Exogenous carbon monoxide suppresses LPS-induced platelet SNAREs complex assembly and α-granule exocytosis via integrin αIIbβ3-mediated PKCθ/Munc18a pathway

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ABSTRACT

Activation of coagulation occurs in sepsis and contributes to the development of thrombosis. Platelet α-granule exocytosis plays an important role in septic coagulation abnormalities. The present study aimed to investigate the effects and the underlying mechanisms of exogenous carbon monoxide, carbon monoxide-releasing molecules II (CORM-2)-liberated CO, on suppressing platelet α-granule exocytosis in sepsis. It was shown that CORM-2 weakened α-granule membrane fusion with platelet plasma membrane and attenuated α-granule contents exocytosis in LPS-Induced platelet. Further studies revealed that CORM-2 suppressed the expression of integrin αIIbβ3 in platelets stimulated by LPS. This was accompanied by a decrease in production and phosphorylation of PKCθ and Munc18a, SNAREs complex assembly and subsequently platelet α-granule exocytosis. Taken together, we suggested that the potential mechanism of suppressive effect of CORM-2 on LPS-induced platelet SNAREs complex assembly and α-Granule Exocytosis might involve integrin αIIbβ3-mediated PKCθ/Munc18a pathway activation.

INTRODUCTION

Sepsis is characterized by life-threatening organ dysfunction with an uncontrolled and abnormal host reaction to microbial infection, and sepsis shock is a condition of sepsis with more severe circulation disorders and cellular metabolism abnormalities [1, 2]. Even though the true incidence is unknown, conservative assessment manifests that sepsis is also a dominating cause of mortality and clinical critical illness all over the world [3]. The pathogenesis of sepsis is gradually being revealed, including microbial infection, inflammatory cytokines release and coagulation disorders [2, 4]. Notably, disorders of coagulation system in sepsis are emerging concerned [5].

It is well known that platelets are involved in the coagulation system. In addition, recent molecular and cellular evidence displayed that platelets have other pathological functions in inflammation, angiogenesis, and malignant tumor [6, 7]. Platelets participate in all these varied functions, which is mainly by exocytosis of their granule contents. There are three granule types (α-granules, dense granules, and lysosomes) in platelets. Of these granule types, the α-granules are the most abundant granules with 50 to 80 granules/platelet, compared with 3 to 6 dense granules/platelet and 0 to 3 lysosomes/platelet [8]. During sepsis, lipopolysaccharides (LPS) and inflammatory cytokines (e.g., TNF-α) accelerate the activation of platelets, which further leads to the formation of micro-thrombi in the capillaries [4, 9]. Meanwhile, activated platelets promote α-granule secretion and release plenty of functional proteins, such as P-selectin, platelet derived growth factor (PDGF), tissue inhibitor of metalloproteinases (TIMP), matrix metalloproteinases (MMP) and so on, which as secondary activators aggravate the inflammatory damage in sepsis [10, 11].
The mechanisms that soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) and SNARE accessory proteins mediate platelet α-granule exocytosis have recently been demonstrated at the molecular level [12, 13]. SNAREs proteins that are localized in opposing membranes drive membrane fusion by the formation of a four-helix bundle complex [14]. It appears to be that VAMP-8, syntaxin-2, and SNAP-23 are the key SNAREs that are involved in platelet α-granule secretion [15, 16]. Studies reveal that Sec/Munc18-like (SM) proteins function as clamps to ‘prime’ SNAREs proteins for assembly into tight SNAREs complex, which destine to fuse and release platelet α-granule. SM protein isoforms were found in platelets containing Munc18a, b, and c and Munc13–4 [17, 18]. Of these, Munc18a has been found to function in platelet α-granule exocytosis. Thus, these studies provide novel insights of antiplatelet therapy in the cure of sepsis by regulating platelet α-granule exocytosis.

Carbon monoxide (CO) is traditionally regarded as a toxic gas, which mainly for the reason by its ability to combine hemoglobin with a much higher affinity than oxygen. However, recently researches suggest that endogenous CO, a product of inducible heme oxygenase (HO-1), is able to modulate inflammation, and suppresses LPS-induced production of inflammatory cytokines both in vivo and in vitro, which exhibits important cyto-protective and anti-inflammatory functions [19–22]. Transition metal carbonyls up to date have been known as potential CO-releasing molecules (CORMs), which have the potential to assist the pharmaceutical use of CO by a controlled way to deliver it to tissues without changing carboxyhemoglobin levels [23]. It has been demonstrated that CORMs could improve the survival rate of sepsis mice induced by LPS or cecal ligation and puncture (CLP) models [24–27].

