All-Trans Retinoic Acid Impairs Platelet Function and Thrombus Formation and Inhibits Protein Kinase CßI/δ Phosphorylation

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Abstract

All-trans retinoic acid (ATRA) is widely used for induction of complete remission in patients with acute promyelocytic leukemia (APL). ATRA also regulates protein kinase C (PKC) activity. Therapeutic use of ATRA reportedly interferes with hemostatic function in APL patients, including effects on coagulation or other vascular cells, although effects of ATRA on platelets remain unclear. This study aims to investigate the effect of therapeutic-relevant doses of ATRA on platelet function. Human platelets were preincubated with ATRA (0–20 μM) for 1 hour at 37°C, followed by analysis of aggregation, granule secretion, receptor expression by flow cytometry, platelet spreading, or clot retraction. Additionally, ATRA (10 mg/kg) was injected intraperitoneally into mice and tail bleeding time and arterial thrombus formation were evaluated. ATRA inhibited platelet aggregation and adenosine triphosphate release induced by collagen (5 μg/mL) or thrombin (0.05 U/mL) in a dose-dependent manner without affecting P-selectin expression or surface levels of glycoprotein (GP) Ibα, GPVI, or $\alpha_{IIb}\beta_3$. ATRA-treated platelets demonstrated reduced spreading on immobilized fibrinogen or collagen and reduced thrombin-induced clot retraction together with reduced phosphorylation of Syk and PLCγ2. In addition, ATRA-treated mice displayed significantly impaired hemostasis and arterial thrombus formation in vivo. Further, in platelets stimulated with either collagen-related peptide or thrombin, ATRA selectively inhibited phosphorylation of PKCßI (Ser661) and PKCδ (Thr505), but not PKCα or PKCßII phosphorylation (Thr638/641). In conclusion, ATRA inhibits platelet function and thrombus formation, possibly involving direct or indirect inhibition of PKCßI/δ, indicating that ATRA might be beneficial for the treatment of thrombotic or cardiovascular diseases.

Keywords
- all-trans retinoic acid
- platelet
- hemostasis
- thrombus formation
- protein kinase CßI/δ

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Introduction

Platelets play critical roles in thrombosis and hemostasis. At site of vascular injury, platelets attach to the subendothelial matrix through binding to exposed von Willebrand factor (vWF) or collagen via surface receptors glycoprotein (GP) Ibα or GPVI.[1,2] Engagement of platelet receptors triggers transduction of intraplatelet signaling, resulting in activation of integrin $\alpha_{IIb}\beta_3$ which binds fibrinogen and mediates platelet aggregation. This process is called $\alpha_{IIb}\beta_3$ "inside-out" signaling.[3] Ligand binding to aIIIb3 also initiates $\alpha_{IIb}\beta_3$ "outside-in" signaling and subsequent activation of c-Src, Syk, or PLCγ2, which mediates platelet spreading, clot retraction, and thrombus formation to prevent blood loss.[4] Therefore, abnormal platelet function is closely associated with bleeding or thrombosis under pathological conditions.

As the synthetic and natural forms of vitamin A, retinoids have been shown to regulate cell differentiation, growth, or apoptosis through binding to their cognate nuclear receptors,[5,6] which include retinoic acid receptor (RAR) and retinoid X receptor (RXR) family members. RARs can bind to all-trans retinoic acid (ATRA) and 9-cis RA, whereas RXRs bind specifically to 9-cis RA.[7] ATRA is an active metabolite of vitamin A under the family retinoid and has been widely used for induction of complete remission in patients with acute promyelocytic leukemia (APL) as the only well-established differentiation therapy.[8,9] In APL, RARα on chromosome 17 is fused with promyelocytic leukemia (PML) protein on chromosome 15, leading to formation of a fusion protein PML-RARα, which exerts negative effect on RA signaling and blocks cell differentiation.[10] Pharmacological concentrations of ATRA are capable to bind PML-RARα and overcome its inhibitory effects, resulting in promotion of terminal differentiation of leukemic promyelocytes.[11] ATRA has been reported to have effects on the hemostatic system of APL patients as demonstrated by decrease or normalization of clotting and fibrinolytic variables[12–14] as well as reduced proteolysis of the vWF.[15] In addition, ATRA has also been reported to interfere with the hemostatic properties of different cells, such as promyelocytic blast cells, normal human endothelial cells, and monocytes.[16] Furthermore, a previous study has demonstrated that rapid cytoskeletal events (platelet spreading) and actin-dependent morphological changes (extended cell body formation) were all significantly inhibited in ATRA-treated human platelets.[17] However, whether ATRA affects platelet function remains unclear.

In the present study, through incubation of human platelets with different concentrations of ATRA, we aim to investigate the role of ATRA in platelet function.

Materials and Methods

Reagents

ATRA was purchased from Sigma-Aldrich (St. Louis, Missouri, United States) and dissolved in 0.5% v/v (final concentration) dimethyl sulfoxide. Collagen and thrombin (≥ 10 NIH units/vial) were from Chrono-log Corporation (Havertown, Pennsylvania, United States). Collagen-related peptide (CRP) was prepared as previously described.[18] Fluorescein isothiocyanate (FITC)-conjugated mouse antihuman CD41a was from BD Biosciences (San Jose, California, United States). Phycoerythrin (PE)-conjugated antihuman/mouse CD62p (P-selectin) and antihuman GPVI purified antibody were purchased from eBioscience (San Diego, California, United States). FITC-conjugated anti-CD42b antibody was from Abcam (Cambridge, Massachusetts, United States). FITC-conjugated goat antimouse immunoglobulin G (IgG) was purchased from ZSGB-BIO (Beijing, China). β-actin antibody and antirabbit IgG (horseradish peroxidase-linked) antibody were purchased from Cell Signaling Technology (Danvers, Massachusetts, United States). Tirofiban was purchased from Grand Pharma (China) CO. LTD.

Animals

All experimental procedures involving animals were complied with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines and approved by the Ethic Committee of Xuzhou Medical University. C57BL/6 mice, aged 8 to 10 weeks and weighted 24 to 28 g, were purchased from SLAC Laboratory Animal Co., Ltd. (Shanghai, China). All mice were housed in specific pathogen-free grade environment with 12-hour light/dark cycle and free access to food and water.

Platelet Preparation

All experimental procedures involving collection of human and mouse blood were approved by the Ethic Committee of Xuzhou Medical University. Informed consent was obtained from all participants. Platelets were prepared from human and mouse blood as described previously.[19] For human platelets, venous blood was collected into a tube anticoagulated with trisodium citrate, glucose, and citric acid (ACD) and centrifuged for 20 minutes at 120 × *g* at room temperature to obtain platelet-rich plasma (PRP). Platelet pellets were then collected by centrifugation of PRP at 1,350 × *g* for 15 minutes, followed by washing three times in citrate-glucose-saline (CGS) buffer and resuspended in Tyrode's buffer. Mouse platelets were isolated from ACD anticoagulated blood, washed using CGS buffer, and resuspended in Tyrode's buffer.

