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Original Article

Exogenous carbon monoxide suppresses LPS-stimulated platelet activation by Interfering with the TLR4/PKCα/integrin $\alpha_{IIb}\beta_3$ pathway in vitro

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Abstract: To investigate whether exogenous carbon monoxide (CO) can suppress lipopolysaccharide (LPS)-stimulated platelet activation and clarify its potential mechanisms. An LPS-stimulated platelet model was used to mimic platelet activation in sepsis. Exogenous CO liberated from CO-releasing molecules II (CORM-2) was administrated. Platelet adhesion, aggregation and granule secretion were measured separately. Immunoprecipitation and western blot were adopted to measure the activity of toll-like receptor 4 (TLR4) and protein kinase C (PKC)-α. Expression of integrin $\alpha_{IIb}\beta_3$ was detected by flow cytometry. LPS stimulation significantly increased platelet adhesion, aggregation and granule secretion, while exogenous CO significantly ameliorated these responses. The elevated activity of TLR4 and PKCα were effectively suppressed by exogenous CO. Furthermore, platelet expression of integrin $\alpha_{IIb}\beta_3$ was significantly elevated by LPS stimulation, while exogenous CO abolished this increase. Inhibition of PKCα activity with RO31-8220 decreased, but not completely abolished, LPS-stimulated platelet reactions. LPS stimulated platelet activation in vitro. Exogenous CO suppressed this reaction through interference of the TLR4/PKCα/Integrin $\alpha_{IIb}\beta_3$ pathway.

Keywords: Sepsis, platelets, carbon monoxide, integrin $\alpha_{IIb}\beta_3$, TLR4, PKCα

Introduction

Sepsis is defined as life-threatening organ dysfunction caused by deregulation of host responses to infection and, to date remains one of the leading causes of mortality among critically ill patients [1-3]. Septic patients often present with thrombocytopenia, as well as circulating platelet-leukocyte aggregates [4-6], and may develop disseminated intravascular coagulation. The latter of these complications involves the systemic activation of the clotting cascade, fibrin deposition and thrombosis formation, and may result in pulmonary embolism, multiple organ failure and death [4].

Emerging evidence reveals that platelet overactivation during sepsis accelerates microthrombi formation and amplifies inflammatory responses [7-9]. The protein kinase C (PKC) family has been implicated in this process, and functions downstream of Toll-like receptors (TLRs) [10-12] thereby regulating platelet activation [13]. PKCα, one of the classical PKC isoforms, plays a key role in platelet activation [14], regulating platelet function in both humans and mice [15, 16]. TLR4, the major subtype of TLRs located on the platelet surface, induce trans-membrane signaling [12] and are associated with host defense against microbial invasion, and furthermore, regulate platelet functional responses [12, 17]. Studies have suggested that integrin $\alpha_{IIb}\beta_3$, the most abundant platelet surface protein, regulates platelet adhesion, aggregation and granule release [18]. Integrin β_3, the functional subunit of integrin $\alpha_{IIb}\beta_3$, is responsible for key signal transduction steps [19]. We believe that is reasonable to suggest that the TLR4/PKCα/Integrin $\alpha_{IIb}\beta_3$ pathway may be responsible for platelet activation in sepsis.

It is well known that endogenous carbon monoxide (CO) can modulate inflammation [20-22]:

administration of exogenous CO, liberated from CO-releasing molecules (CORMs), inhibits the production of cytokines and protects vital organ function [23-26]. Our earlier studies have shown that CO possesses anti-inflammatory effects [27-29], however the mechanisms underpinning this property remain unclear.

Based on our previous findings we hypothesized exogenous CO may decrease LPS-induced platelet activation through TLR4/PKCα/Integrin $\alpha_{IIb}\beta_3$ pathway.

Materials and methods

Materials

CORM-2 and LPS were purchased fromSigma Aldrich (St Louis, USA). FITC-CD41 mAb was purchased from eBioscience (San Diego, USA). PE-integrin β_3 mAb and β-actin goat mAb were purchased from Santa Cruz Biotechnology (Dallas, USA). TLR4 rabbit mAb, PKCα rabbit mAb and phospho-PKCα (Thr638) rabbit mAb were bought from the Cell Signaling Technology (Boston, USA). All other chemical reagents were obtained from the Sigma, unless otherwise stated.

Preparation of LPS-stimulated platelet model

This study was approved by the Ethical Committee at Jiangsu University, Jiansu Province, China. After consent was obtained, blood was withdrawn from healthy drug-free donors' veins. Platelet derivation and LPS-stimulated platelet model were carried out as described previously [30, 31]. Briefly, experimental platelet-rich plasma (PRP) treatments were randomly divided into five groups. Normal group underwent no treatment, LPS group received LPS (10 μg/mL) stimulation for thirty minutes, CORM-2 group and iCORM-2 group received the exact same dose of LPS, plus immediate treatment with CORM-2 (10 or 50 μM) and iCORM-2 (50 μM). Both pre-CORM-2 and post-CORM-2 intervention treatments were performed as additional experiments. RO31-8220 (5 nM), a PKCα inhibitor, was incubated with the platelets for five minutes before stimulation in additional experiments.

Platelet adhesion

A total of 1.5 mL of sample from each group was added into spherical glass bottles. The bottles were rotated at the speed of 3 r/min. One milliliter of blood from pre-rotated and post-rotated samples was collected. An automatic coagulation analyzer was used to count the number of the platelets in the collected samples. The platelet adhesion ratio was calculated with the formula: Platelet adhesion rate (%) = (pre-rotated platelet count-post-rotated count)/pre-rotated platelet count) ×100. Five minutes of data were recorded.