The results of our previous studies had been displayed that CORM-2, one of CO-releasing molecules, could inhibit platelets activation induced by LPS including aggregation, adhesion, spreading and granules secretion [28]. Compared with the LPS group, ulteriorly researches revealed that the levels of platelet surface membrane glycoproteins as well as hematopoietic lineage cell-specific protein 1 (HS1) were gently decreased in the CORM-2 groups. Nevertheless, we know little about whether CORM-2 released CO could decrease platelet α-granule exocytosis in sepsis. In this study, we revealed that integrin α_{in}β_{3} -mediated PKC0/Munc18a pathway activation played a dominating role in platelet SNAREs complex assembly and followed α-granule secretion induced by LPS.

RESULTS

CORM-2 regulates α-granule contents exocytosis in platelets simulated by LPS

Platelet granule exocytosis has a significant effect on promoting platelet activation. To determine whether LPS mediated platelet α-granule exocytosis, the level of P-selectin, which only presented within the α-granule membranes of resting platelets and was translocated to the plasma membrane upon platelet activation, was assessed. As shown in Figure 1A, 1B, the level of P-selectin in LPS-induced platelets was discovered to be obviously increased. Following the treatment of CORM-2 for 30 min, this elevation was significantly attenuated. Platelet α-granule also contains all sorts of pro- and anti-angiogenic molecules. We observed that the levels of PDGF-BB and MMP-2 were notably increased in LPS-stimulated platelets compared with the control group, whereas the levels were markedly downregulated by administration with CORM-2 (Figure 1C, 1D).

CORM-2 regulates the distribution of platelet α-granule in platelets simulated by LPS

We used immunofluorescence microscope and transmission electron microscope (TEM) to assess how platelet α-granules moved following LPS stimulation. Immunofluorescence images showed that the distribution of platelet α-granules (VAMP-8 antibody labelled) was altered immediately after LPS stimulation (0 min) and at the indicated times thereafter. And we found that platelet α-granules exocytosis was significantly obvious at 30 min and changed little from then on (Figure 2A). By means of TEM, resting platelets showed normal morphology with evenly dispersed α-granules in control group. Platelets were stimulated with LPS in the absence and presence of CORM-2, which underwent the similar changes including plasma membrane ruffling, irregularities, and extensions. Meanwhile, α-granules were generally appeared to translocate from the platelet central position to the plasma membrane after LPS challenged, which accelerated α-granule membrane fused with plasma membrane and subsequently released α-granule contents. Following administration of CORM-2 for 30 min, the degree of that α-granules assembled to plasma membrane was markedly reduced (Figure 2B). In parallel, immunofluorescence results showed that distribution changes of α-granules in LPS stimulated- and CORM-2 treated-platelets were similar with TEM results (Figure 2C).

CORM-2 downregulates the expression of platelet integrin α_{in}β_{3}

Integrin α_{in}β_{3} is the most abundant platelet surface protein, which can combine with fibrinogen or von Willebrand factor (vWF) in response to the activation of platelets in impaired blood vessels. Integrin α_{in}β_{3} is essential for normal platelet adhesion, aggregation, spreading and granules release [29, 30]. Researches proved PKC0 as an identified member of an α_{in}β_{3}-based outside-in signaling complex [31]. In our experiment, the expression level of platelet integrin α_{in}β_{3} was assessed
Figure 1: Effects of CORM-2 on platelet α-granule contents exocytosis following LPS stimulation. Platelets were stimulated by LPS (10 μg/mL) for 30 min in the presence or absence of CORM-2 (10, 50 μmol/L). The level of P-selectin was assayed by flow cytometry and concentrations of PDGF-BB and MMP-2 levels were assayed by enzyme linked immunosorbent assay kits. LPS stimulation significantly increased the level of P-selectin as compared with the control groups, while CORM-2 effectively abolished this elevation (A–B). Similar results were also shown that CORM-2 significantly reduced increase of PDGF-BB and MMP-2 levels in LPS-induced platelets (C–D). Data were shown as mean ± SD of five experiments, \( P < 0.01 \) as compared with control; \( \ast P < 0.05 \) as compared with LPS. Note that LPS-induced platelet α-granule exocytosis was inhibited by CORM-2 in a dose-dependent manner.