Treatment of Platelets with ATRA

Isolated human platelets were incubated with different concentrations of ATRA (0, 1, 10, and 20 μM) at 37°C for 1 hour followed by relevant analysis.

Platelet Aggregation and Adenosine Triphosphate Release

Platelet aggregation was performed in the presence of fibrinogen (0.5 mg/mL). After ATRA treatment, platelet aggregation in response to collagen (5 μg/mL) and thrombin (0.05 U/mL) was evaluated in a Lumi-Aggregometer Model 700 (Chrono-log Corporation) at 37°C with stirring (1,000 revolutions per minute). Platelet aggregation was quantified as the percentage of maximum platelet aggregation in the absence of drug. The release of adenosine triphosphate (ATP) was monitored in parallel with platelet aggregation after addition of luciferin/luciferase reagent (Chrono-log Corporation) to the platelet suspension according to the manufacturer's

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instructions. ATP release was quantified relative to the vehicle (0 μM ATRA) treatment.

Platelet α-Granule Release

Platelet α-granule release was assessed through measuring the surface expression of the α-granule GP, P-selectin by flow cytometry as described previously.[20] Briefly, after ATRA treatment, human platelets were stimulated with collagen (5 and 10 μg/mL), thrombin (0.05 and 0.1 U/mL), or CRP (2 μg/mL) in the presence of PE-conjugated anti-P-selectin antibody followed by analyzing platelet P-selectin expression by flow cytometry. P-selectin expression was defined as the percentage of platelets in platelet-specific gate with positive staining of anti-P-selectin antibody.

Expression of Platelet Receptors

After treatment with ATRA, FITC-conjugated anti-CD42b antibody (GPIbα), FITC-conjugated mouse antihuman CD41a antibody (α_{IIb}), or antihuman GPVI antibody (detected by FITC-conjugated goat antimouse IgG) were added and incubated for 30 minutes at room temperature followed by flow cytometry analysis of the expression of platelet receptors as described previously.[21]

Platelet Spreading

Human platelets were placed on glass coverslips which were precoated with fibrinogen (10 μg/mL) or collagen (10 μg/mL) (4°C overnight) at 37°C for 90 minutes followed by washing with phosphate-buffered saline. Then, platelets were fixed, permeabilized, stained with Alexa Fluor-546-labeled phalloidin, and observed under a fluorescence microscopy (Nikon-80i) using an ×100 oil objective. The surface coverage was quantified using Image J software.

Clot Retraction

Human platelets-mediated clot retraction was initiated by addition of thrombin (1 U/mL) in the presence of 2 mM Ca^{2+} and 0.5 mg/mL fibrinogen at 37°C as described previously.[19] Images were captured every 30 minutes.

Tail Bleeding Assay

Mice received intraperitoneal injection of ATRA (10 mg/kg). After 30 minutes, tail bleeding time was measured as described previously.[19,21]

$FeCl_3$-Induced Arterial Thrombosis

After treated with ATRA (20 μM) or vehicle at 37°C for 30 minutes, mouse platelets (10^8) were labeled with calcein and infused into ATRA-treated mice or wild-type mice, respectively, via tail vein injection. After 30 minutes, 10% w/v (final concentration) $FeCl_3$ was used to cause damages to the mesenteric arterioles and thrombus formation was monitored by a fluorescence microscopy (Olympus BX53).[19,21]

Western Blotting

Washed human platelets were treated with CRP (5 μg/mL) or thrombin (under the condition of clot retraction) (1 U/mL) in the presence of different concentrations of ATRA or vehicle for 15 minutes. Levels of total and phosphorylated Syk (anti-Tyr-525 and pan-Syk, Bioworld Technology), PLCγ2 (anti-Tyr-1217 and pan-PLCγ2; Bioworld Technology), protein kinase C (PKC) α/βII (anti-Thr638/641, Cell Signaling Technology, and pan-PKCα, Affinity Biosciences), PKCßI (anti-Ser661 and pan-PKCßI, Affinity Biosciences), or PKCδ (anti-Thr505, Cell Signaling Technology, and pan-PKCδ, Affinity Biosciences) were assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis/Western blot. The density of protein band was quantified using Image J software and the phosphorylation level was presented as a ratio to the total level.

Statistical Analysis

Data are represented as mean ± standard deviation or mean ± standard error and analyzed using GraphPad Prism software. One-way analysis of variance (ANOVA) was conducted for comparison of difference among different groups. Two-way ANOVA with Bonferroni posttests was performed for comparison among different groups over time. A p-value of < 0.05 indicates statistical significance.

Results

ATRA Inhibits Platelet Aggregation and Dense-Granule Secretion

To investigate the effect of ATRA on platelet aggregation, we incubated human platelets with different concentrations of ATRA (0, 1, 10, and 20 μM) followed by measuring collagen- or thrombin-induced platelet aggregation. As seen in ►**Fig. 1A**, since dimethyl sulfoxide has been shown to have an impact on platelet function,[22,23] weak response was observed in vehicle-treated platelets. Compared with vehicle treatment (0 μM ATRA), ATRA significantly impaired platelet aggregation in response to collagen (5 μg/mL) or thrombin (0.05 U/mL) in a dose-dependent manner. To further evaluate whether ATRA affects platelet dense-granule secretion, we also measured ATP release in collagen or thrombin-stimulated platelets and showed ATRA significantly inhibited ATP release from collagen- or thrombin-treated platelets in a dose-dependent manner (►**Fig. 1B**). Interestingly, ATRA did not affect platelet α-granule secretion after collagen or thrombin stimulation even at a higher dose as demonstrated by no significant changes of P-selectin expression after ATRA treatment (►**Fig. 1C**). Taken together, these data show that ATRA inhibits platelet aggregation and dense-granule secretion.

ATRA Does Not Affect the Surface Expression of Platelet Receptors

Platelet surface receptors GPIbα, GPVI, and $\alpha_{IIb}\beta_3$ were demonstrated to play an important role in the regulation of platelet aggregation through engagement of their specific ligand, such as vWF (GPIbα), collagen (GPVI), and fibrinogen ($\alpha_{IIb}\beta_3$).[24,25] Given impaired platelet aggregation in response to agonist stimulation, surface expression of platelet receptors GPIbα, GPVI, and $\alpha_{IIb}\beta_3$ after ATRA treatment was measured by flow cytometry. As shown in ►**Fig. 2**, ATRA treatment did not affect the surface expression of GPIbα (►**Fig. 2A**), GPVI (►**Fig. 2B**), and $\alpha_{IIb}\beta_3$ (►**Fig. 2C**) after being treated with different