Platelet aggregation

An aggregometer was adopted to detect platelet aggregation rate [30, 32]. A total of 300 μL of PRP from each group was incubated with ferrite beads at 37°C whilst stirring, and adenosine diphosphate (10 μmol/L) was used to induce platelet aggregation.

Platelet secretion

Adenosine triphosphate (ATP) release was measured to assess dense granule secretion [33]. Briefly, luciferin-luciferase reagent was added to PRP (final concentration of 100 nM) for 3 s. A luminometer was used to measure luminescence. α-granule secretion was assessed as P-selectin expression and examined by flow cytometry as previous described [34].

Flow cytometry

Both P-selectin and integrin β_3 expression were studied by flow cytometry as previously described [35]. Platelets were collected, fixed and washed. The washed platelets were incubated with CD41-FITC. Then, P-selectin-PE and integrin β_3-PE were independently added into the samples. All incubated samples were analyzed by flow cytometry.

Protein production

Platelets were co-incubated with LPS (10 μg/mL) with or without CORM-2 intervention. RIPA (Radio Immunoprecipitation Assay) buffer containing protease and phosphatase inhibitor cocktails was applied to stop the reaction and split platelets. The lysate was divided equally into two parts. Lysate aliquots were collected by immunoprecipitation for detection of target phosphorylated protein and were processed by immunoblotting for detection of target proteins.

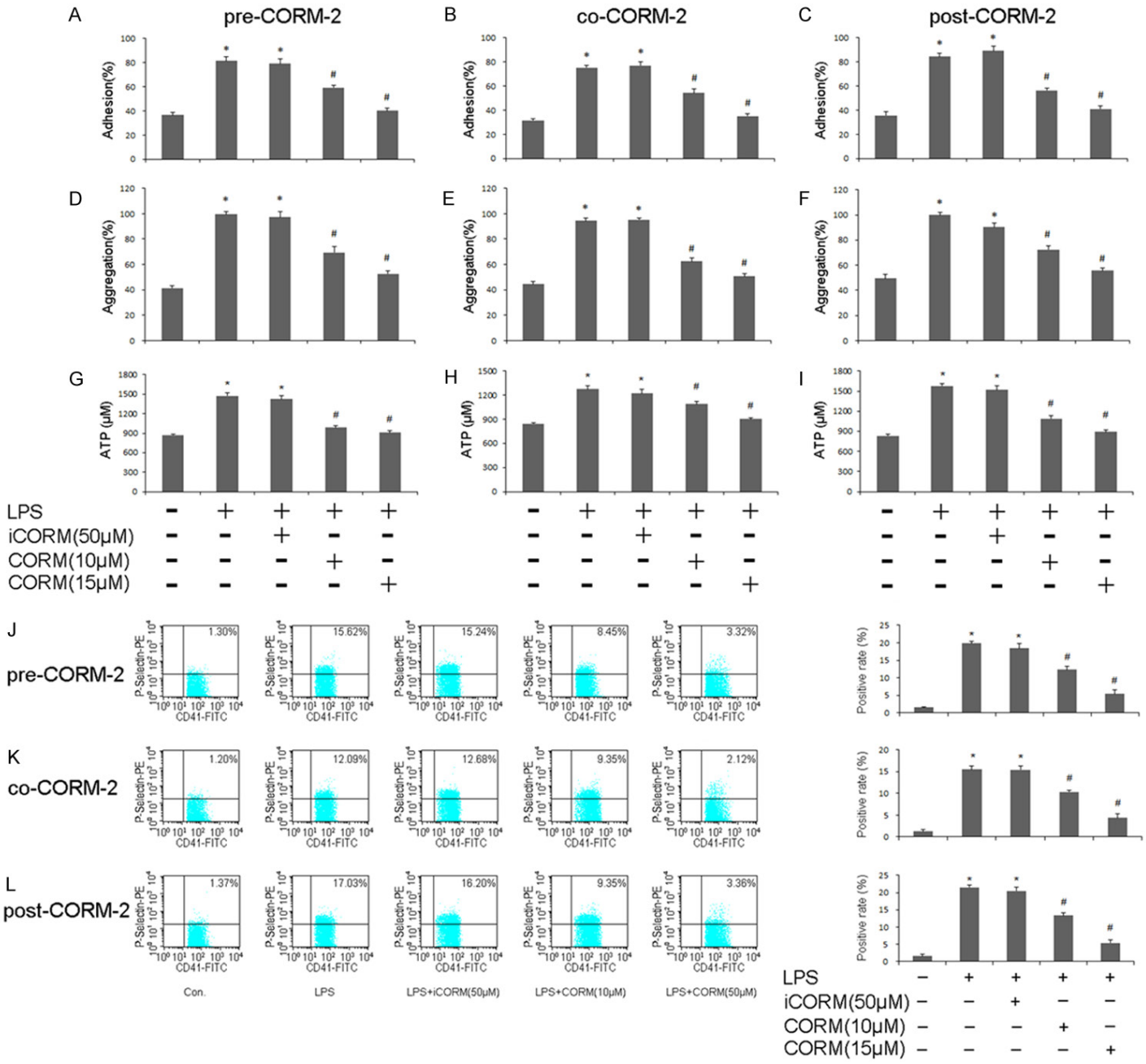
A
pre-CORM-2
B
co-CORM-2
C
post-CORM-2
Adhesion(%)
D
E
F
Aggregation(%)
G
H
I
ATP (μM)
LPS
iCORM(50μM)
CORM(10μM)
CORM(15μM)
J
pre-CORM-2
K
co-CORM-2
L
post-CORM-2
1.30%
15.62%
15.24%
8.45%
3.32%
1.20%
12.09%
12.68%
9.35%
2.12%
1.37%
17.03%
16.20%
9.35%
3.36%
P-Selectin-PE
CD41-FITC
Positive rate (%)
Con.
LPS
LPS+iCORM(50μM)
LPS+CORM(10μM)
LPS+CORM(50μM)

