Exogenous carbon monoxide suppresses LPS-induced platelet SNAREs complex assembly and α -granule exocytosis via integrin α IIB β 3-mediated PKC θ /Munc18a pathway

Mingfeng Zhuang^{1,*}, Mingming Song^{2,*}, Dadong Liu^{1,*}, Jiamin Huang¹, Xiaohan Xu¹, Weiting Qin¹ and Bingwei Sun¹

¹Department of Burns and Plastic Surgery, Affiliated Hospital, Jiangsu University, Zhenjiang 212001, Jiangsu Province, China

²Department of Burns and Plastic Surgery, Suzhou Hospital Affiliated to Nanjing Medical University, Suzhou 215002, Jiangsu Province, China

*These authors contributed equally to this work

Correspondence to: Bingwei Sun, **email:** sunbinwe@hotmail.com

Keywords: sepsis; platelet; SNAREs complex; α -granule; carbon monoxide

Received: August 02, 2017

Accepted: November 03, 2017

Published: January 03, 2018

Copyright: Zhuang et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License 3.0 (CC BY 3.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

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 α IIB β ₃ in platelets stimulated by LPS. This was accompanied by a decrease in production and phosphorylation of PKC θ 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 α IIB β ₃-mediated PKC θ /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 -granules 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 $\alpha_{IIb}\beta_3$ -mediated PKC θ /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 $\alpha_{IIb}\beta_3$

Integrin $\alpha_{IIb}\beta_3$ 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 $\alpha_{IIb}\beta_3$ is essential for normal platelet adhesion, aggregation, spreading and granules release [29, 30]. Researches proved PKC θ as an identified member of an $\alpha_{IIb}\beta_3$ -based outside-in signaling complex [31]. In our experiment, the expression level of platelet integrin $\alpha_{IIb}\beta_3$ was assessed

Figure 1: Effects of CORM-2 on platelet α -granule contents exocytosis following LPS stimulation. Platelets were stimulated by LPS (10 μ g/mL) for 30 min in the presence or absence of CORM-2 (10, 50 μ mol/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 $\pm$ SD of five experiments, $P < 0.01$ as compared with control; $*P < 0.05$ 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 μ mol/L), respectively. Following the treatment with CORM-2 (10 μ mol/L) or CORM-2 (50 μ mol/L), α -granules fused with platelet membrane were decreased. Representative α -granules were indicated by arrows. Scale bars, 0.5 μ m for electron microscopic images and 2 μ m for immunofluorescence images.

by flow cytometry. We found that the expression level of platelet integrin $\alpha_{IIb}\beta_3$ was significantly increased in the LPS and LPS with iCORM (50 $\mu\text{mol/L}$) groups compared with the control group (Figure 3). Interestingly, after administration of CORM-2 (10 or 50 $\mu\text{mol/L}$), this increase was abolished by a dose dependent manner when compared with LPS group.

CORM-2 downregulates the expression of platelet PKC θ

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 PKC θ plays a key role in platelet α -granule secretion response and is phosphorylated after activation of integrin $\alpha_{IIb}\beta_3$ -based ‘outside-in’ signaling in platelet stimulation. In the present study, threonine phosphorylation of PKC θ in LPS-stimulated platelets was measured by Western blotting to assess its activation. Our study showed that the expression levels of PKC θ have significant increase in LPS-induced platelets (Figure 4A). Parallely, threonine-phosphorylated

PKC θ levels were significantly up-regulated in LPS groups (Figure 4B). However, the expression levels of PKC θ and phosphorylated PKC θ 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 $\mu\text{mol/L}$), (Figure 5A, 5B).

In order to further explore the effect of PKC θ in platelet exocytosis, AEB071 (sotrastaurin), a small molecule PKC θ inhibitor [33, 34], was used to inhibit PKC θ 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 $\alpha_{IIb}\beta_3$. 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 $\alpha_{IIb}\beta_3$ -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 $\alpha_{IIb}\beta_3$ 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 $\pm$ SD of five experiments, * $P < 0.01$ as compared with control; * $P < 0.05$ as compared with LPS. Note that LPS-induced platelet Integrin $\alpha_{IIb}\beta_3$ 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 $\mu\text{mol/L}$). And there was no difference between the AEB071 group and CORM-2 (50 $\mu\text{mol/L}$) 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 VAMP8, 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 $\mu\text{mol/L}$) 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 $\pm$ 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 amount 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 $\alpha_{IIb}\beta_3$ 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

