Lipopolysaccharide-stimulated Leukocytes Contribute to Platelet Aggregative Dysfunction, Which is Attenuated by Catalase in Rats

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Endotoxemia causes several hematological dysfunctions, including platelet degranulation or disseminated intravascular coagulation, which lead to thrombotic and hemorrhagic events. Here, we tested the hypothesis that bacterial lipopolysaccharide (LPS)-stimulated leukocytes contribute to platelet aggregative dysfunction, and this function is attenuated by antioxidants. Platelet-rich plasma (PRP) was prepared from whole blood of normal and endotoxemic rats. The ability of platelet aggregation was measured by an aggregometer. LPS (50–100 μg/mL) was incubated with PRP, whole blood and PRP with polymorphonuclear leukocytes (PMNs) for 30 minutes, 60 minutes and 90 minutes, and platelet aggregation was detected. LPS-induced platelet aggregative dysfunction was undetectable in intact PRP which was isolated from normal whole blood, whereas it was detected in PRP isolated from endotoxemic rats and LPS-treated whole blood. Moreover, the effect of LPS-induced platelet aggregative dysfunction on intact PRP was observed when the PMNs were added. LPS-induced platelet aggregative dysfunction was significantly attenuated by catalase alone and in combination with N^G-nitro-L-arginine methyl ester, but not by N^G-nitro-L-arginine methyl ester alone. These results indicate that LPS-stimulated PMNs modulate platelet aggregation during LPS treatment and the effects are reversed by antioxidants. PMNs serve as an approach to understand LPS-induced platelet aggregative dysfunction during endotoxemia. During this process, the generation of reactive oxygen species, hydrogen peroxide especially, from LPS-stimulated PMNs could be an important potential factor in LPS-induced platelet aggregative dysfunction. Catalase contributes to the prevention of platelet dysfunction during LPS-induced sepsis.

Key Words: catalase, lipopolysaccharide, platelet aggregation, polymorphonuclear leukocytes, sepsis

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Lipopolysaccharide (LPS) is derived from the outer membrane of Gram-negative bacteria, and is one of the major active components that stimulate a series of inflammatory responses during infection, which initiates septic syndrome, multiple organ dysfunction and systemic failure [1–3]. In the course of this, coagulation activation that leads to disseminated intravascular

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coagulation (DIC) could be regarded as an important factor in promoting multiple organ failure. DIC is a systemic thrombotic–hemorrhagic disorder that leads to microvascular failure by obstructing the blood supply in various organs [4–6]. After the development of pathogenic cascades, hemorrhage is the most frequent and common presentation in DIC [7,8]. Coagulation abnormalities and thrombocytopenia are commonly observed in severe sepsis. Platelets could be considered to be closely associated with blood coagulation failure. Bleeding patients with consumption coagulopathy are most frequently treated with platelet transfusions and various plasma products including fresh frozen plasma and coagulation factor concentrates [9,10]. However, the effects are limited and the mortality rate in severe sepsis remains high.

Platelets as key players in coagulation might have been somewhat neglected with regard to these new insights into the pathogenesis of coagulation abnormalities in sepsis. Therefore, it is essential to understand further the response of platelets during severe infection, when we are searching for a better therapeutic intervention. Platelets have a complex function in numerous inflammatory and infective conditions, including sepsis; they are able to modulate not only their own function, but also that of other cells around them.

Platelets can combine with leukocytes to form platelet–leukocyte aggregates [11]. Such an association is regarded as an important initiating factor in the inflammatory cascade [12,13]. Activated leukocytes also can influence platelet function. Levine et al have reported that phagocytosis of granulocytes exerts a marked inhibitory effect on platelet function. The granulocytes could release factors that are capable of crossing a dialysis membrane and inhibiting platelet aggregation [14]. Furthermore, polymorphonuclear leukocytes (PMNs) can exert an antithrombotic influence by inhibition of platelet activation [15–17]. Therefore, to clarify the role of leukocytes in modulating platelet function during sepsis, it is helpful to explore new avenues in bleeding diathesis of septicemic and endotoxemic patients.