Figure 1. Platelet adhesion, aggregation and granule exocytosis in LPS stimulation and CORM-2 intervention groups. Upon stimulation with LPS, an increase in platelet adhesion was demonstrated, and co-CORM-2 intervention effectively abolished this increase in a dose-dependent manner (B). Similar phenomena were detected in the pre-CORM-2 intervention (A) and post-CORM-2 intervention groups (C). Upon stimulation with LPS, a markedly increase in platelet aggregation was demonstrated, and co-CORM-2 intervention effectively abolished this increase in a dose-dependent manner (E). Similar phenomena were also detected in the pre-CORM-2 intervention (D) and post-CORM-2 intervention groups (F). After LPS stimulation, a significant increase in platelet ATP release was detectable, and co-CORM-2 intervention effectively abolished this increase in a dose-dependent manner (H). Similar phenomena were also detected in the pre-CORM-2 intervention (G) and post-CORM-2 intervention groups (I). The positive rate of P-selectin was detected via flow cytometry. LPS stimulation lead to a markedly increase in P-selectin expression and co-CORM-2 intervention effectively abolished this increase in a dose-dependent manner (K). Similar phenomena were also detected in the pre-CORM-2 intervention (J) and post-CORM-2 intervention groups (L). One-way ANOVA was used to assess the difference among groups. SNK was applied to assess the difference between two groups. *P<0.05 as compared to normal, #P<0.05 as compared to LPS.

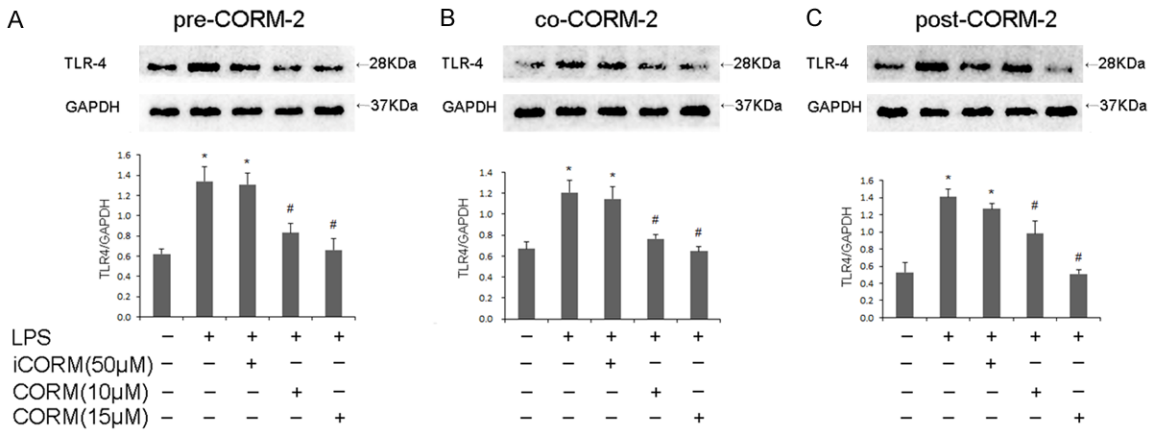

Figure 2. TLR4 expression in LPS stimulation and CORM-2 intervention. LPS stimulation lead to a markedly increase in TLR4 expression and co-CORM-2 intervention significantly reduced this increase (B). Similar phenomena were also detected in the pre-CORM-2 intervention (A) and post-CORM-2 intervention groups (C). One-way ANOVA was used to assess the difference among groups. SNK was applied to assess the difference between two groups. *P<0.05 as compared to normal, #P<0.05 as compared to LPS.

Immunoprecipitation

For detection of phospho-PKCα, immunoprecipitation was adopted as previously described [36]. Briefly, platelet lysates were centrifuged at 12,000 g for ten minutes. Non-specifically bound proteins were removed by incubating platelet lysates with the PKCα antibody alone overnight, and rotated with protein A-sepharose beads for four hours. All procedures were performed at 4°C. The mixture was washed twice, and the bound proteins were eluted and subjected to western blotting with the indicated antibodies as described below.

Western blot

Proteins were subjected to electrophoresis on 12% SDS-polyacrylamide gels, transferred onto nitrocellulose membranes and incubated with different dilutions of primary and secondary antibodies. The bands were visualized by the use of ECL reagent. Bands were scanned and quantified by Basic Quantifier software.

Statistical analyses

Data was presented as the mean ± standard deviation (SD). One-way ANOVA was used to assess the difference among groups. The post-hoc test (SNK) was applied to assess the difference between two groups. P<0.05 was considered statistically significant. Data analysis was performed with SPSS 16.0 (Chicago, IL, USA).