Figure 5: Effects of CORM-2 on platelet Munc18a expression and α -granule Contents Exocytosis in LPS-induced Platelets. The platelets were pretreated with AEB071 for 5min followed by LPS stimulation with or without CORM-2 (50 μ mol/L) for an additional 30 min. Platelet Munc18a serine phosphorylation was detected by using Western blotting. The representative images were shown in (A) and (B). The average ratio of Munc18a/GAPDH and p-Munc18a/t-Munc18a were also shown. The level of P-selectin (C–D) was assayed by flow cytometry and levels of PDGF-BB (E) and MMP-2 (F) levels were assayed by ELISA kits. LPS stimulation significantly increased levels of P-selectin, PDGF-BB and MMP-2 as compared with the control groups, while AEB071 used with or without CORM-2 (50 μ mol/L), effectively abolished this elevation. And there was no difference between the AEB071 group and CORM-2 group. Data were shown as mean $\pm$ SD of five experiments, * $P < 0.01$ as compared with control; * $P < 0.05$ as compared with LPS.

platelets triggers ‘outside-in’ signals, which contribute to actin polymerization, cell spreading and exocytosis and secretion of α -granules [41]. PKC θ , the downstream of integrin $\alpha_{IIb}\beta_3$, plays a crucial role in platelet α -granule exocytosis [37, 38]. Research showed that PKC θ inhibited by PKC- θ RACK peptide significantly impaired platelet secretion [42]. In this study, we found that the expression of integrin $\alpha_{IIb}\beta_3$ in LPS-stimulated platelets was significantly increased. This increase was effectively suppressed by CORM-2 treatment. Likewise, changes of PKC θ and PKC θ phosphorylation levels in LPS-stimulated platelets with or without CORM-2 were consistent with the expression of integrin $\alpha_{IIb}\beta_3$. Our results are in agreement with the fact that PKC θ is phosphorylated after activation

of integrin $\alpha_{IIb}\beta_3$ -based ‘outside-in’ signaling in platelet stimulation. It is also suggested that CORM-2 can inhibit the expression of integrin $\alpha_{IIb}\beta_3$ and phosphorylation of PKC θ , 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 $\pm$ 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 μ m.

[36]. 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 transduced cell signaling via its phosphorylation on Ser313 by PKC θ [48–50]. In LPS-stimulated platelets, we found both production and phosphorylation of PKC θ and Munc18a were significantly increased, while they were significantly attenuated after co-incubated with CORM-2. These results suggested that the PKC θ /Munc18a pathway was involved in platelet α -granule exocytosis induced by LPS. Administration of CORM-2 was able to affect the PKC θ /Munc18a pathway. Furthermore, using AEB071, as a valid PKC θ 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 PKC θ /Munc18a pathway

Figure 7: Effects of CORM-2 on SNAREs complex assembly in LPS-induced platelets. 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 $\pm$ 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 $\alpha_{IIb}\beta_3$, prevented activation of PKC θ /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 $\alpha_{IIb}\beta_3$ mAb and PE-labeled

Figure 8: Model of α -granule exocytosis in LPS-induced platelets. A schematic model depicted carbon monoxide and integrin $\alpha_{IIb}\beta_3$ mediated PKC θ /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 PKC θ , which subsequently enhances the level of integrin $\alpha_{IIb}\beta_3$ via the 'inside-out' signaling. Upon fibrinogen binding to integrin $\alpha_{IIb}\beta_3$, in turn, promotes PKC θ phosphorylation of Thr538 via the 'outside-in' signaling. PKC θ 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 $\alpha_{IIb}\beta_3$ mediated PKC θ /Munc18a pathway activation and SNAREs complex assembly in our experiment.

IgG1 were obtained from Santa Cruz Biotechnology (Dallas, Texas, USA). PKC θ (E117Y) rabbit mAb, Phospho-PKC θ (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 Dickinson Corporation (San Jose, CA, USA). Incubator-CO $_2$ (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 $\times$ g for 10 min. Platelets were isolated by centrifuging at 678 $\times$ 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 before used [34]. For experiments with PRP, the platelet density was maintained at 2×10^8 /mL for every experiment.