The aim of the present study was to investigate the effect of interaction of platelets with leukocytes, especially PMNs, on LPS-treated platelet aggregative response and the possible effects of antioxidants. We dealt with two questions. First, could leukocytes influence platelet function in sepsis? Second, could such an interaction be mediated, at least in part, by leukocyte production of hydrogen peroxide and nitric oxide?

Methods

Materials

ADP, catalase, *Escherichia-coli*-derived LPS, N^G-nitro-L-arginine methyl ester (L-NAME), and pentobarbital were obtained from Sigma (St. Louis, MO, USA).

Animals

We adhered to the guidelines of the National Institutes of Health for the use of experimental animals and all experiments were approved by the Committee for the Use of Experimental Animals of Kaohsiung Medical University. Male Sprague–Dawley rats (350–400 g) were purchased from the National Experimental Animal Center (Nan-Kang, Taipei, Taiwan). The rats were anesthetized by intraperitoneal injection of pentobarbital (25 mg/kg). Blood samples were drawn from the carotid artery.

Ex vivo experiments

Rats were injected through the femoral vein with either LPS (5 mg/kg) or an equal volume of phosphate-buffered saline for 90 minutes. Blood samples were drawn from the carotid artery and collected into a tube that contained sodium citrate (3.8%; 1 volume for 9 volumes of blood).

In vitro experiments

The experimental conditions were divided into five groups: (1) control: platelets from intact whole blood ($n=12$); (2) Platelet+LPS: platelets from intact whole blood treated with LPS (final concentration 50 μg/mL and 100 μg/mL) for 30 minutes, 60 minutes and 90 minutes ($n=8$ for each condition); (3) Platelet-WB+LPS: platelets from whole blood treated with LPS for 30 minutes, 60 minutes and 90 minutes ($n=10$ for each condition); (4) Platelet+PMN (3,500 cells/μL, $n=4$); (5) Platelet+LPS+PMN: LPS and PMNs (3,500 cells/μL) from intact whole blood were added to intact platelets during LPS treatment for 30 minutes, 60 minutes and 90 minutes ($n=4$ for each condition). The whole blood, platelets and PMNs were collected and utilized within 2.5 hours.

Isolation of PMNs

Peripheral blood was obtained from the carotid artery. Fresh heparinized blood was diluted 1:1 with phosphate-buffered saline and layered over Ficoll-Paque Plus solution (Amersham Biosciences Corp.,

Piscataway, NJ, USA) and centrifuged at 1,500 rpm for 30 minutes at room temperature. The erythrocyte/PMN-containing pellet was diluted 1:1 with normal saline. Erythrocyte sedimentation was achieved by diluting the suspension 1:10 with 6% dextran and incubating for 30 minutes at 37°C. Red blood cells were lysed with Tris–NH_4Cl solution. PMNs were collected and counted by an electronic counter.

Detection of platelet aggregation

Platelet-rich plasma (PRP) with a leukocyte count of 0–100/μL was prepared by centrifugation at 1,000 rpm for 10 minutes at room temperature. Platelet-poor plasma (PPP) was prepared by centrifugation at 3,000 rpm for 10 minutes. Platelet count was determined by an electronic counter (Sysmex microcell-counter F-800; Toa Medical Electronics, Kobe, Japan). The platelet count in the PRP was adjusted to 400,000/μL by dilution with PPP as needed [18]. Platelet aggregation in PRP was induced by 2.5 μM ADP and measured with an aggregometer (PACKS-4 platelet aggregation chromogenic kinetic system; Helena Laboratories, Beaumont, TX, USA). The tests were performed at 37°C with 250 μL PRP in a siliconized cuvette with continuous stirring. Catalase (25 U/mL, 50 U/ml or 100 U/mL) and L-NAME (0.1 mg/mL or 0.5 mg/mL) were added to the reaction tube 90 minutes after incubation with platelets, PMNs and LPS. Catalase and L-NAME were added to the reaction tube 3 minutes before triggering with 2.5 μM ADP.

Statistical analysis

The data for platelet aggregation were presented as percentage aggregation relative to PPP. Data were expressed as mean ± standard error of the mean. Significance was determined by the *t* test and $p < 0.05$ was considered to be statistically significant.