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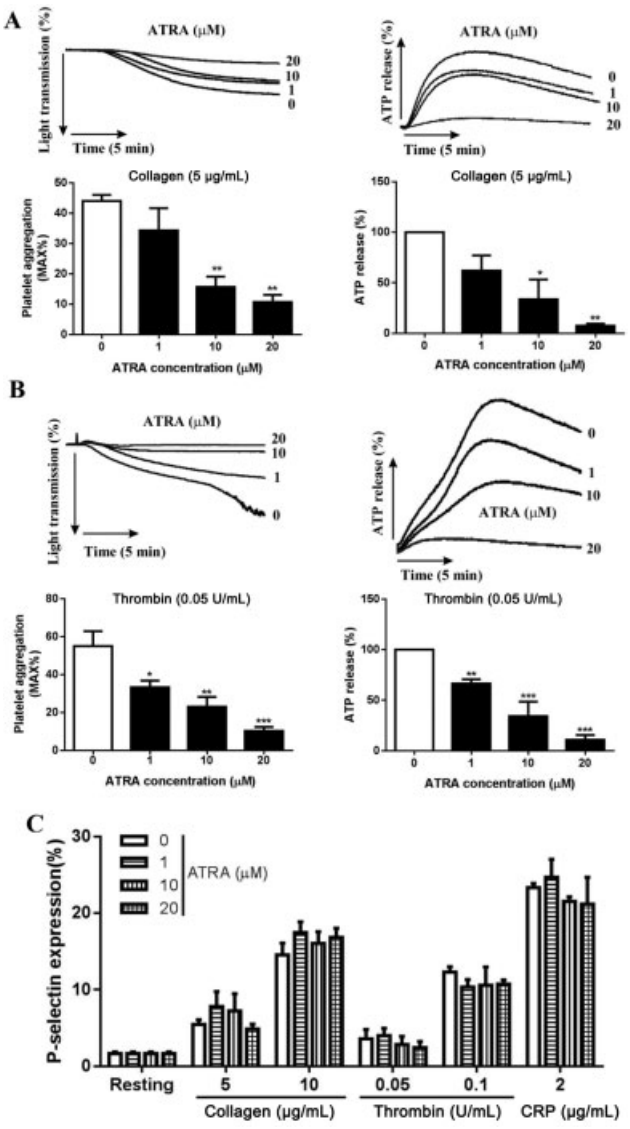

Fig. 1 Platelet aggregation and adenosine triphosphate (ATP) release. Washed human platelets were incubated with different concentrations of all-trans retinoic acid (ATRA) (0, 1, 10, and 20 μM) at 37°C for 1 hour followed by analysis of platelet aggregation in response to collagen (5 μg/mL) (**A**) or thrombin (0.05 U/mL) (**B**) in a Lumi-Aggregometer. Meanwhile, ATP release was measured simultaneously using luciferin/luciferase reagent. ATP release was presented relative to 0 μM ATRA which was defined as 100%. Platelet P-selectin expression was measured in ATRA-treated platelets after collagen or thrombin stimulation by flow cytometry (**C**). Data were presented as mean ± standard error (SE) ($n = 3$–4) and analyzed by one-way analysis of variance (ANOVA). Compared with 0, $^{*}p < 0.05$; $^{**}p < 0.01$.

concentrations of ATRA even at a higher dose as indicated by no significant changes of the expression of these platelet receptors compared with vehicle treatment.

Impaired Platelet Spreading and Clot Retraction after ATRA Treatment

To assess whether ATRA affects platelet spreading, after being treated with different concentrations of ATRA, human platelets were allowed to spread on immobilized fibrinogen or collagen and found that platelet spreading was significantly impaired in ATRA-treated platelets in a dose-dependent manner as the surface coverage area of spread platelets on fibrinogen (►**Fig. 3A**) or collagen (►**Fig. 3B**) after ATRA treatment was significantly smaller than platelets after vehicle treatment, consistent with a previous study showing platelet spreading was inhibited in ATRA-treated human platelets.[17] In addition, we also evaluate the effect of ATRA on platelet-mediated clot retraction, a process regulated by $\alpha_{IIb}\beta_3$ outside-in signaling.[4,26] Consistent with platelet spreading, clot retraction in ATRA-treated platelets was significantly inhibited in a dose-dependent manner as demonstrated by the significantly higher clot volume of ATRA-treated platelets than vehicle-treated platelets (►**Fig. 3C**). These data suggest that ATRA inhibits platelet $\alpha_{IIb}\beta_3$ outside-in signaling transduction.

Reduced Phosphorylation of Syk and PLCγ2 in ATRA-Treated Platelets

Since ATRA inhibits platelet aggregation, spreading, and clot retraction, we next assessed the effect of ATRA on platelet intracellular signaling transduction through measuring the phosphorylation level of Syk and PLCγ2, which have been shown to be important in the regulation of platelet function and $\alpha_{IIb}\beta_3$ signaling transduction.[4] Our data showed that the phosphorylation of Syk and PLCγ2 in CRP (5 μg/mL)-stimulated platelets was significantly impaired after ATRA treatment in a dose-dependent manner (►**Fig. 4A**). To investigate whether the impaired Syk phosphorylation is due to reduced GPVI signaling or integrin $\alpha_{IIb}\beta_3$ outside-in signaling, platelets were treated with tirofiban to block integrin $\alpha_{IIb}\beta_3$ followed by analysis of Syk phosphorylation and found that there was no difference of Syk phosphorylation in platelets treated with ATRA compared with that in platelets treated with vehicle in the presence of tirofiban (►**Supplementary Fig. S1**, available in the online version), indicating that the impaired Syk phosphorylation is due to impaired integrin $\alpha_{IIb}\beta_3$ outside-in signaling. Since thrombin-mediated clot retraction was inhibited in ATRA-treated platelets, the phosphorylation level of Syk and PLCγ2 which have been demonstrated to regulate clot retraction[27] was also measured in thrombin (1 U/mL)-treated platelets under the conditions of clot retraction and found reduced phosphorylation of Syk and PLCγ2 after ATRA treatment (►**Fig. 4B**). Taken together, these data show that ATRA inhibits $\alpha_{IIb}\beta_3$ outside-in signaling transduction possibly through inhibition of the phosphorylation of Syk and PLCγ2.

ATRA Impairs Hemostasis and Arterial Thrombosis In Vivo

To investigate the effect of ATRA on mouse platelet function in vivo, ATRA (10 mg/kg) was intraperitoneally injected into mice followed by analysis of platelet aggregation and ATP release as well as tail bleeding time. As seen in ►**Fig. 5**, ATRA-treated mice displayed significantly reduced CRP- or thrombin-induced platelet aggregation (►**Fig. 5A**) and ATP release (►**Fig. 5B**) compared with vehicle-treated mice. Regarding the effect of ATRA on in vivo hemostasis and thrombosis, mice receiving injection of ATRA showed a significantly prolonged tail bleeding time (265.80 ± 46.93 seconds) compared with mice receiving vehicle (96.00 ± 23.59 seconds) ($p < 0.05$) (►**Fig. 5C**).