Figure 2: Effects of CORM-2 on platelet α-granule distribution in LPS-induced platelets. The platelets were stimulated by LPS and treated with CORM-2 as described in Figure 1 and were then fixed for transmission electron microscope (TEM) and immunofluorescence analysis as described under Materials and Methods. Platelets were labeled with antibodies to VAMP8 followed by staining with secondary antibodies labeled with Alexa Fluor 594. Images were taken immediately after LPS stimulation (0 min) and at the indicated times thereafter. A series of representative images demonstrated LPS-induced platelet α-granule distribution at different time points: 0 min, 5 min, 10 min, 15 min, 30 min, 45 min, 60 min (A). Representative electron microscopic images (B) and immunofluorescence images (C) showed obvious changes of platelet α-granule distribution under LPS stimulation and co-incubated LPS with iCORM (50 μmol/L), respectively. Following the treatment with CORM-2 (10 μmol/L) or CORM-2 (50 μmol/L), α-granules fused with platelet membrane were decreased. Representative α-granules were indicated by arrows. Scale bars, 0.5 μm for electron microscopic images and 2 μm for immunofluorescence images.
by flow cytometry. We found that the expression level of platelet integrin α<sub>IIb</sub>β<sub>3</sub> was significantly increased in the LPS and LPS with iCORM (50 μmol/L) groups compared with the control group (Figure 3). Interestingly, after administration of CORM-2 (10 or 50 μmol/L), this increase was abolished by a dose dependent manner when compared with LPS group.

**CORM-2 downregulates the expression of platelet PKCθ**

The protein kinase C (PKC) family is the member of serine/threonine kinases, involving in a variety of platelet functions containing the secretion of granule, activation of integrin, platelet aggregation and spreading, and pro-coagulation [32]. Researches turn out that PKCθ plays a key role in platelet α-granule secretion response and is phosphorylated after activation of integrin α<sub>IIb</sub>β<sub>3</sub>-based ‘outside-in’ signaling in platelet stimulation. In the present study, threonine phosphorylation of PKCθ in LPS-stimulated platelets was measured by Western blotting to assess its activation. Our study showed that the expression levels of PKCθ have significant increase in LPS-induced platelets (Figure 4A). Parallely, threonine-phosphorylated PKCθ levels were significantly up-regulated in LPS groups (Figure 4B). However, the expression levels of PKCθ and phosphorylated PKCθ were apparently down-regulated when platelets were co-incubated with LPS and CORM-2.

**Effects of CORM-2 on the expression of platelet Munc18a**

Sec1/Munc18-like (SM) proteins, as SNARE chaperones, are involve in degranulation in secretory cells [18]. Munc18a, as an important member of SM proteins, exerts a vital effect on platelet membrane fusion and platelet α-granule exocytosis through its interactions with syntaxins. Protein expression of both Munc18a and serine-phosphorylated Munc18a in LPS-stimulated platelets was significantly enhanced when compared with the control group. However, expression of Munc18a and serine-phosphorylated Munc18a was significantly decreased while CORM-2 administration (50 μmol/L), (Figure 5A, 5B).

In order to further explore the effect of PKCθ in platelet exocytosis, AEB071 (sotrastaurin), a small molecule PKCθ inhibitor [33, 34], was used to inhibit PKCθ phosphorylation at a concentration of 50 nmol/L according to our pre-experiments in vitro (Figure 4C).

![Figure 3: Effects of CORM-2 on expression of platelet integrin α<sub>IIb</sub>β<sub>3</sub>](image)

**Figure 3: Effects of CORM-2 on expression of platelet integrin α<sub>IIb</sub>β<sub>3</sub>.** The platelets were stimulated by LPS and treated with CORM-2 as described in Figure 1. Samples were collected and fixed in 1% paraformaldehyde for 15 min at room temperature and incubated with FITC-labeled CD41 (CD41-FITC). Integrin α<sub>IIb</sub>β<sub>3</sub>-PE was added to the samples. All samples were incubated in the dark for 30 min, washed three times and analyzed by flow cytometry. The expression level of integrin α<sub>IIb</sub>β<sub>3</sub> was markedly increased in LPS-induced platelets compared with the control group, while it was significantly decreased by treatment with CORM-2. Data were shown as mean ± SD of five experiments, *P < 0.01 as compared with control; †P < 0.05 as compared with LPS. Note that LPS-induced platelet Integrin α<sub>IIb</sub>β<sub>3</sub> expression was inhibited by CORM-2 in a dose-dependent manner.
Furthermore, Munc18a expression and its phosphorylation were significantly decreased compared with the LPS group, following AEB071 treatment with or without CORM-2 (50 μmol/L). And there was no difference between the AEB071 group and CORM-2 (50 μmol/L) group (Figure 5A, 5B). Simultaneously, we detected platelet α-granule contents release with AEB071 by flow cytometry and ELISA kits. And we found changes of P-selectin, PDGF-BB and MMP-2 were similarly consistent with that of Munc18a phosphorylation (Figure 5D, 5E, 5F).