Results

Platelet adhesion, aggregation and granules exocytosis in LPS stimulation and CORM-2 intervention

The rate of platelet adhesion and aggregation was significantly increased with LPS stimulation, and treatment with CORM-2 effectively

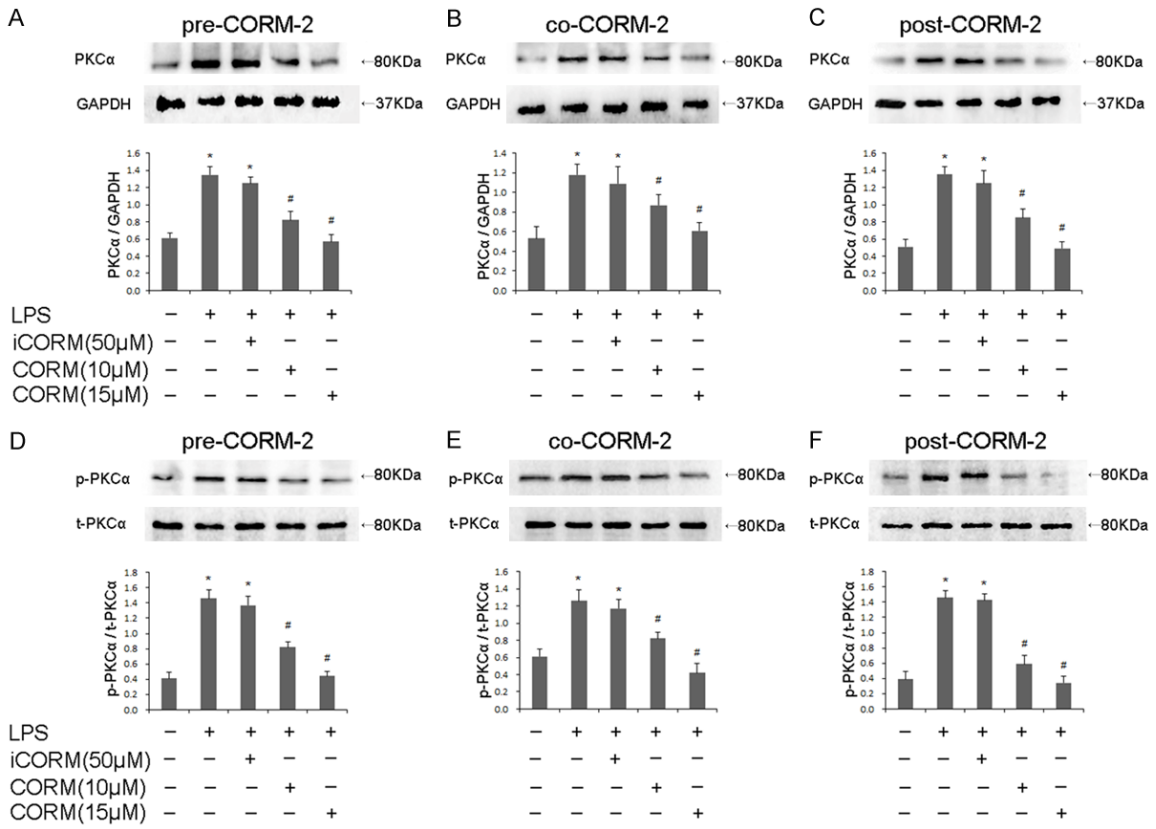

Figure 3. PKCα production and phosphorylation in LPS stimulation and CORM-2 intervention. Upon stimulation with LPS, a markedly increase in PKCα production and co-CORM-2 intervention significantly reduced this effect (B). Similar phenomena were also detected in the pre-CORM-2 intervention (A) and post-CORM-2 intervention groups (C). LPS stimulation lead to a markedly increase in PKCα phosphorylation and co-CORM-2 intervention significantly reduced this increase (E). Similar phenomena were also detected in the pre-CORM-2 intervention (D) and post-CORM-2 intervention groups (F). One-way ANOVA was used to assess the difference among groups. SNK was applied to assess the difference between two groups. *P<0.05 as compared to normal, #P<0.05 as compared to LPS.

abolished this increase in a dose-dependent manner (**Figure 1B**, **1E**). Similar observations in pre-CORM-2 intervention (**Figure 1A**, **1D**) and post-CORM-2 intervention groups (**Figure 1C**, **1F**) were also noted.

Both ATP release and P-selectin expression were significantly increased after LPS stimulation, and CORM-2 treatment showed a dose-dependent reduction in ATP release and P-selectin expression (**Figure 1H**, **1K**). Similar alterations were found in pre-CORM-2 intervention (**Figure 1G**, **1J**) and post-CORM-2 intervention groups (**Figure 1I**, **1L**).

TLR4 production in LPS stimulation and CORM-2 intervention

LPS stimulation increased the expression of TLR4. However, the level of TLR4 expression was down-regulated by co-CORM-2 intervention (**Figure 2B**). Similar phenomena were observed in pre-CORM-2 intervention (**Figure 2A**) and post-CORM-2 intervention groups (**Figure 2C**). Results suggested that platelet over-activation induced by LPS-stimulation may be involved in TLR4 expression, and exogenous CO effectively reduced this effect.