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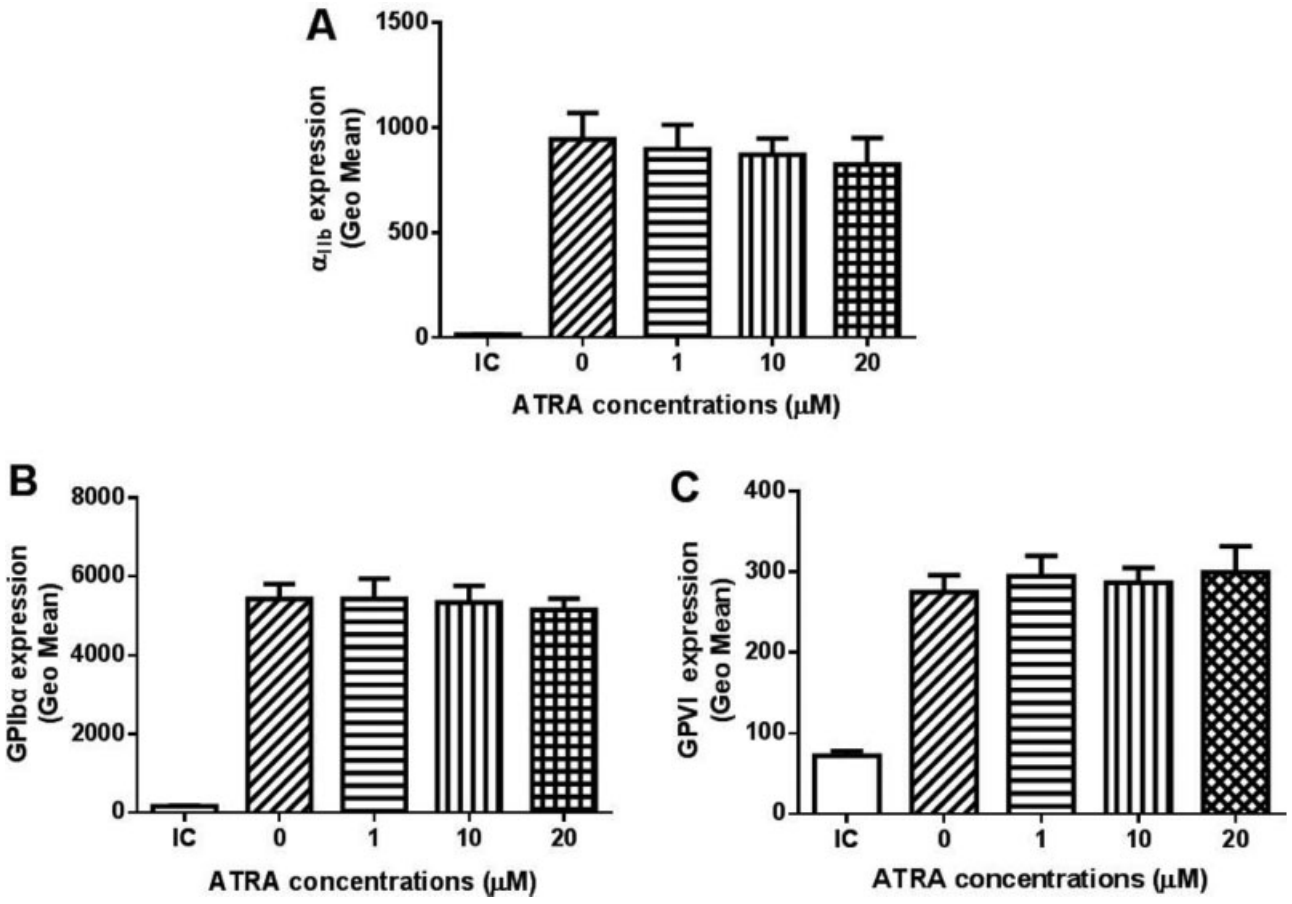

Fig. 2 Expression of platelet surface receptors. After treated with difference concentrations of all-trans retinoic acid (ATRA), the expression of platelet surface receptors $\alpha_{IIb}\beta_3$ (**A**), glycoprotein (GP) Ibα (**B**), and GPVI (**C**) was measured by flow cytometry. Data were presented as mean ± standard error (SE) ($n = 4$) and analyzed by one-way analysis of variance (ANOVA). IC, isotype control.

Meanwhile, the arterial thrombus formation which was induced by $FeCl_3$ was also significantly delayed in ATRA-treated mice receiving infusion of ATRA-treated platelets (29.67 ± 3.51 minutes) compared with that in mice receiving vehicle-treated platelets (16.50 ± 1.36 minutes) ($p < 0.01$) (►**Fig. 5D**). Taken together, these data show that ATRA impairs hemostasis and arterial thrombosis in vivo.

ATRA Inhibits the Phosphorylation of PKCßI and PKCδ

A previous study has demonstrated a direct interaction of ATRA with PKC,[28] and PKC has been shown to play an important role in the regulation of platelet aggregation, granule secretion, and spreading.[29] To evaluate whether ATRA affects platelet function through regulation of PKC, we measured the phosphorylation level of PKCα, PKCßI/II, and PKCδ in CRP (5 μg/mL)- or thrombin (1 U/mL)-stimulated platelets in the presence of different doses of ATRA, and found that the phosphorylation level of PKCßI (Ser661) and PKCδ (Thr505) was significantly reduced in CRP- (►**Fig. 6A**) and thrombin-treated (►**Fig. 6B**) platelets in the presence of ATRA in a dose-dependent manner. However, ATRA did not affect the phosphorylation of PKCα or PKCßII (Thr638/641). Consistently, ATRA administration into mice also decreased the phosphorylation of PKCßI and PKCδ in CRP-stimulated platelets (►**Fig. 6C**).

Discussion

As an active metabolite of vitamin A under the family retinoid, ATRA has been demonstrated to be widely applied for induction of complete remission in APL patients as the only well-established differentiation therapy.[8,9] However, therapeutic use of ATRA is reported to interfere with the hemostatic function in APL patients,[13,16] including effects on coagulation or other vascular cells, such as promyelocytic blast cells, normal human endothelial cells, and normal human monocytes, although effects of ATRA on platelets remain poorly understood. In the present study, through incubating human platelets with different doses of ATRA, we investigated the effect of ATRA on platelet function and showed ATRA inhibits platelet aggregation, spreading, clot retraction, as well as hemostasis and arterial thrombosis in vivo.

ATRA has been previously shown to downregulate tumor necrosis factor-induced expression of tissue factor in vascular endothelial cells,[30] increase tissue plasminogen activator activity in rats, as well as thrombomodulin expression in human U937 monoblast-like cells and human MEGO1 megakaryocyte-like cells.[31,32] In addition, ATRA has also been demonstrated to reduce the cancer procoagulant activity in APL cells[33] and improve the coagulopathy in APL patients[14] as demonstrated by decreased markers of clotting activation and

