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ORIGINAL ARTICLE

Drospirenone enhances GPIb-IX-V-mediated platelet activation

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Summary. *Background:* Epidemiologic studies recently revealed that using drospirenone (DRSP)-containing contraceptives is associated with an increased risk of thrombosis in women. However, the underlying causality is unclear. *Objective:* To study the effects of DRSP on coagulation *in vitro* and the probable mechanisms involved. *Methods:* First, the effects of DRSP on the activated partial thromboplastin time (APTT), prothrombin time (PT), thrombin time (TT) and fibrinogen (FIB) were measured. Then, the effects of DRSP on platelet activation were investigated in response to low levels of collagen, adenosine 5′-diphosphate (ADP), thrombin, U46619, adrenaline and botrocetin/von Willebrand factor (VWF). *Results:* DRSP has no direct effect on APTT, PT, TT, FIB and platelet aggregation induced by low levels of collagen, ADP, thrombin, U46619 or adrenaline. However, DRSP enhances botrocetin/VWF-induced platelet aggregation and VWF receptor glycoprotein Ib-IX-V (GPIb-IX-V)-mediated signaling. This enhancement can be blocked by the progesterone receptor membrane component 1 (PGRMC1) inhibitor AG205, or by the ADP scavenger apyrase and the cyclooxygenase inhibitor indomethacin. *Conclusions:* Although DRSP did not directly induce platelet activation, it obviously facilitated VWF receptor GPIb-IX-V-mediated platelet activation. The potential DRSP-binding protein PGRMC1 may play a role in this process. Our study also suggested that the inhibition of thromboxane A2 production and the activation of ADP receptors might prevent the side-effects of DRSP.

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Introduction

Drospirenone (DRSP) is a synthetic compound with pharmacologic properties similar to natural progesterone, which has anti-mineralocorticoid and androgen resistance effects [1]. The new generation of oral contraceptives containing DRSP as the steroidal component had low side-effects and were widely used by women for contraception [2]. Recent epidemiologic studies, however, have demonstrated that taking DRSP-containing contraceptive pills may cause some side-effects, including increased risks of venous and arterial thrombotic events such as deep and superficial venous thrombosis, pulmonary embolism, myocardial infarction, stroke and thrombophlebitis [3–6]. In particular, some studies indicated that the risk of venous thromboembolism for women using DRSP-containing contraceptives increased 1.5-3 times, compared with levonorgestrel-containing contraceptives [5,7–10]. Although several studies have shown conflicting results regarding the effects of DRSP on the risk of elevated thromboembolism [11–13], accumulating evidence from more and more epidemiologic studies has revealed that DRSP is correlated with the risk of thrombosis. Therefore, it is necessary to clarify the effects of DRSP on blood coagulation. In this study, we investigate the effects of DRSP on blood coagulation and platelet activation and elucidate the possible underlying mechanisms of these effects.

Materials and methods

Materials

DRSP was obtained from Selleckchem (Houston, TX, USA). Apyrases (adenosine diphosphatase), indomethacin, PGE1, adenosine 5′-diphosphate (ADP), the TXA2 analog U46619, adrenaline, the progesterone receptor membrane component 1 (PGRMC1) inhibitor AG205

and the anti-α-tubulin antibody were obtained from Sigma-Aldrich (St Louis, MO, USA). α-Thrombin was obtained from Enzyme Research Laboratories (South Bend, IN, USA). Collagen was purchased from Chrono-Par Aggregation Reagents (Chrono-Log Corporation, Havertown, PA, USA). Anti-phospho-Akt (Ser473s), anti-phospho-p44/42 MAPK (ERK1/2), anti-phospho-p38 (Thr180/Tyr182) and anti-GAPDH antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-PGRMC1 was obtained from Abcam (Cambridge, MA, USA). The horseradish peroxidase-conjugated goat anti-rabbit antibody was from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). Botrocetin and human von Willebrand factor (VWF) were supplied by Professor Michael Berndt (Curtin University, Perth, Western Australia). Ristocetin was purchased from Chrono-Log. The human cervical cancer cell line HeLa was obtained from the cell bank of the Shanghai Institutes for Biological Sciences.