CORM-2 regulates SNAREs complex assembly in LPS-stimulated platelets

α-granule contents begin to be released when the α-granule membrane fuses with the platelet plasma membrane or the open canalicular system (OCS). It have been identified that SNAREs and SNARE accessory proteins control the process of α-granule secretion [11, 35]. Known V AMP8, syntaxin2 and SNAP23 are highlighted by reports showing enrichment at sites of platelet membrane fusion [36]. Based on this, we measured protein expression of VAMP8, syntaxin2 and SNAP23 by western blotting. The expression of these three SNARE proteins in LPS-stimulated platelets was dramatically increased when compared with the control group, while significantly decreased after co-incubated with LPS and CORM-2 (50 μmol/L) for 30 min (Figure 6A). Researches suggest that Munc18a is able to bind to syntaxins with three different conditions: ‘closed’, ‘open’ or ‘in SNAREs complex’ [35]. To identify association of Munc18a with SNARE proteins in platelets, the method of immunoprecipitation was used. Different stimulated platelet extracts were subjected to immunoprecipitation with anti-Munc18a antibody and both bound and unbound fractions were analysed by western blotting for Munc18a and above-mentioned SNARE proteins, including VAMP8, syntaxin2 and SNAP23. In this precipitate, those three SNARE proteins were found associated with Munc18a in significant quantities. In addition, the number of those three SNARE proteins combined with Munc18a in the LPS group was discovered to be markedly increased when compared to

Figure 4: Effects of CORM-2 on platelet PKCθ expression and PKCθ threonine phosphorylation. The platelets were stimulated by LPS and treated with CORM-2 as described in Figure 1. The platelets were lysed in RIPA supplemented with protease and phosphatase inhibitor cocktails. Platelet PKCθ threonine phosphorylation was detected by using SDS-polyacrylamide gel electrophoresis and Western blotting. The representative images were shown in (A) and (B). Pretreated with AEB071 for 5min, the image of PKCθ threonine phosphorylation was shown in (C) The average ratio of PKCθ/GAPDH and p-PKCθ/t-PKCθ were also shown. Data were shown as mean ± SD of three independent experiments, *P < 0.01 as compared with control; **P < 0.05 as compared with LPS.
the control group. However, the elevation in amounts of VAMP8, syntaxin2 and SNAP23 were significantly abolished by CORM-2 treatment. In parallel, associations of SNARE proteins with Munc18a were attenuated stimulated by LPS in the presence of AEB071 (Figure 6B). As well, for syntaxin2 immunoprecipitation, the elevation in amounts of VAMP8, SNAP23 and Munc18a in LPS-stimulated platelets were significantly abolished by CORM-2 treatment with or without AEB071 (Figure 7).

Immunofluorescence microscopy was also adopted to detect the distribution of VAMP8 and Munc18a in LPS-stimulated platelets (Figure 6C). We discovered that VAMP8 and Munc18a transferred to the plasma membrane in LPS stimulated platelet, while CORM-2 with or without AEB071 attenuated this distribution trend.

**DISCUSSION**

Platelet activation is a vital event in early sepsis. Upon activation by extracellular matrix components or soluble agonists, platelet α-granules release hundreds of different types of active molecules, which further activate platelets and thus participate in a series of responses to aggravate the inflammatory damage in sepsis. Platelet α-granule contents include coagulation proteins (e.g., fibrinogen), protease inhibitors (e.g., plasminogen activator inhibitor-1), growth factors (e.g., PDGF), soluble adhesion molecules (e.g., vWF), and membrane adhesion molecules (e.g., P-selectin) [11]. Platelet α-granule heterogeneity and differential release possibly account for opposing actions of physiological and pathological correlates in sepsis [37]. Our previous studies have suggested that CORM-2 attenuated the activation of LPS-induced platelets such as adhesion, spreading, aggregation, and granules release [28]. While our studies showed that CORM-2 could decrease platelet α-granule contents exocytosis in LPS-stimulated platelets, such as P-selectin, PDGF-BB and MMP-2. From the results of TEM and immunofluorescence microscopy, subcellular distribution of α-granules in LPS-stimulated platelets was visually observed. Following the treatment of CORM-2, a decreased tendency was shown in α-granule movement to periphery membrane in LPS-stimulated platelets. Hence, the mechanisms of spatial regulation of platelet secretion could be in place to allow platelets to perform their functions. However, how this regulation is achieved remains to be understood.