PKCα production and phosphorylation in LPS stimulation and CORM-2 intervention

Both the production and phosphorylation of PKCα were increased upon LPS stimulation. However, this increase was abolished by co-CORM-2 intervention (**Figure 3B**, **3E**). Similar phenomena were observed in pre-CORM-2 intervention (**Figure 3A**, **3D**) and post-CORM-2 intervention groups (**Figure 3C**, **3F**).

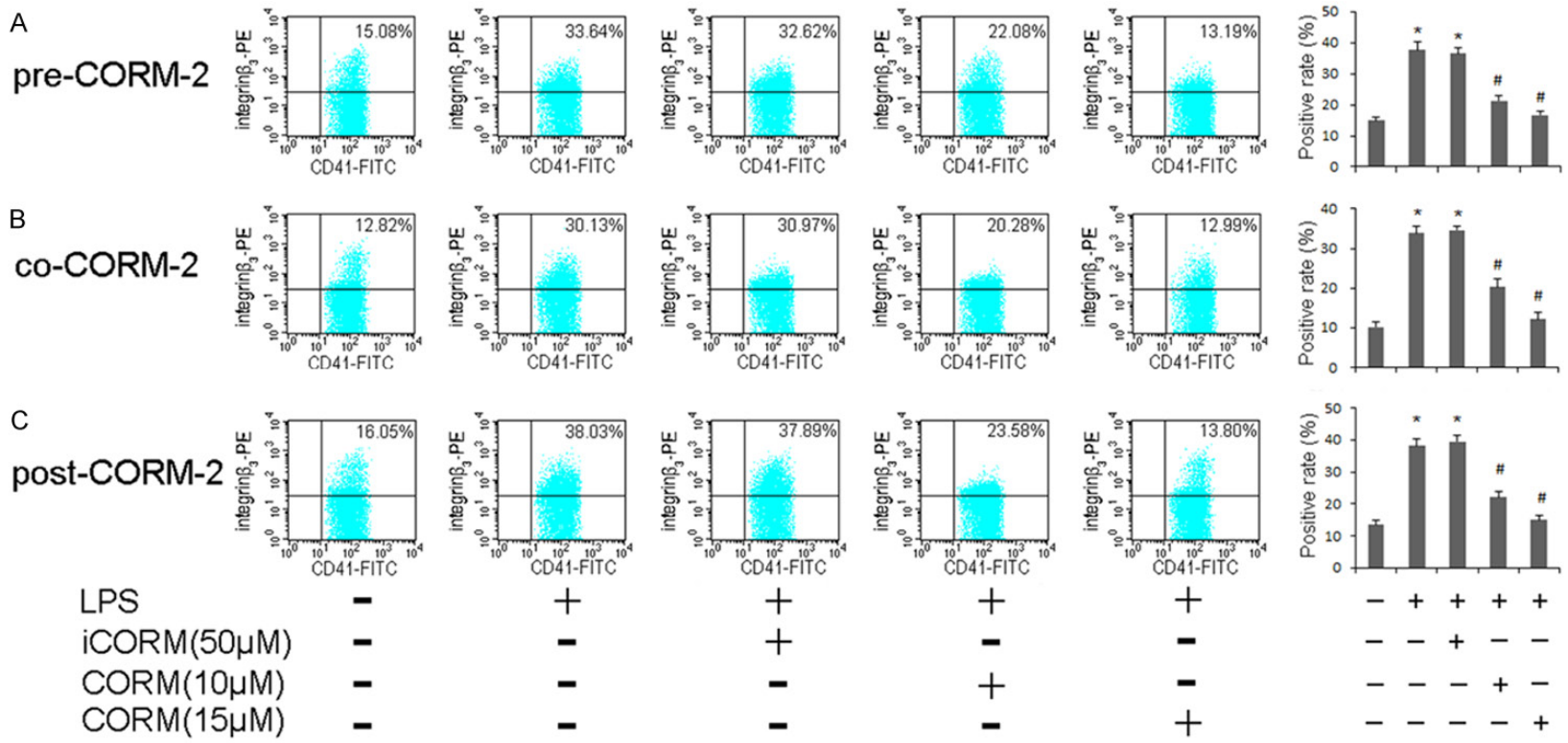

Figure 4. Integrin β_3 exposure in LPS stimulation and CORM-2 intervention. LPS stimulation lead to a markedly increase in integrin β_3 expression and co-CORM-2 intervention significantly reduced this increase (B). Similar phenomena were also detected in the pre-CORM-2 intervention (A) and post-CORM-2 intervention groups (C). One-way ANOVA was used to assess the difference among groups. SNK was applied to assess the difference between two groups. *P<0.05 as compared to normal, #P<0.05 as compared to LPS.