Results

Catalase prevents ex vivo platelet aggregative dysfunction in endotoxemic rats

Platelet aggregation was evaluated at 90 minutes after treatment with 5 mg/kg LPS. ADP-induced aggregation was inhibited significantly in platelets derived from endotoxemic rats compared with those derived from control rats (Figure 1). Catalase, at a dose of at least 50 U/mL, contributed to the attenuation of platelet hyporesponsiveness in endotoxemic rats, in a dose-dependent manner.

Platelet aggregative dysfunction cannot be induced directly in vitro by LPS

Platelets were isolated from normal whole blood of rats. The effects of LPS on platelet aggregation were detected *in vitro* (Figure 2). The platelet aggregative responses of 50 μg/mL LPS were 65.8 ± 7.6%, 69.0 ± 9.6% and 67.9 ± 11.8% after 30 minutes, 60 minutes and 90 minutes incubation, respectively. The platelet aggregative responses of 100 μg/mL LPS were 71.4 ± 10.3%, 69.3 ± 9.8% and 68.6 ± 10.3% after 30 minutes, 60 minutes and 90 minutes incubation, respectively. There were no differences between the experimental and control groups. LPS by itself did not lead to platelet aggregative dysfunction within 90 minutes.

LPS-induced aggregative inhibition on platelets isolated from LPS-treated whole blood

Whole blood was derived from normal rats and treated with 100 μg/mL LPS for 30 minutes, 60 minutes and 90 minutes. Platelets were isolated from normal and LPS-treated whole blood and platelet aggregation was determined. LPS did not inhibit aggregation of platelets isolated from normal whole blood. Aggregation

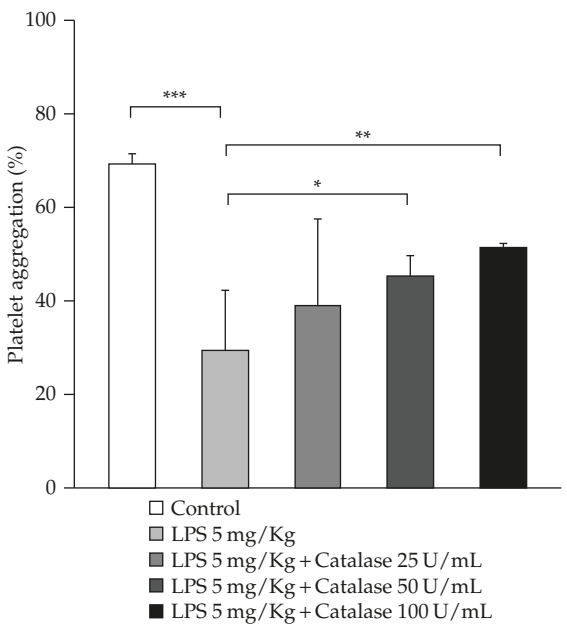

Figure 1. *Effect of catalase on platelet aggregation ex vivo. Platelets were isolated from rats with lipopolysaccharide (LPS)-induced endotoxemia (n = 4). *p < 0.05, **p < 0.01 versus LPS, ***p < 0.001.*

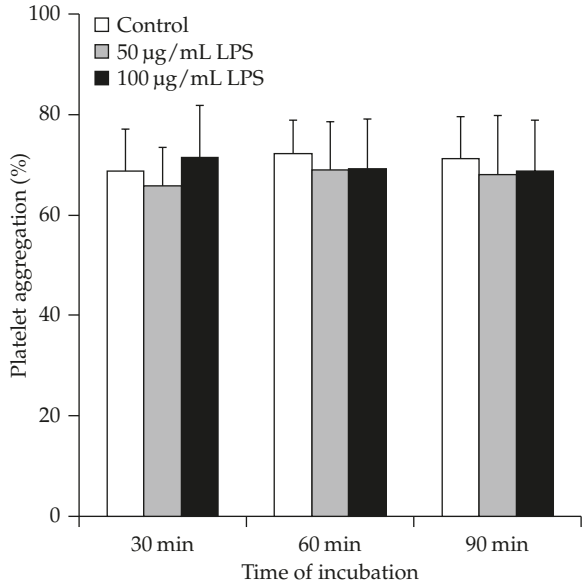

Figure 2. *The timing and dose effect of lipopolysaccharide (LPS) on platelet aggregation in vitro. Various doses of LPS (50 μg/mL and 100 μg/mL) were utilized and incubated with platelets for 30 minutes, 60 minutes and 90 minutes (n = 10).*