The platelet integrin αIIbβ3 exerts an important role in the formation of thrombus during sepsis [38, 39]. Stimulation by adhesion or agonist, a series of ‘inside-out’ signals occurring within the platelets in sepsis ultimately results in platelet aggregation [40]. Fibrinogen binding to...
platelets triggers ‘outside-in’ signals, which contribute to actin polymerization, cell spreading and exocytosis and secretion of α-granules [41]. PKCθ, the downstream of integrin αIIbβ3, plays a crucial role in platelet α-granule exocytosis [37, 38]. Research showed that PKCθ inhibited by PKC-θ RACK peptide significantly impaired platelet secretion [42]. In this study, we found that the expression of integrin αIIbβ3 in LPS-stimulated platelets was significantly increased. This increase was effectively suppressed by CORM-2 treatment. Likewise, changes of PKCθ and PKCθ phosphorylation levels in LPS-stimulated platelets with or without CORM-2 were consistent with the expression of integrin αIIbβ3. Our results are in agreement with the fact that PKCθ is phosphorylated after activation of integrin αIIbβ3-based ‘outside-in’ signaling in platelet stimulation. It is also suggested that CORM-2 can inhibit the expression of integrin αIIbβ3 and phosphorylation of PKCθ, and consequently decrease abnormal platelet α-granule exocytosis in sepsis.

Initial studies demonstrated that platelet α-granule exocytosis was based on its fusion with the platelet plasma membrane or OCS. Studies discovered that SNAREs and SNAREs regulators mediated the reaction of platelets release [43]. According to their membrane location, SNAREs were divided into two isoforms: vesicle- or target-SNAREs. More recently, vesicle-associated membrane protein (VAMP) 8 is regarded as the primary and most abundant v-SNARE in platelet α-granule

**Figure 6: Effects of CORM-2 on SNAREs complex assembly in LPS-induced platelets.** The platelets were stimulated by LPS and treated with CORM-2 as described in Figure 4 for Western blotting. The representative images of VAMP8, SNAP23 and syntaxin2 were shown in (A) The average ratio of VAMP8/GAPDH, SNAP23/GAPDH and syntaxin2/GAPDH were also shown. For Munc18a immunoprecipitations, platelets were stimulated by LPS and treated with CORM-2 and AEB071 as described in Figure 5. The representative images of Munc18a immunoprecipitations were shown in (B) The average ratio of VAMP8/Munc18a, SNAP23/Munc18a and syntaxin2/ Munc18a were also shown. Data were shown as mean±SD of three independent experiments, *P < 0.01 as compared with control; **P < 0.05 as compared with LPS. Distributions of VAMP8 and Munc18a were determined in LPS-induced platelets using double staining immunofluorescence microscopy. Platelets were labeled with antibodies to VAMP8 (C row 1), Munc18a (C row 2) followed by staining with secondary antibodies labeled with Alexa Fluor 594 and Alexa Fluor 488, respectively. Merged images were evaluated to determine the distribution of VAMP8 relative to Munc18a in LPS-induced platelets (C row3). After LPS stimulation, VAMP8 and Munc18a localized to the platelet plasma membrane, whereas CORM-2 with or without AEB071 attenuated this distribution change. Scale bars, 2 μm.
Syntaxin2, a t-SNARE, plays an important role in platelet exocytotic core complex [44]. As well, SNAP (synaptosomal-associated protein) 23, another family of t-SNAREs, is a main SNAP family member and also essential for the release of granules in platelets [16]. Thus, VAMP8, syntaxin2 and SNAP23 appear to be essential for SNAREs complex formation in platelet α-granule exocytosis. In this present study, we found the expression levels of those three SNARE proteins in LPS-stimulated platelets were significantly increased. We thereby speculated SNAREs complex synthesis and assembly were increased and then accelerated platelet α-granule exocytosis in sepsis. In contrast, CORM-2 decreased the expression levels of those three SNARE proteins in LPS-stimulated platelets, possibly inhibited formation of SNAREs complex and α-granule exocytosis in sepsis. However, the specific mechanism of how CORM-2 regulated the formation of SNAREs complex should be clarified.