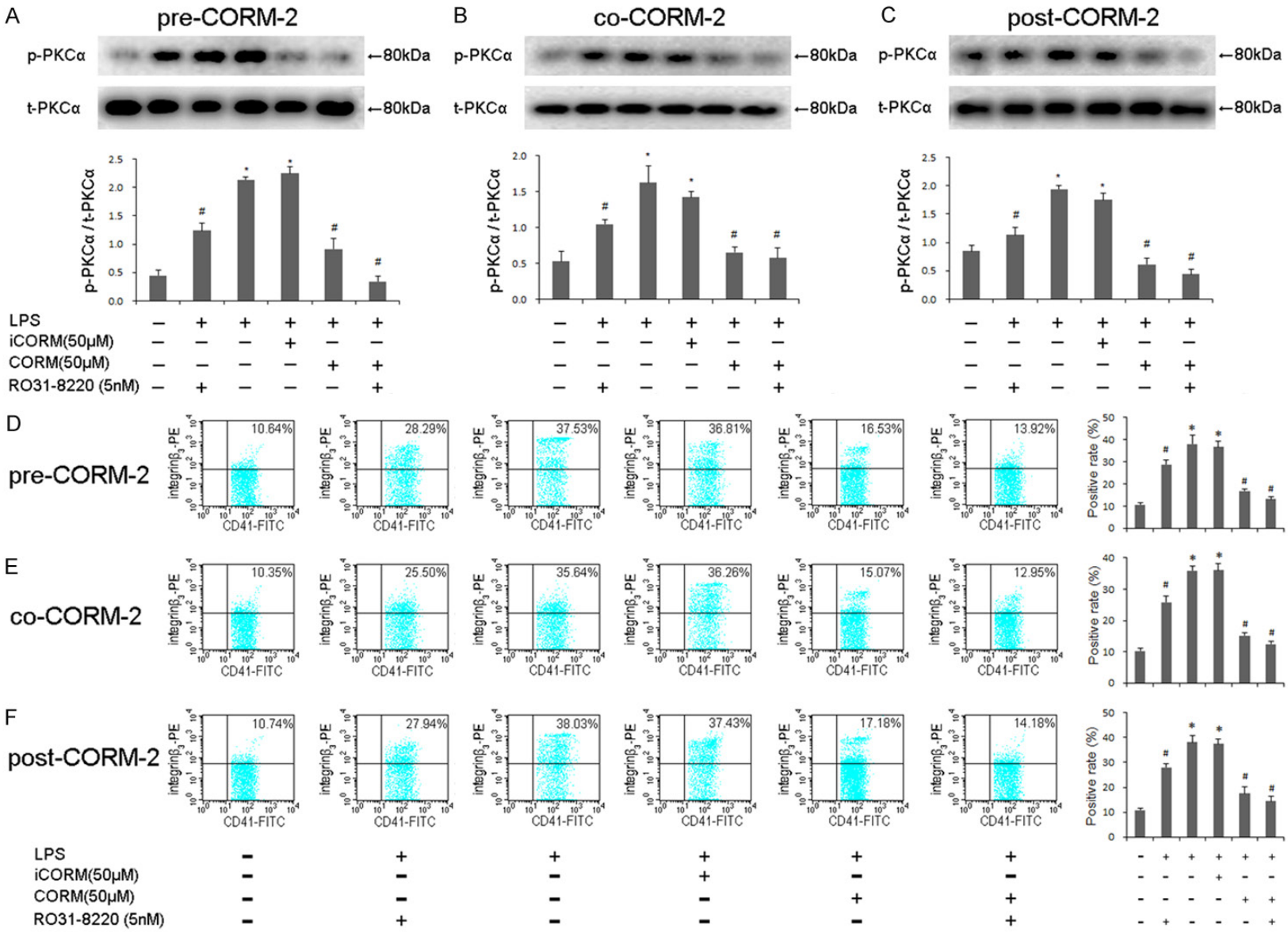

Figure 5. PKCα phosphorylation and integrin β_3 expression with RO31-8220 treatment and CORM-2 intervention. LPS stimulation lead to a markedly increase in PKCα phosphorylation. Co-incubation with RO31-8220 and CORM-2 significantly reduced this increase (B). Similar phenomena were also detected in the pre-

CORM-2 intervention (A) and post-CORM-2 intervention groups (C). LPS stimulation lead to a markedly increase in integrin β_3 expression, RO31-8220 treatment mildly reduced this increase, and CORM-2 treatment significantly abolished this increase (E). Similar phenomena were also detected in the pre-CORM-2 intervention (D) and post-CORM-2 intervention groups (F). One-way ANOVA was used to assess the difference among groups. SNK was applied to assess the difference between two groups. *P<0.05 as compared to normal, #P<0.05 as compared to LPS.

Integrin β_3 exposure in LPS stimulation and CORM-2 intervention

In the LPS group, integrin β_3 expression was significantly up-regulated. However, integrinβ_3 expression was markedly decreased upon CORM-2 co-incubation (**Figure 4B**). Similar phenomena were observed in pre-CORM-2 intervention (**Figure 4A**) and post-CORM-2 intervention groups (**Figure 4C**).

Platelet activation in RO31-8220 precondition

In order to further explore the role of the TLR4/PKCα/Integrin $\alpha_{IIb}\beta_3$ pathway in platelet activation, RO31-8220 was applied to inhibit PKCα activity at a concentration of 5 nmol/L. The phosphorylation of PKCα was decreased compared with the LPS group following RO31-8220 treatment, with or without CORM-2 treatment (50 μmol/L) (**Figure 5B**). Similar phenomena were observed in the pre-CORM-2 intervention (**Figure 5A**) and post-CORM-2 intervention groups (**Figure 5C**).

An increase in integrin β_3 expression was reduced upon RO31-8220 treatment, and CORM-2 treatment abolished this increase with or without RO31-8220 treatment (**Figure 5E**). Similar phenomena were observed inthe pre-CORM-2 intervention (**Figure 5D**) and post-CORM-2 intervention groups (**Figure 5F**). However, there was no effect on TLR4 expression following RO31-8220 treatment.