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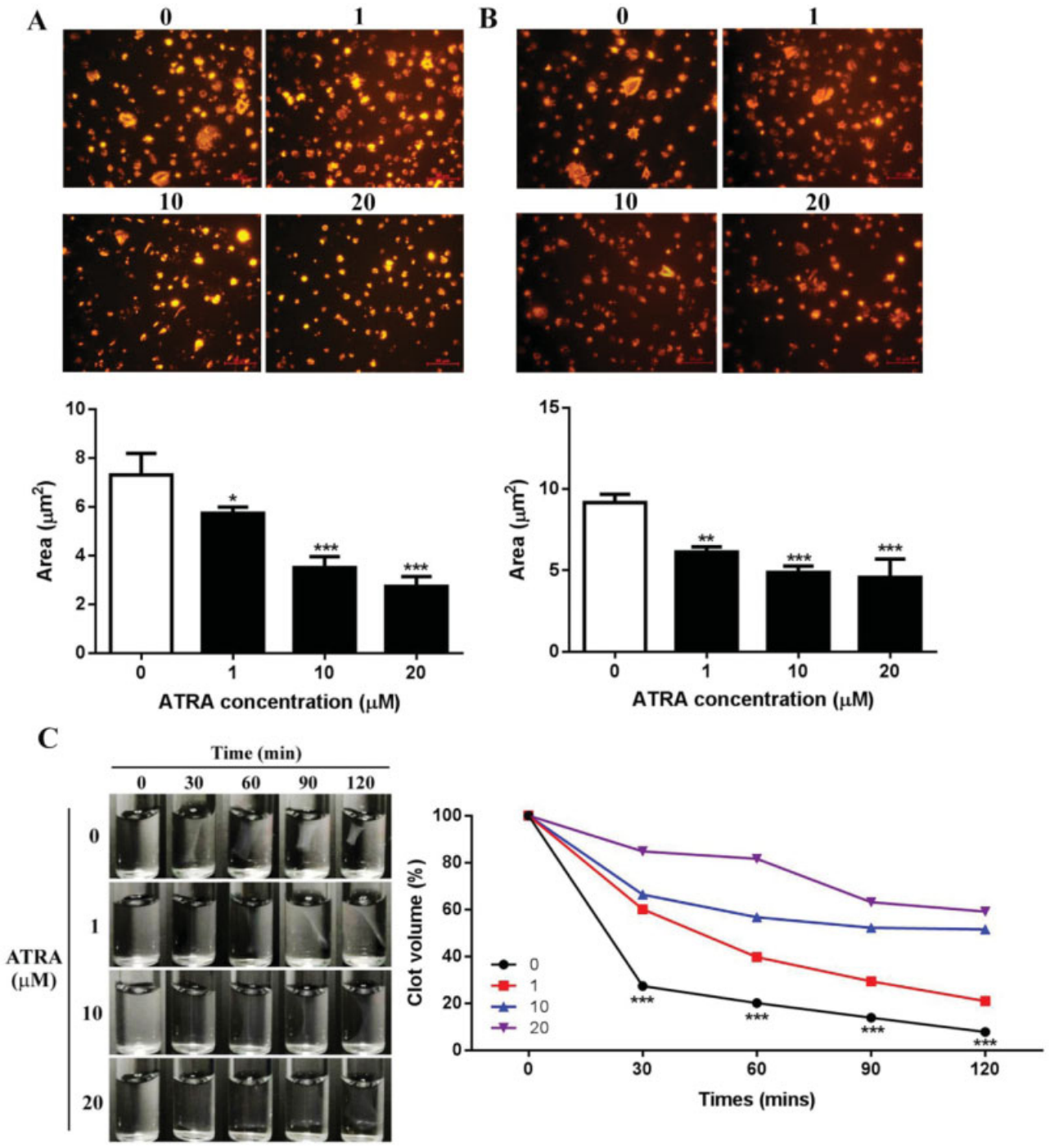

Fig. 3 Platelet spreading and clot retraction. After all-trans retinoic acid (ATRA) treatment, washed human platelets were placed on fibrinogen- (**A**) or collagen (**B**)-coated glass coverslips and allowed to spread at 37°C for 90 minutes followed by staining with Alexa Fluor-546-labeled phalloidin (mean ± standard deviation [SD], $n = 3$). ATRA-treated platelets were supplemented with 2 mM Ca^{2+} and 0.5 mg/mL fibrinogen and clot retraction was initiated after addition of thrombin (1 U/mL). Images were captured every 30 minutes (mean ± SD, $n = 3$) (**C**). For **A** and **B**, data were analyzed by one-way analysis of variance (ANOVA). Compared with 0, $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. For **C**, data were analyzed by two-way ANOVA. Compared with 1, 10, or 20, $^{***}p < 0.001$.

fibrin degradation, increased protein C, as well as reduced proteolysis of vWF after ATRA treatment, which was paralleled the improvement of clinical signs of the coagulopathy in APL patients.[16] Furthermore, ATRA has been reported to interfere with the hemostatic function of promyelocytic blast cells, normal human endothelial cells, and normal human monocytes to exert beneficial antithrombotic effects.[16] Consistent with the antithrombotic role of ATRA, in the present study, we showed that ATRA inhibits platelet function as demonstrated by reduced platelet aggregation, spreading, clot retraction, as well as impaired in vivo hemostasis and arterial thrombus formation after ATRA treatment, suggesting ATRA might also

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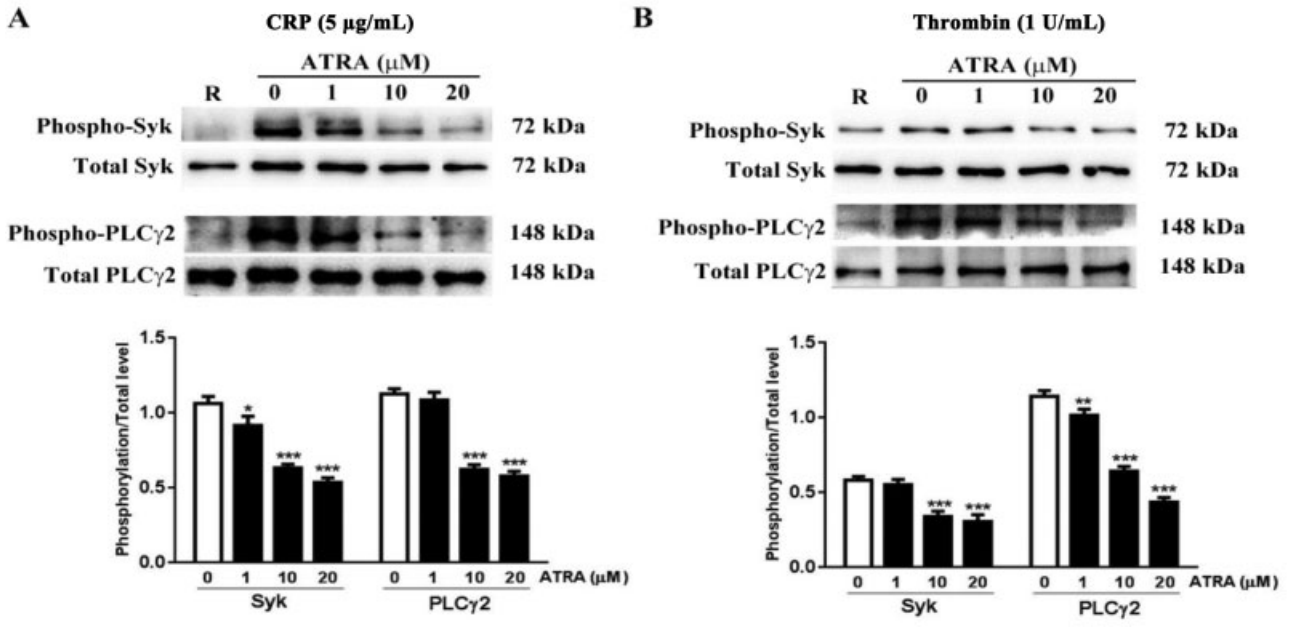