of platelets from whole blood treated with LPS for 30 minutes and 60 minutes was unaffected. However, platelet aggregation was significantly decreased to 37.5 ± 11.4% ($n = 8$) at 90 minutes after treatment with LPS (Figure 3). The platelet aggregative response to ADP was significantly inhibited when platelets were isolated from LPS-treated whole blood.

Catalase prevents LPS-induced inhibition of platelet aggregation from LPS-treated whole blood

Platelets were isolated from normal and LPS-treated whole blood. LPS-induced inhibition of aggregation was detected in platelets isolated from LPS-treated-whole blood (37.5 ± 11.4%) and the effect was significantly attenuated (67.5 ± 5.9%) by 100 U/mL catalase (Figure 4). Catalase by itself did not affect platelet aggregation.

LPS-induced inhibition of platelet aggregation is associated with PMNs

PMNs and platelets were isolated from normal whole blood of rats. To clarify the influence of PMNs on platelet function during LPS-induced endotoxemic sepsis, PMNs were added to the platelet aggregative reactions (Figure 5). Addition of PMNs alone did not affect platelet aggregation (69.6 ± 4.8%). However, when the PMNs were added, platelet aggregation was

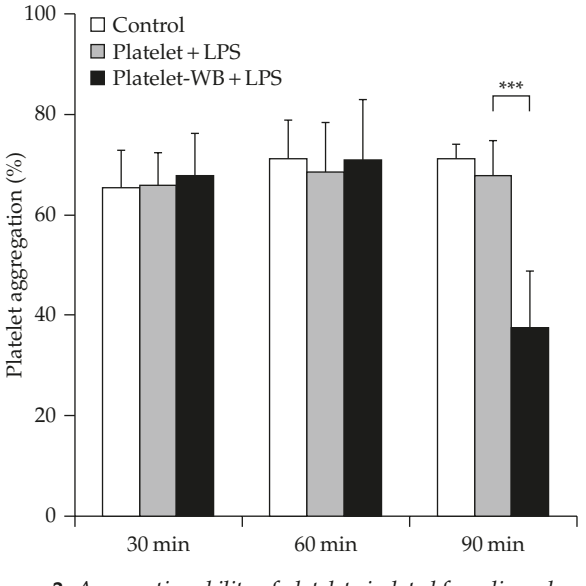

Figure 3. *Aggregative ability of platelets isolated from lipopolysaccharide (LPS)-treated whole blood. LPS (100 μg/mL) was added to whole blood for 30 minutes, 60 minutes and 90 minutes (n = 8). Control: platelets from intact whole blood. Platelet + LPS: platelets from intact whole blood treated with LPS. Platelet-WB + LPS: platelets from LPS-pretreated whole blood. ***p < 0.001.*