Except for the core SNAREs machinery, α-granule secretion is also regulated by SNARE regulators. Sec/Munc18-like (SM) proteins are phosphorylated in platelets (Munc18a, Munc18b and Munc18c) and, even though the significance of this is not yet clear, phosphorylation does affect SM proteins binding both the v-SNARE and t-SNARE components and ‘prime’ them for assembly into the SNAREs complex, as well as regulate the dynamics of α-granule secretion [45, 46]. Munc18a, originally characterized as a key syntaxin2 regulator, has a vital effect on platelet α-granule exocytosis [47]. By means of immunoprecipitation, our study revealed that the amount of VAMP8, syntaxin2 and SNAP23 associated with Munc18a were markedly elevated in LPS-stimulated platelets, while CORM-2 inhibited these elevations. In parallel, CORM-2 attenuated the elevation in amounts of VAMP8, SNAP23 and Munc18a associated with syntaxin2 in LPS-induced platelets. As well, immunofluorescence images showed that CORM-2 attenuated the trend of VAMP8 and Munc18a assembly to the platelet plasma membrane in LPS-stimulated platelets. Those results further proved speculation that SNAREs complex assembly was markedly enhanced after LPS stimulation, which was decreased by treatment with CORM-2. Initial researches showed that Munc18a transducted cell signaling via its phosphorylation on Ser313 by PKCθ [48–50]. In LPS-stimulated platelets, we found both production and phosphorylation of PKCθ and Munc18a were significantly increased, while they were significantly attenuated after co-incubated with CORM-2. These results suggested that the PKCθ/Munc18a pathway was involved in platelet α-granule exocytosis induced by LPS. Administration of CORM-2 was able to affect the PKCθ/Munc18a pathway. Furthermore, using AEB071, as a valid PKCθ inhibitor, we found that Munc18a phosphorylation was significantly decreased compared with the LPS group. What’s more, there is no difference between the AEB071 group and CORM-2 group. Taken together, those data further indicated the PKCθ/Munc18a pathway

![Figure 7: Effects of CORM-2 on SNAREs complex assembly in LPS-induced platelets.](image)

For syntaxin2 immunoprecipitations, platelets were stimulated by LPS and treated with CORM-2 and AEB071 as described in Figure 5. The representative images of syntaxin2 immunoprecipitations were shown in (A) The average ratio of Munc18a/syntaxin2, SNAP23/syntaxin2 and VAMP8/syntaxin2 were also shown in (B–D) Data were shown as mean ± SD of three independent experiments, *P < 0.01 as compared with control; **P < 0.05 as compared with LPS.
was involved in platelet SNAREs complex assembly and α-granule exocytosis in sepsis.

It is well known that endogenous CO, serving as an endogenous messenger molecule, which can activate sGC (soluble guanylyl cyclase) and thus promote the formation of cGMP (cyclic guanosine 3′,5′-monophosphate). Our previous study indicated that exogenous CO were capable of increasing cGMP levels in platelets stimulated by LPS with a dose-dependent manner, and thus inhibited inflammatory responses and protected vital organ functions against sepsis [51, 52].

In conclusion, this study served to clarify the effects of CORM-2 on the mechanisms of suppression of platelet α-granule exocytosis during sepsis. Administration of CORM-2 down-regulated the expression of integrin αIIbβ3, prevented activation of PKCθ/Munc18a pathway, and subsequently attenuated the SNAREs complex assembly and α-granule exocytosis in LPS-stimulated platelets (Figure 8). In parallel, levels of P-selectin, PDGF-BB and MMP-2 released from platelet α-granule stimulated by LPS were markedly decreased following the treatment of CORM-2. However, the potential application value of CORM-2 in platelet α-granule exocytosis during clinical sepsis should be further explored.

**MATERIALS AND METHODS**

**Ethics statement**

The Medical Ethical Committee of Affiliated Hospital of Jiangsu University approved the study. After written informed consent, blood specimens were obtained from the cubital veins of healthy donors. The Medical Ethical Committee of Affiliated Hospital of Jiangsu University gave consent for the use of these samples.