Fig. 4 Phosphorylation level of Syk and PLCγ2. After all-trans retinoic acid (ATRA) treatment, human platelets were treated with 5 μg/mL collagen-related peptide (CRP) (**A**) or 1 U/mL thrombin (under the conditions of clot retraction) (**B**) for 15 minutes followed by analysis of the phosphorylation level of Syk and PLCγ2 by Western blot. The protein expression was quantified using Image J software and represented as a ratio of phosphorylation to the total level (mean ± standard deviation [SD], $n = 3$). Data were analyzed by one-way analysis of variance (ANOVA). Compared with 0, $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

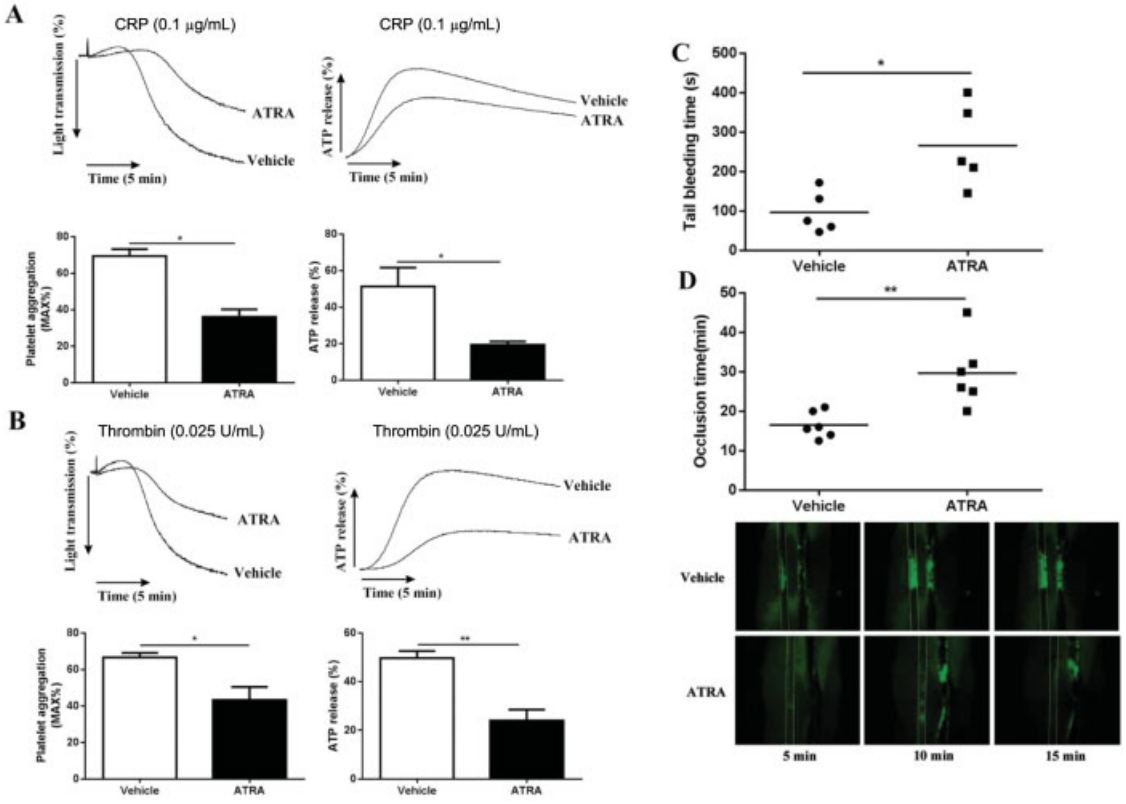

Fig. 5 Effect of all-trans retinoic acid (ATRA) administration on mouse platelet function. Mice received intraperitoneal injection of ATRA (10 mg/kg) followed by analysis of platelet aggregation (**A**), adenosine triphosphate (ATP) release (**B**), and tail bleeding time (mean, $n = 5$) (**C**). After treated with ATRA (20 μM) or vehicle, mouse platelets were labeled with calcein and infused into ATRA-treated or vehicle-treated mice, respectively, via tail vein injection followed by challenge with 10% $FeCl_3$ to induce arterial thrombus formation which was monitored by a fluorescence microscopy (Olympus BX53) and the vessel occlusion time was then measured (mean, $n = 6$) (**D**). For **A** and **B**, data were presented as mean ± standard error (SE) ($n = 3$). Data were analyzed by Student's *t*-test. $^{*}p < 0.05$; $^{**}p < 0.01$.

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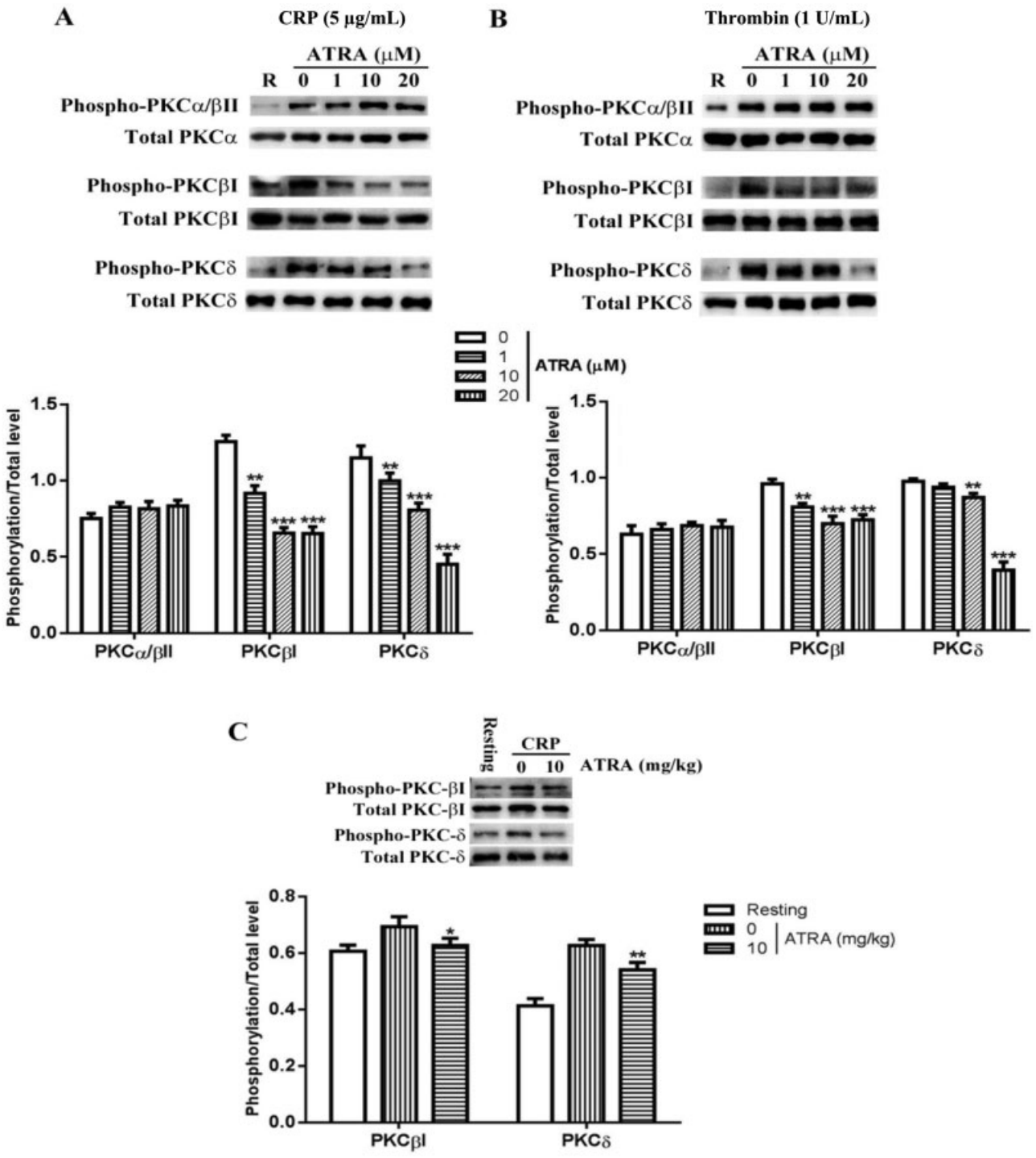