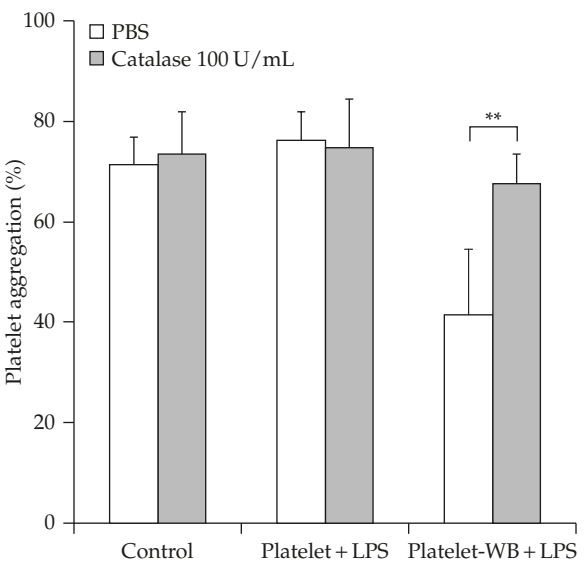

Figure 4. *Catalase on aggregative response of platelets isolated from lipopolysaccharide (LPS)-treated whole blood. LPS (100 μg/mL) was added to whole blood for 90 minutes (n = 8). PBS: volume control. Catalase: catalase-treated group. Control: platelets from intact whole blood. Platelet + LPS: platelets from intact whole blood treated with LPS. Platelet-WB + LPS: platelets from LPS-pretreated whole blood. **p < 0.01.*

inhibited to 40.5 ± 6.4% ($n = 4$) in the platelets containing 100 μg/mL LPS. PMNs might contribute to LPS-induced platelet aggregative inhibition at 90 minutes after platelets co-mixing with PMNs and LPS.

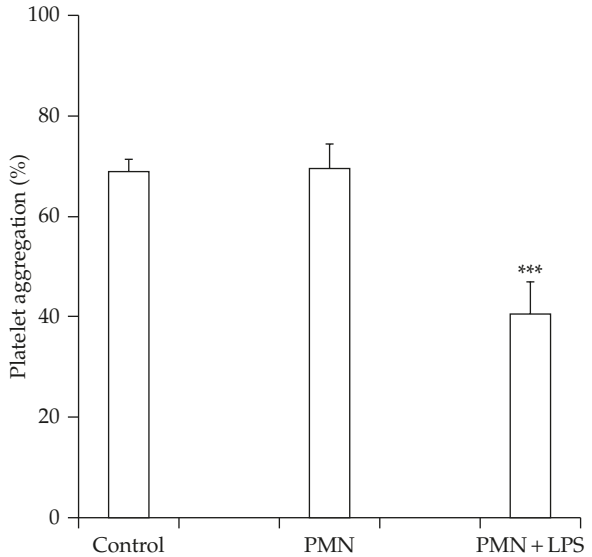

Figure 5. *Effect of polymorphonuclear leukocytes (PMNs) on LPS-induced platelet aggregative inhibition. Platelets from intact whole blood were collected (n = 4). The PMNs (3,500 cells/μL) were contained during LPS treatment for 90 minutes. ***p < 0.001 versus controls.*

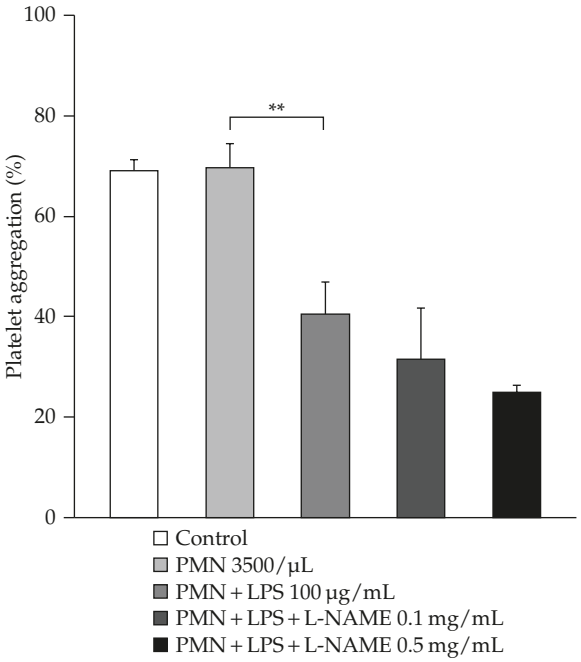

Figure 6. *Effect of N^G-nitro-L-arginine methyl ester (L-NAME) on lipopolysaccharide (LPS)-induced platelet aggregative inhibition. L-NAME was added to the incubation tube at 90 minutes after platelets, polymorphonuclear leukocytes and LPS (n = 4). **p < 0.01.*

Effect of L-NAME on platelet aggregative inhibition induced by LPS-treated PMNs

Different doses of L-NAME were added to platelets, PMNs and LPS. Platelet aggregative inhibition induced by LPS treatment and PMNs was not improved by the addition of 0.1 mg/mL and 0.5 mg/mL L-NAME (31.6 ± 10.2% and 25 ± 1.3%, respectively) (Figure 6).