Platelets stimulation model

LPS (10 μ g/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 stimulation for 30 min, the CORM-2 group and iCORM-2 group underwent the same stimulation and immediate administration of CORM-2 or iCORM-2 with different doses (10 and 50 μ mol/L), respectively. PKC θ inhibitor, AEB071 (sotrastaurin), was incubated with the platelets for 5 minutes before stimulation in additional experiments. Samples were incubated in a CO $_2$ -incubator at 37°C, 95% humidity, and 5% CO $_2$. After the intervention, the correlated indices were detected.

Transmission electron microscopy

Platelet α -granule distribution was observed by a transmission electron microscope (TEM) as previous

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 spinned at 800 $\times$ 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 $\alpha_{IIb}\beta_3$ -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 $\times$ 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% SDSpolyacrylamidegels, 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, 2 mmol/L EDTA, 100 mmol/L HEPES, 150 mmol/L NaCl, 2 mmol/L Na₃VO₃ 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 4 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. Sncees 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.

FUNDING

This study was supported by the National Natural Science Foundation of China, No. 81071546, No. 81272148 and No. 81471903; by the Jiangsu Natural Science Foundation, No. BE2017695.

REFERENCES

1. Chang HJ, Lynm C, Glass RM. JAMA patient page. Sepsis. JAMA. 2010; 304:1856.
2. Remick DG. Pathophysiology of sepsis. Am J Pathol. 2007; 170:1435–44.
3. Angus DC, van der Poll T. Severe sepsis and septic shock. N Engl J Med. 2013; 369:2063.
4. Tymk K. Critical role for oxidative stress, platelets, and coagulation in capillary blood flow impairment in sepsis. Microcirculation. 2011; 18:152–62.
5. de Stoppelaar SF, van't Veer C, van der Poll T. The role of platelets in sepsis. Thromb Haemost. 2014; 112:666–77.
6. Blair P, Flaumenhaft R. Platelet alpha-granules: basic biology and clinical correlates. Blood Rev. 2009; 23:177–89.
7. Leslie M. Cell biology. Beyond clotting: the powers of platelets. Science. 2010; 328:562–4.
8. Peters CG, Michelson AD, Flaumenhaft R. Granule exocytosis is required for platelet spreading: differential sorting of alpha-granules expressing VAMP-7. Blood. 2012; 120:199–206.
9. Soni HM, Jain MR, Mehta AA. Mechanism(s) Involved in Carbon Monoxide-releasing Molecule-2-mediated Cardioprotection During Ischaemia-reperfusion Injury in Isolated Rat Heart. Indian J Pharm Sci. 2012; 74:281–91.
10. Maynard DM, Heijnen HFG, Horne MK, White JG, Gahl WA. Proteomic analysis of platelet alpha-granules using mass spectrometry. J Thromb Haemost. 2007; 5:1945–55.
11. Golebiewska EM, Poole AW. Secrets of platelet exocytosis - what do we really know about platelet secretion mechanisms? Br J Haematol. 2014; 165:204–216.
12. Xu X, Sun B. Platelet granule secretion mechanisms: Are they modified in sepsis? Thromb Res. 2015; 136:845–50.
13. Hong W. SNAREs and traffic. Biochim Biophys Acta. 2005; 1744:120–44.
14. Fasshauer D. Structural insights into the SNARE mechanism. Biochim Biophys Acta. 2003; 1641:87–97.
15. Marks MS. SNAREing platelet granule secretion. Blood. 2012; 120:2355–7.
16. Koseoglu S, Flaumenhaft R. Advances in platelet granule biology. Curr Opin Hematol. 2013; 20:464–71.
17. Misura KM, Scheller RH, Weis WI. Three-dimensional structure of the neuronal-Sec1-syntaxin 1a complex. Nature. 2000; 404:355–62.
18. Carr CM, Rizo J. At the junction of SNARE and SM protein function. Curr Opin Cell Biol. 2010; 22:488–95.

