported that the aggregation ability did not change after pretreating the rats with L-NAME before LPS. It was suggested that NO seems unlikely to affect the response directly.

Recently, H_2O_2 has been reported to be a potent factor affecting the platelet aggregation ability. Exposure to larger (but not toxic) concentrations of exogenous H_2O_2 may lead to inhibit aggregation of platelets by ADP and several agonists (23). In addition, if NO and H_2O_2 were mixed before the addition to platelets, the inhibitory effect remained but still depended on the presence of NO and suggested an enhanced sensitivity of the platelets to residual NO in the presence of H_2O_2 via stimulation of guanylate cyclase and increase cGMP formation. H_2O_2 may play a positive physiological role by amplification and/or prolongation of the action of NO (24–26). LPS activates granulocytes and subsequently, H_2O_2 was released by the activated granulocytes. This fact may support our hypothesis that H_2O_2 generated by LPS may be involved in the platelet hyporesponsiveness observed only in the presence of endotoxic plasma. Furthermore, the addition of catalase (a hydrogen peroxide scavenger) significantly regained the aggregation ability of platelets in endotoxemia, making it nearly reach the level of intact rats. On the contrary, only little activity was gained in intact or preheated group in addition of catalase. These results provide the clue that a larger amount of H_2O_2 might be produced in the nonheated, LPS-injected rat and it may play a crucial role influencing the aggregation ability. Heat shock pretreatment may attenuate the serum H_2O_2/NO content, or H_2O_2/NO synergistic effect, and finally the hyporesponsiveness of platelet.

Although the pathophysiology of sepsis has not been fully elucidated, oxidative stress associated with excessive systemic inflammation plays an important role in its pathogenesis. Leukocyte-dependent endothelial injury may occur as a consequence of leukocyte adhesion to endothelial cells and subsequent migration into tissue injury during the inflammation response. Such leukocytes may be activated in the process. Endothelial cell injury is frequently mediated by activated neutrophils involving the generation of reactive oxygen metabolites, principally H_2O_2 within the target cells (27). Thermotolerance could prevent LPS-activated, neutrophil-mediated endothelial cell injury and could significantly reduce leukocyte adhesion and migration at 30, 60, and 90 min after LPS administration and attenuate LPS-induced microvascular injury (28). The present study showed that LPS induced a marked decrease in WBC count in rats with sepsis, whereas heat-pretreatment improved the LPS-induced neutropenia. It means that the decrease of H_2O_2 and protective effect by heat shock pretreatment might not only be due to the number of leukocytes, but also to the activation state of leukocytes. Besides, an increasing number of reports have revealed that the presence of HSPs might influence the synthesis of various cytokine mediators *in vivo* or *in vitro* during a cascade of sepsis (29–32). Some reports have shown that exposure to hyperthermia induced transient inhibition of the superoxide-generating enzyme NADPH oxidase in neutrophils (29–32) and induce the increase of the cytoprotective protein catalase (31, 32). It was suggested that inhibition of NADPH oxidase and increase of catalase activity by heat shock in sepsis might represent an intrinsic cellular mechanism to down-regulate H_2O_2 production. The real mechanism needs further investigation.

In conclusion, these findings showed that previous heat shock treatment attenuated LPS-induced platelet hyporesponsiveness. We suggested that the possible protection on platelet function is mediated by an inhibition in the production of H_2O_2 and NO circulating in the endotoxemic plasma, H_2O_2/NO synergistic effects, and finally the hyporesponsiveness of platelets.

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