Synergistic effect of L-NAME and catalase in preventing platelet aggregation inhibition induced by LPS-treated PMNs

When catalase and L-NAME were applied separately at a dose of 25 U/mL and 0.1 mg/mL, respectively, they had no effect on LPS-induced inhibition of platelet aggregation in the presence of PMNs. However, when catalase and L-NAME were applied concomitantly, they synergistically prevented inhibition of platelet aggregation (58.7 ± 4.5%) (Figure 7).

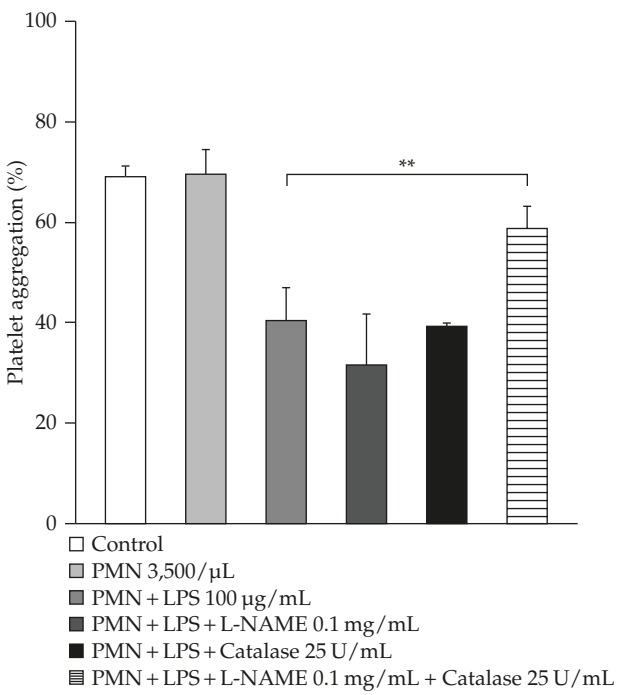

Figure 7. *Effect of N^G-nitro-L-arginine methyl ester (L-NAME) and catalase on LPS-induced platelet aggregative inhibition. L-NAME and catalase were added to the incubation tube at 90 minutes after platelets, polymorphonuclear leukocytes and LPS (n = 4). **p < 0.01.*

Discussion

The present study showed that ADP-induced aggregation of platelets from endotoxemic rats was significantly inhibited *ex vivo*. LPS was considered to be a crucial factor for induction of hyporesponsiveness of

platelet aggregation. LPS-induced aggregative hyporesponsiveness was undetectable on intact platelets. The platelets contained in whole blood and mixed with PMNs were more susceptible to LPS treatment than platelets alone. LPS-induced inhibition of platelet aggregation *ex vivo* and *in vitro* was significantly blocked by catalase. We conclude that LPS-activated PMNs could influence platelet function through reactive oxygen species (ROS) during LPS treatment.

Coagulation abnormalities and thrombocytopenia are common features in severe sepsis. Decreased platelet count could be regarded as a pivotal factor in the pathogenesis of sepsis [9,19]. However, although the functions of platelets in sepsis have been determined, they have yielded conflicting results. The aggregability of platelets is consistently decreased in septic patients compared with controls, regardless of platelet count, thrombin generation, and overt DIC status. The severity of sepsis is correlated with defective platelet aggregation [20]. However, contradictory opinions have been stated. Gawaz et al noted the timing of the increased platelet aggregability in patients [21]. Platelet aggregability is markedly increased in patients with a complicated septic course, but is decreased over time in patients who develop sepsis. The effects of bacterial products on platelet function vary according to the species, timing of the study, and sepsis pathogenesis [22]. Thirty-five minutes after a rabbit received endotoxin (100 μg/kg intravenously), *in vivo* microscopy of the microcirculation discovered massive aggregations of platelets and adhesion of leukocytes in a precapillary that showed pre-stasis [23]. By 1 hour, numerous granulocytes were firmly adhered to the endothelium and paved the venular endothelial lining. Erythrocytes and platelets also adhered to the granulocytes to form mixed thrombi, and platelet aggregates and microthrombi were also seen at that time. The granulocytes released some of their granules and began to undergo diapedesis through the injured blood vessel walls. The leukocytes could have been followed by extravasation of erythrocytes and the appearance of massive micro-hemorrhages [23]. The present study proved that the platelet aggregability induced by ADP was inhibited when platelets were isolated from endotoxemic rats 90 minutes after LPS administration. Endotoxemia-induced downregulation of platelet aggregability can be prevented by catalase in a dose-dependent manner [18]. Oxidative stress during endotoxemia can also participate in modulation of platelet function. LPS induces the accumulation of ROS, especially hydrogen peroxide, in plasma during endotoxemia, and might contribute to reduction of ADP-induced platelet aggregability from endotoxemic rats *ex vivo* [18].