19. Coburn RF. The measurement of endogenous carbon monoxide production. *J Appl Physiol* (1985). 2012; 112:1949–55.
20. Ozaki KS, Yoshida J, Ueki S, Pettigrew GL, Ghonem N, Sico RM, Lee LY, Shapiro R, Lakkis FG, Pacheco-Silva A, Murase N. Carbon monoxide inhibits apoptosis during cold storage and protects kidney grafts donated after cardiac death. *Transpl Int*. 2012; 25:107–17.
21. Ryter SW, Choi AM. Carbon monoxide: present and future indications for a medical gas. *Korean J Intern Med*. 2013; 28:123–40.
22. Sun BW, Chen X. Carbon monoxide releasing molecules: new insights for anticoagulation strategy in sepsis. *Cell Mol Life Sci*. 2009; 66:365–9.
23. Motterlini R, Mann BE, Johnson TR, Clark JE, Foresti R, Green CJ. Bioactivity and pharmacological actions of carbon monoxide-releasing molecules. *Curr Pharm Des*. 2003; 9:2525–39.
24. Tsoyi K, Lee TY, Lee YS, Kim HJ, Seo HG, Lee JH, Chang KC. Heme-oxygenase-1 induction and carbon monoxide-releasing molecule inhibit lipopolysaccharide (LPS)-induced high-mobility group box 1 release *in vitro* and improve survival of mice in LPS- and cecal ligation and puncture-induced sepsis model *in vivo*. *Mol Pharmacol*. 2009; 76:173–82.
25. Cepinskas G, Katada K, Bihari A, Potter RF. Carbon monoxide liberated from carbon monoxide-releasing molecule CORM-2 attenuates inflammation in the liver of septic mice. *Am J Physiol Gastrointest Liver Physiol*. 2008; 294:G184–91.
26. Wang X, Cao J, Sun BW, Liu DD, Liang F, Gao L. Exogenous carbon monoxide attenuates inflammatory responses in the small intestine of septic mice. *World J Gastroenterol*. 2012; 18:5719–28.
27. Sun B, Zou X, Chen Y, Zhang P, Shi G. Preconditioning of carbon monoxide releasing molecule-derived CO attenuates LPS-induced activation of HUVEC. *Int J Biol Sci*. 2008; 4:270–8.
28. Liu D, Liang F, Wang X, Cao J, Qin W, Sun B. Suppressive effect of CORM-2 on LPS-induced platelet activation by glycoprotein mediated HS1 phosphorylation interference. *PLoS One*. 2013; 8:e83112.
29. Phillips DR, Charo IF, Scarborough RM. GPIIb-IIIa: the responsive integrin. *Cell*. 1991; 65:359–62.
30. Smyth SS, Reis ED, Väänänen H, Zhang W, Collier BS. Variable protection of beta 3-integrin--deficient mice from thrombosis initiated by different mechanisms. *Blood*. 2001; 98:1055–62.
31. Smyth SS, Reis ED, Väänänen H, Zhang W, Collier BS. A role for PKC θ in outside-in α (IIb) β 3 signaling. *J Thromb Haemost*. 2006; 4:648–55.
32. Cohen S, Braiman A, Shubinsky G, Isakov N. Protein kinase C- θ in platelet activation. *FEBS Lett*. 2011; 585:3208–15.
33. Anderson I, Low JS, Weston S, Weinberger M, Zhyvoloup A, Labokha AA, Corazza G, Kitson RA, Moody CJ, Marcello A, Fassati A. Heat shock protein 90 controls HIV-1 reactivation from latency. *Proc Natl Acad Sci USA*. 2014; 111:E1528–37.
34. Evenou JP, Wagner J, Zenke G, Brinkmann V, Wagner K, Kovarik J, Welzenbach KA, Weitz-Schmidt G, Guntermann C, Towbin H, Cottens S, Kaminski S, Letschka T, et al. The potent protein kinase C-selective inhibitor AEB071 (sotrastaurin) represents a new class of immunosuppressive agents affecting early T-cell activation. *J Pharmacol Exp Ther*. 2009; 330:792–801.
35. Sudhof TC, Rothman JE. Membrane fusion: grappling with SNARE and SM proteins. *Science*. 2009; 323:474–7.
36. Fitch-Tewfik JL, Flaumenhaft R. Platelet granule exocytosis: a comparison with chromaffin cells. *Front Endocrinol (Lausanne)*. 2013; 4:77.
37. Italiano JE Jr, Battinelli EM. Selective sorting of α -granule proteins. *J Thromb Haemost*. 2009; 7:173–6.
38. Hagemeyer CE, Peter K. Targeting the platelet integrin GPIIb/IIIa. *Curr Pharm Des*. 2010; 16:4119–33.
39. Joo SJ. Mechanisms of Platelet Activation and Integrin α IIb β 3. *Korean Circ J*. 2012; 42:295–301.
40. Nieswandt B, Varga-Szabo D, Elvers M. Integrins in platelet activation. *J Thromb Haemost*. 2009; 1:206–9.
41. Shattil SJ, Newman PJ. Integrins: dynamic scaffolds for adhesion and signaling in platelets. *Blood*. 2004; 104:1606–15.
42. Nagy B Jr, Bhavaraju K, Getz T, Bynagari YS, Kim S, Kunapuli SP. Impaired activation of platelets lacking protein kinase C- θ isoform. *Blood*. 2009; 113:2557–67.
43. Jahn R, Scheller RH. SNAREs—engines for membrane fusion. *Nat Rev Mol Cell Biol*. 2006; 7:631–43.
44. Flaumenhaft R. Molecular basis of platelet granule secretion. *Arterioscler Thromb Vasc Biol*. 2003; 23:1152–60.
45. Zilly FE, Sørensen JB, Jahn R, Lang T. Munc18-bound syntaxin readily forms SNARE complexes with synaptobrevin in native plasma membranes. *PLoS Biol*. 2006; 4:e330.
46. Rickman C, Duncan RR. Munc18/Syntaxin interaction kinetics control secretory vesicle dynamics. *J Biol Chem*. 2010; 285:3965–72.
47. Schraw TD, Lemons PP, Dean WL, Whiteheart SW. A role for Sec1/Munc18 proteins in platelet exocytosis. *Biochem J*. 2003; 374:207–17.
48. Barclay JW, Craig TJ, Fisher RJ, Ciuffo LF, Evans GJ, Morgan A, Burgoyne RD. Phosphorylation of Munc18 by protein kinase C regulates the kinetics of exocytosis. *J Biol Chem*. 2003; 278:10538–45.
49. Burgoyne RD, Barclay JW, Ciuffo LF, Graham ME, Handley MT, Morgan A. The functions of Munc18–1 in regulated exocytosis. *Ann N Y Acad Sci*. 2009; 1152:76–86.
50. Morgan A, Burgoyne RD, Barclay JW, Craig TJ, Prescott GR, Ciuffo LF, Evans GJ, Graham ME. Regulation of exocytosis by protein kinase C. *Biochem Soc Trans*. 2005; 33:1341–4.
51. Liu D, Wang X, Qin W, Chen J, Wang Y, Zhuang M, Sun B. Suppressive effect of exogenous carbon monoxide on endotoxin-stimulated platelet over-activation via the