RO31-8220 mildly inhibited platelet adhesion and reduced the effects of aggregation induced by LPS. The co-CORM-2 treatment group significantly abolished this increase with or without RO31-8220 (**Figure 6B**, **6E**). Similar phenomena were shown in the pre-CORM-2 intervention (**Figure 6A**, **6D**) and post-CORM-2 intervention groups (**Figure 2C**, **2F**). Furthermore, RO31-8220 intervention mildly inhibited ATP release and P-selectin expression induced by LPS, and co-CORM-2 treatment strongly abolished this increase in both the presence and absence of RO31-8220 (**Figure 6H**, **6K**). Similar results were shown in the pre-CORM-2 intervention (**Figure 2G**, **2J**) and post-CORM-2 intervention groups (**Figure 2I**, **2L**).

Discussion

The main findings of our study showed that CO released from CORM-2 altered the pathological and physiological changes of LPS-induced platelet function, namely adhesion, aggregation, and granulesecretion, mediated by the TLR4/PKCα/Integrin $\alpha_{IIb}\beta_3$ pathway. Inhibition of PKCα activity with RO31-8220 decreased, but not completely abolished, LPS-stimulated platelet aggregation.

Recent studies have revealed that abnormal platelet activation is related to a poor prognostic outcome in septic patients. During early stages of sepsis, platelets are activated by extracellular matrix components or soluble agonists [37-40]. Activated platelets adhere to leucocytes, endothelial cells and aggregate. In addition, various chemokines are produced and are released into the circulation from activated platelet granules during sepsis. These mediators, including ADP, ATP and TXA_2, act as secondary agonists, which can induce further platelet activation. Others act as inflammatory mediators, such as PF4, β-TG and P-selectin, which increase inflammatory responses. All of these aggravate the damage caused by sepsis and reduce patient survival rates. We confirmed that LPS-stimulated platelet adhesion, aggregation, and granule secretion, and these effects were effectively abolished by exogenous CO, released from CORM-2, in both preconditioning and delayed groups [30, 31]. Further, we also found that inhibition of PKCα decreased, although did not completely abolish LPS-stimulated platelet function. Thus, we propose that exogenous CO interventioncould inhibit LPS-induced platelet over activation and PKCα plays a key role in platelet over activation stimulated by LPS.

PKCα, one of the classical PKC isoforms, plays a key role in controlling platelet formation and regulating platelet function [41-43]. Recent studies have indicated that PKCα is subse-

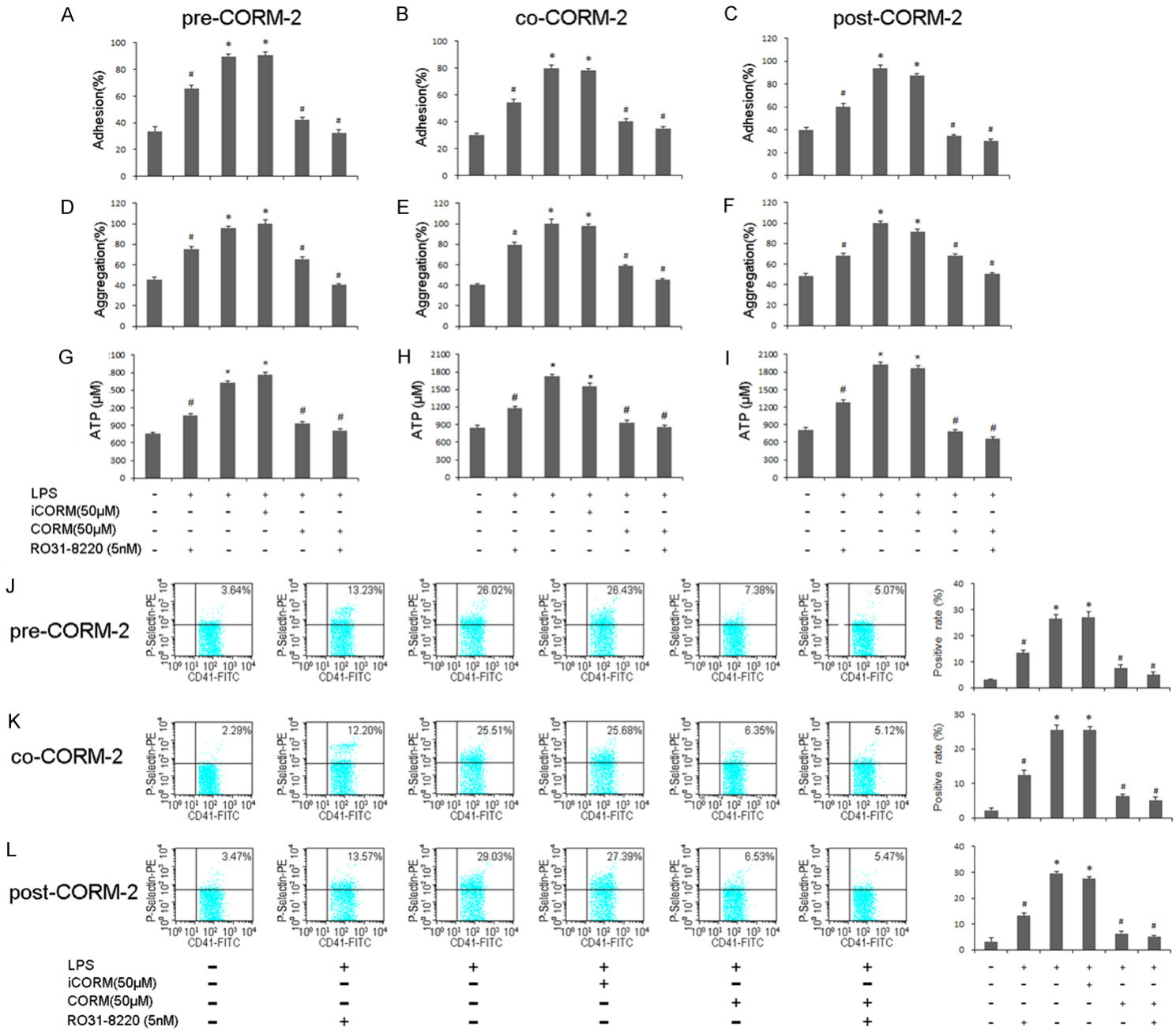
A
pre-CORM-2
B
co-CORM-2
C
post-CORM-2
Adhesion(%)
D
E
F
Aggregation(%)
G
H
I
ATP (μM)
LPS
iCORM(50μM)
CORM(50μM)
RO31-8220 (5nM)
J
pre-CORM-2
K
co-CORM-2
L
post-CORM-2
P-Selectin-PE
CD41-FITC
3.64%
13.23%
26.02%
26.43%
7.38%
5.07%
2.29%
12.20%
25.51%
25.68%
6.35%
5.12%
3.47%
13.57%
29.03%
27.39%
6.53%
5.47%
Positive rate (%)