Moreover, the hemostatic function of platelets can be influenced, and coagulation abnormalities and thrombocytopenia are common in severe sepsis [20]. The direct effects of LPS in modulating platelet function need to be clarified. Despite toll-like receptors (TLRs) being activated on platelets and appearing to be responsible for mediating LPS-induced thrombocytopenia *in vivo* [24,25], platelet aggregability cannot be influenced directly by LPS *in vitro*. A previous study showed LPS led to platelet hyporesponsiveness to ADP only in the presence of endotoxemic plasma. Intermediated mediators secreted in plasma are necessary for LPS-induced platelet hyporesponsiveness [18]. The aggregability of platelets isolated from whole blood pre-treated with LPS was decreased. This finding implies that an interaction between blood cells and platelets is involved in sepsis. Moreover, our results showed that the platelet aggregability was significantly decreased when platelets were co-mixed with PMNs and LPS concomitantly. LPS-activated PMNs exerted a marked inhibitory effect on platelet aggregability, whereas no significant inhibitory effect on intact PMNs was seen. We consider that LPS-activated PMNs contribute to the modulation of platelet aggregability in sepsis.

PMNs are cardinal cellular effectors in the innate host response to injury or infection. Besides participation in immune function, leukocytes, such as PMNs, can also be activated by activated platelets and participate in modulation of coagulation and interaction with platelets during inflammatory diseases [11,12]. PMNs can also be activated by adhesive molecules, primarily by P-selectin expressed on platelets. The formation of PMN–platelet complexes enhances inflammation and pathogenesis [12,13]. Moreover, phagocytosing granulocytes have been shown to exert a marked inhibitory effect on platelet function. Granulocytes can release factors that are capable of crossing dialysis membranes and inhibiting platelet aggregation [14]. The present study showed that PMNs contributed to platelet hyporesponsiveness during LPS treatment. The mediators, especially ROS, that were secreted from PMNs were involved in stimulation of platelet hyporesponsiveness during LPS treatment.

PMNs express almost the entire repertoire of TLRs [26]. Among these, TLR4 can recognize the cell-wall constituents of Gram-negative bacteria and is upregulated in sepsis [26,27]. After TLR stimulation, the bactericidal mechanisms in PMNs are initiated, and highly reactive oxygen intermediates are produced and released [28]. These intermediaries are highly reactive with crucial biological molecules and can cause lipid peroxidation and irreversible protein structural modifications. Among these, hydrogen peroxide production is important for platelet hyporesponsiveness during endotoxemia. PMNs can be regarded as an important source of hydrogen peroxide in plasma. Catalase, a hydrogen peroxide scavenger, administered *ex vivo* and *in vitro* prevents downregulation of platelet aggregability in response to ADP after LPS treatment.