- glycoprotein-mediated PI3K-Akt-GSK3 β pathway. *Sci Rep*. 2016; 6:23653.
52. Stojanovic A, Marjanovic JA, Brovkovich VM, Peng X, Hay N, Skidgel RA, Du X. A phosphoinositide 3-kinase-AKT-nitric oxide-cGMP signaling pathway in stimulating platelet secretion and aggregation. *J Biol Chem*. 2006; 281:16333–9.
 53. Sun B, Sun Z, Jin Q, Chen X. CO-releasing molecules (CORM-2)-liberated CO attenuates leukocytes infiltration in the renal tissue of thermally injured mice. *Int J Biol Sci*. 2008; 4:176–83.
 54. Zhang G, Han J, Welch EJ, Ye RD, Voyno-Yasenetskaya TA, Malik AB, Du X, Li Z. Lipopolysaccharide stimulates platelet secretion and potentiates platelet aggregation via TLR4/MyD88 and the cGMP-dependent protein kinase pathway. *J Immunol*. 2009; 182:7997–8004.
 55. Ohtsuka M, Sasaki K, Ueno T, Seki R, Nakayoshi T, Koiwaya H, Toyama Y, Yokoyama S, Mitsutake Y, Chibana H, Itaya N, Okamura T, Imaizumi T. Platelet-derived microparticles augment the adhesion and neovascularization capacities of circulating angiogenic cells obtained from atherosclerotic patients. *Atherosclerosis*. 2013; 227:275–82.
 56. Al Hawas R, Ren Q, Ye S, Karim ZA, Filipovich AH, Whiteheart SW. Munc18b/STXBP2 is required for platelet secretion. *Blood*. 2012; 120:2493–500.
 57. Woronowicz K, Dilks JR, Rozenvayn N, Dowal L, Blair PS, Peters CG, Woronowicz L, Flaumenhaft R. The platelet actin cytoskeleton associates with SNAREs and participates in alpha-granule secretion. *Biochemistry*. 2010; 49:4533–42.
 58. Karim ZA, Mukhopadhyay S, Ramars AS, Dash D. Sustained stimulation of platelet thrombin receptor is associated with tyrosine dephosphorylation of a novel p67 peptide in a manner regulated by extracellular calcium. *Biochim Biophys Acta*. 2004; 1693:147–57.