Fig. 6 Phosphorylation of protein kinase C (PKC) α/βII, PKCßI and PKCδ. All-trans retinoic acid (ATRA)-treated human platelets were stimulated with 5 μg/mL collagen-related peptide (CRP) (**A**) or 1 U/mL thrombin (under the conditions of clot retraction) (**B**) for 15 minutes and the phosphorylation level of PKCα/βII, PKCßI, and PKCδ was measured by Western blot. In addition, platelets were isolated from mice treated with ATRA (10 mg/kg) and treated with 5 μg/mL CRP followed by analysis of the phosphorylation of PKCßI and PKCδ (**C**). The protein expression was quantified using Image J software and represented as a ratio of phosphorylation to the total level (mean ± standard deviation [SD], $n = 3$). Data were analyzed by one-way analysis of variance (ANOVA). Compared with 0, $^{**}p < 0.01$; $^{***}p < 0.001$.

exert antithrombotic effects through inhibition of platelet function.

The platelet surface GP receptors, such as $\alpha_{IIb}\beta_3$, GPIbα, and GPVI, play an important role in the regulation of platelet function.[34] In response to vascular injury, platelet receptors GPIbα and GPVI adhere to the damaged blood vessel wall through recognition of exposed vWF and collagen, respectively, leading to transduction of intraplatelet signaling

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pathway and subsequent activation of $\alpha_{IIb}\beta_3$, which binds to fibrinogen or vWF and mediates platelet aggregation and thrombus formation.[2,34] Considering the inhibition of platelet aggregation and thrombosis by ATRA, as well as importance of platelet receptors $\alpha_{IIb}\beta_3$, GPIbα, and GPVI in platelet function, we evaluated whether ATRA affects the surface expression of these receptors and found no changes of the surface expression of these receptors in ATRA-treated platelets, suggesting ATRA does not affect the surface expression of platelet GP receptors.

PKC belongs to a family of serine/threonine kinases and are classified into three subfamilies, which are conventional isoforms (α, βI/II, and γ), novel isoforms (δ, ε, η, and θ), and atypical isoforms (ζ and ι/λ). Human platelets express PKCα, PKCβ, PKCδ, and PKCθ, which are demonstrated to play important role in the regulation of platelet aggregation, granule secretion, integrin activation, spreading, as well as procoagulant activity.[29] A previous study has demonstrated a direct interaction of ATRA with PKC,[26] showing ATRA binding site in three PKC isozymes (α, βI, and γ) after amino acid alignments and comparison of the crystal structures of several ATRA-utilizing proteins. Using photoaffinity labeling assay, they further showed that other PKC isozymes (α, βI, βII, γ, δ, ε, and ζ) were also photolabeled with PKCßII having the highest affinity for ATRA and PKCζ having the lowest affinity. Consistent with the interaction between ATRA and PKC, in the present study, we showed that ATRA treatment selectively inhibited phosphorylation of PKCßI (Ser661) and PKCδ (Thr505), but not PKCα or PKCßII phosphorylation (Thr638/641) in CRP- or thrombin-stimulated platelets. In addition, we also demonstrated that ATRA significantly reduced dense-granule secretion (ATP release) from collagen- or thrombin-treated platelets, which was consistent with a previous study showing that inhibition of PKCα/β or PKCδ significantly reduced dense-granule release from human platelets after treatment with convulxin or thrombin receptor (protease-activated receptor [PAR]) agonists, respectively.[35] Interestingly, inhibition of both classical and novel PKC isoforms completely abolished dense-granule secretion from convulxin- or PAR agonists-treated platelets.[35] In the present study, both classical and novel PKC isoforms (PKCßI and PKCδ) phosphorylation was significantly reduced in CRP- or thrombin-stimulated platelets in the presence of ATRA, which might be the reason why dense-granule secretion was both inhibited in collagen- or thrombin-treated platelets. Surprisingly, ATRA treatment did not affect α-granule secretion (P-selectin expression) from collagen- or thrombin-treated platelets, which was in accordance with a previous study showing that PKCδ had minimal effect on platelet P-selectin expression.[36]

In conclusion, therapeutic dose of ATRA inhibits platelet function, hemostasis, and arterial thrombus formation in vivo, which might be through direct or indirect inhibition of the phosphorylation of PKCßI/δ, suggesting that it may represent a novel inhibitor of platelet function and thrombus formation.

What is known about this topic?

- All-trans retinoic acid (ATRA) has been widely used for induction of complete remission in patients with acute promyelocytic leukemia (APL).
- ATRA has effects on the hemostatic system of APL patients as demonstrated by decrease or normalization of clotting and fibrinolytic variables.
- ATRA inhibits cytoskeletal events (platelet spreading) and actin-dependent morphological changes (extended cell body formation) in human platelets.

What does this paper add?

- ATRA inhibits platelet aggregation, ATP release, spreading on fibrinogen or collagen, and clot retraction.
- ATRA impairs in vivo hemostasis and arterial thrombus formation.
- ATRA selectively inhibits phosphorylation of PKCßI (Ser661) and PKCδ (Thr505).

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Conflict of Interest

None declared.