Additionally, another PMN-derived factor for the platelet inhibition that has been identified is nitric oxide (NO) [15]. PMN-derived NO can directly inhibit platelet activation by increasing platelet cGMP content [16]. LPS significantly increases nitrate production within a 10-minute incubation period. LPS-induced platelet aggregative inhibition is involved in the activation of a NO/cGMP pathway in platelets [29,30]. NO can react with superoxide to produce peroxynitrite. Peroxynitrite inhibits aggregation of human platelets stimulated by ADP (5 μM), collagen, thrombin and U46619, in a dose-dependent manner (50–200 μM) [31]. Our previous study also has found that the amount of plasma NO is higher than in the controls after intravenous LPS injection [18]. However, L-NAME and catalase were shown to have a synergistic effect in *in vitro* LPS-induced septic study. Platelet hyporesponsiveness to ADP was not attenuated when 20 mg/kg L-NAME combined with 5 mg/kg LPS intravenous injection into rats (16.4 ± 7.6%, LPS with L-NAME *vs.* 25.6 ± 5.5%, LPS; $n = 6$). We suggest that the synergistic effect of L-NAME and catalase in preventing LPS-induced platelet hyporesponsiveness is associated with inhibition of peroxynitrite production. However, the mechanism needs further investigation.

The experiments reported here are an attempt to define a potential interaction between platelets and leukocytes. PMNs can be used to understand LPS-induced platelet aggregative dysfunction during endotoxemia. ROS, especially hydrogen peroxide, that are derived from LPS-treated PMNs could be an important contributing factor to LPS-induced platelet hyporesponsiveness. Catalase contributes to the prevention of platelet functional failure during LPS treatment. The investigation of leukocyte–platelet association offers a new approach for the therapy of sepsis.

In conclusion, the present study indicates that PMNs can be used to help our understanding of LPS-induced platelet aggregative dysfunction during endotoxemia. The generation of ROS, especially hydrogen peroxide, from LPS-stimulated PMNs could be an important factor in LPS-induced platelet aggregative dysfunction. Catalase contributes to the prevention of platelet dysfunction during LPS-induced sepsis.

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過氧化氫酶可以降低脂多醣刺激白血球所導致的血小板凝集功能異常

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導致敗血症最常見的致病菌是格蘭氏陰性桿菌，內毒素（endotoxin）是這些細菌細胞壁上面的脂多醣（LPS）。敗血症會導致血液循環系統障礙，包含血小板去顆粒作用或瀰漫性血管內凝血病變，當產生血管血栓或出血現象時，是導致敗血症的主要死亡原因。因為血液中的白血球和所接觸的血小板無論在生理狀態或病理狀態下，彼此之間皆互有非常強烈的互動關係。所以，在這個實驗中我們想要利用體外實驗來探究：內毒素所誘發的敗血症中，進行吞噬作用的白血球和血小板凝集能力的相互關係。實驗中我們分別將內毒素置入在富含血小板的血漿、全血以及富含血小板的血漿中加入白血球等三種不同樣品於 30 分鐘、60 分鐘、90 分鐘由血小板凝集檢驗儀器測定血小板由 ADP 刺激產生的凝集能力。體外實驗研究結果中，我們發現內毒素（50 μg/mL、100 μg/mL）置入富含血小板的血漿後 30、60、90 分鐘後均不會影響老鼠血小板的凝集功能反應，然而將內毒素分別置入全血及富含血小板的血漿加入白血球 90 分鐘後，卻發現老鼠的血小板的凝集功能反應有顯著下降的現象。然而，當單獨外加一氧化氮合成酶抑制劑 L-NAME 並無法改善敗血症血小板凝集功能下降的現象；但是，當外加過氧化氫酶 Catalase 時，發現可以顯著改善敗血症血小板凝集功能下降的現象。而且，當一氧化氮合成酶抑制劑 L-NAME 合併過氧化氫酶 Catalase 一起作用時，發現可以改善敗血症血小板凝集功能下降的現象更為顯著。由這些研究結果中指出：白血球對敗血症血小板凝集功能下降的現象中扮演一個很重要的角色，白血球影響敗血症血小板凝集功能下降的主要機制很可能是經由活化的白血球所釋放至血漿的過氧化氫（H_2O_2）的作用和過氧化氫影響一氧化氮（NO）的作用。總結：由此實驗發現在敗血症中白血球和血小板有更進一步的互動關係，而且白血球、血小板間的相互關係，可以藉由氧化自由基中過氧化氫的量來調節。

關鍵詞：過氧化氫酶，內毒素，血小板凝集，一氧化氮合成酶抑制劑，敗血症

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