**Materials**

CORM-2, dimethyl sulfoxide (DMSO), tyrodes solution, protease inhibitor cocktail, LPS, RIPA buffer and VAMP8 (V7514) rabbit antibody were obtained from Sigma-Aldrich (St. Louis, MO, USA). CORM-2 was solubilized in DMSO to obtain a 40 mmol/L stock. The inactive form of CORM-2 (negative control) was prepared as described previously [53]. FITC-labeled CD41 monoclonal antibody (mAb), GAPDH rabbit polyclonal IgG, PE-labeled P-Selectin were obtained from eBioscience (San Diego, CA, USA). Phosphatase inhibitor cocktail, PE-labeled integrin αIIbβ3 mAb and PE-labeled P-Selectin were obtained from eBioscience (San Diego, CA, USA). Phosphatase inhibitor cocktail, PE-labeled integrin αIIbβ3 mAb and PE-labeled

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**Figure 8: Model of α-granule exocytosis in LPS-induced platelets.** A schematic model depicted carbon monoxide and integrin αIIbβ3 mediated PKCθ/Munc18a pathway involved in the regulation of α-granule exocytosis in LPS-induced platelets. Binding of LPS to the Toll-like (TLR) 4 receptor triggers receptor-linked signaling pathways that possibly result in the activation of PKCθ, which subsequently enhances the level of integrin αIIbβ3 via the ‘inside-out’ signaling. Upon fibrinogen binding to integrin αIIbβ3, in turn, promotes PKCθ phosphorylation of Thr538 via the ‘outside-in’ signaling. PKCθ can phosphorylates the Munc18a protein, which may enhance association with VAMP8, syntaxin2 and SNAP23 and thereby promote engagement of the v-SNARE and t-SNARE to form a 4-helix bundle. Then α-granule fuses with the plasma membrane and the contents are released to the extracellular space. Carbon monoxide, derived from CORM-2, suppressed α-granule exocytosis in LPS-induced platelets, involving integrin αIIbβ3 mediated PKCθ/Munc18a pathway activation and SNAREs complex assembly in our experiment.
IgG1 were obtained from Santa Cruz Biotechnology (Dallas, Texas, USA). PKCδ (E117Y) rabbit mAb, Phospho-PKCδ (Thr538) antibody, Munc18a (D406V) rabbit mAb and anti-rabbit IgG HRP-linked antibody were obtained from Cell Signaling Technology (Boston, MA, USA). Phospho-Munc18a (Ser313) antibody, Syntaxin2 and SNAP23 rabbit polyclonal antibody were obtained from Abcam (Cambridge, MA, USA). AEB071 (sotrastaurin) were obtained from Selleckchem (Houston, Texas, USA). Other reagents and instruments included PDGF-BB, MMP-2, VEGF and TIMP-1 ELISA kits (Joyee, Shanghai, China), protein A-Sepharose beads (Beyotime, Jiangsu, China). A FACSCalibur BD Flow Cytometer was obtained from Becton Dickison Corporation (San Jose, CA, USA). Incubator-CO₂ (Napco 5400) and high-speed cryogenic desktop centrifuges were obtained from Beckman Coulter Corporation (Pasadena, CA, USA). Image-Pro plus 6.0 software was obtained from Media Cybernetics (Maryland, USA).

Preparation of washed platelets

Blood was extracted from healthy volunteers, after informed consent, and in accordance with the Medical Ethical Committee of Jiangsu University. Blood was collected into vacuum tubes and anti-coagulated with one-nine of 129 mmol/L trisodium citrate. Platelet rich plasma (PRP) was obtained by centrifuging at 120×g for 10 min. Platelets were isolated by centrifuging at 678×g for 10 min and then washed twice with CGS buffer. The platelet poor plasma (PPP) was used to measure platelet aggregation. The platelets were resuspended with Tyrode’s buffer for further use [34]. For experiments with PRP, the platelet density was maintained at 2 × 10^9/mL for every experiment.

Platelets stimulation model

LPS (10 μg/mL) was used to stimulate PRP to imitate the condition of coagulation under sepsis. This final concentration of LPS was used according to the published results in our laboratory and others [27, 54]. The PRP was assigned to five groups randomly. The control group did not undergo any treatment, whereas the LPS group received LPS simulation for 30 min, the CORM-2 group and iCORM-2 group underwent the same simulation and immediate administration of CORM-2 or iCORM-2 with different doses (10 and 50 μmol/L), respectively. PKCδ inhibitor, AEB071 (sotrastaurin), was incubated with the platelets for 5 minutes before stimulation in additional experiments. Samples were incubated in a CO₂ incubator at 37°C, 95% humidity, and 5% CO₂. After the intervention, the correlated indices were detected.