Figure 6. Platelet adhesion, aggregation and granule exocytosis with RO31-8220 treatment and CORM-2 intervention. LPS stimulation lead to a markedly increase in platelet adhesion. Co-incubation with RO31-8220 and CORM-2 significantly reduced this increase (B). Similar phenomena were also detected in the pre-CORM-2 intervention (A) and post-CORM-2 intervention groups (C). LPS stimulation lead to a markedly increase in platelet aggregation. Co-incubation with RO31-8220 and CORM-2 significantly reduced this increase (E). Similar phenomena were also detected in the pre-CORM-2 intervention (D) and post-CORM-2 intervention groups (F). LPS stimulation lead to a markedly increase in platelet ATP release. Co-incubation with RO31-8220 and CORM-2 significantly reduced this increase (H). Similar phenomena were also detected in the pre-CORM-2 intervention (G) and post-CORM-2 intervention groups (I). LPS stimulation lead to a markedly increase in P-selectin expression. Co-incubation with CORM-2 significantly reduced this increase (K). Similar phenomena were also detected in the pre-CORM-2 intervention (J) and post-CORM-2 intervention groups (L).One-way ANOVA was used to assess the difference among groups. SNK was applied to assess the difference between two groups. $^{*}P<0.05$ as compared to normal, $^{\#}P<0.05$ as compared to LPS.

quently phosphorylated by TLR4 activation and regulates inflammatory activation [44]. TLR4, the major subtype of TLRs which belong to a family of receptors that recognize pathogen-associated molecular pattern recognition molecules and play a crucial role in against microbial infection, are activated by LPS combined into the platelet surface and induced transmembrane signaling [12, 17]. Both in vitro and in vivo studies have indicated that LPS-induced TLR4 signaling plays a significant role in platelet activation [45-47]. However, it is not clear whether the TLR4/PKCα pathway is associated with abnormal platelet activation during sepsis. In this study, the production of TLR4 and PKCα in LPS-stimulated platelets was significantly elevated. Interestingly, administration of CORM-2 effectively inhibited this elevation, in both preconditioning and delayed settings. In addition, LPS-stimulation up-regulated the activity of PKCα, and CORM-2 incubation could inhibit PKCα activation, in both preconditioning and delayed treatment conditions. The inhibition of PKCα did not influence TLR4 levels. This suggests that PKCα is downstream of this signal pathway. Thus, we propose that exogenous CO intervention could inhibit LPS-induced plateletactivation and PKCα may play a key role in LPS-induced platelet activation.

Platelet membrane glycoproteins are associated with platelet activation [48-50]. There are over ten species of glycoproteins. Integrin $\alpha_{IIb}\beta_3$ (also named CD41/CD61, GPIIb/IIIa), the most abundant platelet surface protein, taken charge offibrinogen and von Willebrand factor (vWF) binding and is required for normal platelet adhesion, aggregation and granule release [51-53]. Recent studies have shown that integrin $\alpha_{IIb}\beta_3$ is activated by both intracellular and extracellular signaling and exerts an essential role in thrombus formation in sepsis [54-56]. Integrin β_3 is the functional subunit of integrin $\alpha_{IIb}\beta_3$ and is responsible for key signal transduction [57]. In the present study, the expression of integrin β_3 in LPS-stimulated platelets was significantly up-regulated. Administration of CORM-2 effectively inhibited this increase in expression, in both CORM-2 preconditioned and delayed treatment groups. Furthermore, inhibition of PKCα further decreased integrin β_3 expression. This indicates that integrin β_3 is downstream of PKCα.

However, there are some limitations to our study. Firstly, our study only involved in vitro experiments, and our results need to be confirmed in in vivo models. Secondly, we only focused on the role of PKCα, while other important regulators remain to be elucidated.

Conclusion

In summary, data from the present study indicated that CORM-2 released CO could suppress LPS-induced platelet over activation, and the TLR4/PKCα/Integrin $\alpha_{IIb}\beta_3$ pathway activation plays an important role in this process.

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Disclosure of conflict of interest

None.

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