References

1 Qiao JL, Shen Y, Gardiner EE, Andrews RK. Proteolysis of platelet receptors in humans and other species. Biol Chem 2010;391(08): 893–900
2 Qiao J, Arthur JF, Gardiner EE, Andrews RK, Zeng L, Xu K. Regulation of platelet activation and thrombus formation by reactive oxygen species. Redox Biol 2018;14:126–130
3 Li Z, Delaney MK, O'Brien KA, Du X. Signaling during platelet adhesion and activation. Arterioscler Thromb Vasc Biol 2010;30 (12):2341–2349
4 Durrant TN, van den Bosch MT, Hers I. Integrin $\alpha_{IIb}\beta_3$ outside-in signaling. Blood 2017;130(14):1607–1619
5 Siddikuzzaman GC, Guruvayoorappan C, Berlin Grace VM. All trans retinoic acid and cancer. Immunopharmacol Immunotoxicol 2011;33(02):241–249

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6 Napoli JL. Retinoic acid biosynthesis and metabolism. FASEB J 1996;10(09):993–1001
7 Allenby G, Janocha R, Kazmer S, Speck J, Grippo JF, Levin AA. Binding of 9-cis-retinoic acid and all-trans-retinoic acid to retinoic acid receptor-alpha, receptor-beta, and receptor-gamma - retinoic acid receptor-gamma binds all-trans-retinoic acid preferentially over 9-cis-retinoic acid. J Biol Chem 1994;269(24): 16689–16695
8 Cicconi L, Lo-Coco F. Current management of newly diagnosed acute promyelocytic leukemia. Ann Oncol 2016;27(08):1474–1481
9 Wang ZY, Chen Z. Acute promyelocytic leukemia: from highly fatal to highly curable. Blood 2008;111(05):2505–2515
10 Dos Santos GA, Kats L, Pandolfi PP. Synergy against PML-RARa: targeting transcription, proteolysis, differentiation, and self-renewal in acute promyelocytic leukemia. J Exp Med 2013;210(13): 2793–2802
11 Warrell RP Jr, de Thé H, Wang ZY, Degos L. Acute promyelocytic leukemia. N Engl J Med 1993;329(03):177–189
12 Falanga A, Iacoviello L, Evangelista V, et al. Loss of blast cell procoagulant activity and improvement of hemostatic variables in patients with acute promyelocytic leukemia administered all-trans-retinoic acid. Blood 1995;86(03):1072–1081
13 Dombret H, Scrobohaci ML, Ghorra P, et al. Coagulation disorders associated with acute promyelocytic leukemia: corrective effect of all-trans retinoic acid treatment. Leukemia 1993;7(01):2–9
14 Kawai Y, Watanabe K, Kizaki M, et al. Rapid improvement of coagulopathy by all-trans retinoic acid in acute promyelocytic leukemia. Am J Hematol 1994;46(03):184–188
15 Federici AB, Falanga A, Lattuada A, Di Rocco N, Barbui T, Mannucci PM. Proteolysis of von Willebrand factor is decreased in acute promyelocytic leukaemia by treatment with all-trans-retinoic acid. Br J Haematol 1996;92(03):733–739
16 Barbui T, Finazzi G, Falanga A. The impact of all-trans-retinoic acid on the coagulopathy of acute promyelocytic leukemia. Blood 1998;91(09):3093–3102
17 Rondina MT, Freitag M, Pluthero FG, et al. Non-genomic activities of retinoic acid receptor alpha control actin cytoskeletal events in human platelets. J Thromb Haemost 2016;14(05):1082–1094
18 Arthur JF, Qiao J, Shen Y, et al. ITAM receptor-mediated generation of reactive oxygen species in human platelets occurs via Syk-dependent and Syk-independent pathways. J Thromb Haemost 2012;10(06):1133–1141
19 Qiao J, Wu X, Luo Q, et al. NLRP3 regulates platelet integrin αIIbβ3 outside-in signaling, hemostasis and arterial thrombosis. Haematologica 2018;103(09):1568–1576
20 Qiao J, Wu Y, Liu Y, et al. Busulfan triggers intrinsic mitochondrial-dependent platelet apoptosis independent of platelet activation. Biol Blood Marrow Transplant 2016;22(09):1565–1572
21 Luo Q, Wei G, Wu X, et al. Platycodin D inhibits platelet function and thrombus formation through inducing internalization of platelet glycoprotein receptors. J Transl Med 2018;16(01):311
22 Asmis L, Tanner FC, Sudano I, Lüscher TF, Camici GG. DMSO inhibits human platelet activation through cyclooxygenase-1 inhibition. A novel agent for drug eluting stents? Biochem Biophys Res Commun 2010;391(04):1629–1633
23 Lehuu B, Curtis-Prior PB. Effects of dimethyl sulphoxide (DMSO) on aggregation of human blood platelets. J Pharm Pharmacol 1987;39(01):62–63
24 Gardiner EE, Andrews RK. Structure and function of platelet receptors initiating blood clotting. Adv Exp Med Biol 2014; 844:263–275
25 Bennett JS. Structure and function of the platelet integrin alphaIIbbeta3. J Clin Invest 2005;115(12):3363–3369
26 Flevaris P, Stojanovic A, Gong H, Chishti A, Welch E, Du X. A molecular switch that controls cell spreading and retraction. J Cell Biol 2007;179(03):553–565
27 Suzuki-Inoue K, Hughes CE, Inoue O, et al. Involvement of Src kinases and PLCgamma2 in clot retraction. Thromb Res 2007;120 (02):251–258
28 Radominska-Pandya A, Chen G, Czernik PJ, et al. Direct interaction of all-trans-retinoic acid with protein kinase C (PKC). Implications for PKC signaling and cancer therapy. J Biol Chem 2000;275(29): 22324–22330
29 Harper MT, Poole AW. Diverse functions of protein kinase C isoforms in platelet activation and thrombus formation. J Thromb Haemost 2010;8(03):454–462
30 Ishii H, Horie S, Kizaki K, Kazama M. Retinoic acid counteracts both the downregulation of thrombomodulin and the induction of tissue factor in cultured human endothelial cells exposed to tumor necrosis factor. Blood 1992;80(10):2556–2562
31 Dittman WA, Nelson SC, Greer PK, Horton ET, Palomba ML, McCachren SS. Characterization of thrombomodulin expression in response to retinoic acid and identification of a retinoic acid response element in the human thrombomodulin gene. J Biol Chem 1994;269(24):16925–16932
32 van Giezen JJ, Boon GI, Jansen JW, Bouma BN. Retinoic acid enhances fibrinolytic activity in-vivo by enhancing tissue type plasminogen activator (t-PA) activity and inhibits venous thrombosis. Thromb Haemost 1993;69(04):381–386
33 Falanga A, Consonni R, Marchetti M, et al. Cancer procoagulant in the human promyelocytic cell line NB4 and its modulation by all-trans-retinoic acid. Leukemia 1994;8(01):156–159
34 Rivera J, Lozano ML, Navarro-Núñez L, Vicente V. Platelet receptors and signaling in the dynamics of thrombus formation. Haematologica 2009;94(05):700–711
35 Murugappan S, Tuluc F, Dorsam RT, Shankar H, Kunapuli SP. Differential role of protein kinase C delta isoform in agonist-induced dense granule secretion in human platelets. J Biol Chem 2004;279(04):2360–2367
36 Chari R, Getz T, Nagy B Jr, et al. Protein kinase C[delta] differentially regulates platelet functional responses. Arterioscler Thromb Vasc Biol 2009;29(05):699–705

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