Transmission electron microscopy

Platelet α-granule distribution was observed by a transmission electron microscope (TEM) as previously described [55–57]. Briefly, samples were fixed by mixing them with an equal volume of 3% glutaraldehyde in 0.1 mol phosphate buffer for 1.5 hours at room temperature. After primary fixation, samples were centrifuged and washed three times with phosphate buffer. Then, platelets were post-fixed with 2% osmium tetroxide in phosphate buffer for 30 minutes and dehydrated in a series of graded ethanol solution, infiltrated and embedded in agar 100 epoxy resin, using propylene oxide as a transitional fluid. Finally, ultrathin sections were stained with 0.5% aqueous uranyl acetate and visualized on a JEM 2100 transmission electron microscope (Tokyo, Japan).

Immunofluorescence

Samples of all groups were collected and fixed in 1% paraformaldehyde for 15 min at room temperature. The fixed platelets were spun at 800×g for 5 min and permeabilized using 0.3% Triton-X-100. Samples were washed and blocked for 2 hours in 1% BSA. After washing with PBS, platelets were incubated with the corresponding antibodies overnight. Then primary antibodies were removed and Alexa Fluor conjugated anti–mouse/anti–rabbit antibodies (1:250 dilutions) were applied for 2 hour at room temperature. For visualization of granule distribution, fluorescent microscopy was performed using an Olympus IX71 microscope (Olympus USA). All images were processed using cellSens Standard 1.12 software.

Flow cytometry

Samples of all groups were collected and fixed in 1% paraformaldehyde for 15 min at room temperature. The fixed samples were incubated with FITC-labeled CD41 (CD41-FITC). Then P-Selectin-PE and integrin αIIbβ3-PE were independently added into the above samples. IgG1-PE was applied as isotype control antibody. All samples were incubated in the dark for 30 min at room temperature. Samples were washed three times and then analyzed by flow cytometry.

ELISA

Platelet α-granule contents including PDGF-BB, MMP-2, VEGF and TIMP-1 were detected by using standard ELISA kits. Briefly, different samples after the intervention were immediately centrifuged at 678×g for 10 min. Then concentrations of α-granule contents in serum were assayed by standard ELISA kits following the manufacturer’s instructions.

Protein production and western blotting

Platelet stimulation and CORM-2 intervention were performed as described above. Then platelets were lysed in RIPA supplemented with protease and phosphatase
inhibitor cocktails. The lysates were incubated for 30 min on ice for completion of the lysis action.

Samples (10 μg of protein) were subjected to electrophoresis on 7% SDS-polyacrylamide gels, with the use of the discontinuous system and transferred onto nitrocellulose membranes, which were incubated with different dilutions of primary and secondary antibodies. The bands were visualized by the use of ECL reagent and Hyperfilm ECL (Amersham, Arlington Heights, IL, USA) as described by the manufacturer. Films were scanned using a flatbed scanner and the bands were quantified using BasicQuantifier software (Bio Image, Ann Arbor, MI, USA).

Immunoprecipitation of platelet SNAREs and regulatory proteins

Washed platelets were prepared for immunoprecipitations as previously described [58]. For Munc18a immunoprecipitations, resting and stimulated platelets were incubated with 2×Lysis buffer (2% n-octyl β-D-glucopyranoside, 2 mmol/L EGTA, 2mmol/L EDTA, 100 mmol/L HEPES, 150 mmol/L NaCl, 2 mmol/L Na3VO4, and protease inhibitor cocktail, pH 7.4) and kept on ice for 30 minutes. Platelet lysates were centrifuged at 12000×g for 10 min at 4°C to remove insoluble material. The nonspecific binding proteins were cleared by incubating platelet lysates with anti-Munc18a rabbit polyclonal antibody alone overnight at 4°C, and then rotated with protein A-Sepharose beads for 16 hours at 4°C. The mixture was washed twice with 1× lysis buffer, and the bound proteins were eluted with SDS sample buffer and subjected to Western blotting with the indicated antibodies.

Statistical analyses

Our data were presented as the mean ± standard deviation. Snces between two groups were analyzed by posthoc test (SNK). An alpha value of $P < 0.05$ was considered statistically significant.

Author contributions

MZ, MS and DL contributed equally to the work. BS, MZ, MS and DL conceived the initial concept for the experimental aims and design. JH, DL and XX performed the experiments of platelet α-granule contents exocytosis and platelet function. JH and WQ performed the experiments of protein production and activity. MZ and MS performed the statistical analysis. BS and MZ drafted the manuscript. All authors read and approved the final manuscript.

CONFLICTS OF INTEREST

The authors declare that they have no competing interests.

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