Measurement of PT, APTT, TT and FIB

Platelet-poor plasma (PPP) was collected from 20 healthy donors and prepared by centrifugation ($2500 \times g$ for 10 min) from PRP containing 2.5% sodium citrate. Prothrombin time (PT), activated partial thromboplastin time (APTT), thrombin time (TT) and fibrinogen (FIB) were measured using a Sysmex CA7000 coagulation analyzer (Sysmex, Kobe, Japan).

Preparation and aggregation of human washed platelets

After informed consent was obtained, peripheral blood was collected from over five different healthy donors (with approval from the Institutional Review Board of the Shanghai Jiao Tong University School of Medicine) and then transferred to polypropylene centrifuge tubes containing 100 μL mL^{-1} anticoagulant (3.8% trisodium citrate), 0.1 μg mL^{-1} PGE1 and 1 U mL^{-1} apyrase. Platelet-rich plasma (PRP) was prepared by differential centrifugation. Washed platelets were prepared from the PRP containing 5 mM ethylenediaminetetraacetic acid (EDTA) by differential centrifugation ($1100 \times g$ for 10 min) and resuspended with modified Tyrode's solution (12 mM $NaHCO_3$, 138 mM NaCl, 5.5 mM glucose, 2.9 mM KCl, 2 mM $MgCl_2$, 0.42 mM NaH_2PO_4, 10 mM HEPES [N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid], pH 7.4) to approximately 3×10^8 platelets mL^{-1}.

For the aggregation studies, platelets were stimulated with collagen (1 μg mL^{-1}), ADP (10 μM), thrombin (0.03 U mL^{-1}) and thromboxane A2 analog U46619 (1 μM) or adrenaline (0.3 μg mL^{-1}). Botrocetin (0.3 μg mL^{-1}) or ristocetin (0.4 μg mL^{-1}) and VWF (10 μg mL^{-1}) were used to investigate glycoprotein Ib-IX-V (GPIb-IX-V)-mediated platelet activation. Different concentrations of DRSP were incubated with the platelets with or without inhibitor (AG205; 1 μM, 5 μM, 10 μM) at 37°C for 3 min before the agonists were used. Dimethylsulfoxide (DMSO) was used as the vehicle control. Platelet aggregation was measured in a lumi-aggregometer (Chrono-Log) using 300 μL washed platelets.

Co-immunoprecipitation and Western blot analysis

For the analysis of signaling molecules downstream of GPIb-IX-V/VWF, the platelet aggregation reaction was stopped by adding an equal volume of 2 × SDS sample buffer, containing 1 M Tris-HCl (pH 6.8), 4% SDS, 10% β-mercaptoethanol, 10% glycerol and 2% bromophenol blue.

Platelet lysates and HeLa cell lysates were boiled in sample buffer at 100 °C for 10 min, separated by 10% SDS-PAGE, transferred to a PVDF membrane, and blotted with the indicated antibodies. Blots were developed using SuperSignal chemiluminescent substrate (Pierce, Rockford, IL, USA).

Results

DRSP had no effect on PT, APTT, TT and FIB

Platelets and coagulation factors interact to generate the hemostatic plug. PT, APTT, TT and FIB are four major clinical indicators that reflect the functions of the extrinsic pathway, intrinsic pathway and common pathway in hemostatic plug formation. Prior to analyzing the four indicators, PPP was pre-incubated with the vehicle DMSO or DRSP at 60 ng mL^{-1}, 120 ng mL^{-1} or 360 ng mL^{-1} for 3 min at room temperature. As shown in Fig. 1, there was no significant difference between the DRSP treatment group and the DMSO control group or blank control group at each DRSP concentration, demonstrating that DRSP did not affect the outcome of the assay (PT, APTT, TT and FIB) under the conditions tested. Consequently, it is unlikely that DRSP facilitates thromboembolism by affecting coagulation.

DRSP had no effect on platelet aggregation induced by low levels of collagen, ADP, thrombin, U46619 or adrenaline

Platelet activation is associated with the activation of signaling by several important receptors such as GPVI and a variety of G-protein-coupled receptors [14,15]. Some soluble platelet agonists such as collagen, ADP, thrombin, thromboxane A2 and adrenaline are known to interact with these receptors on platelets and to induce platelet activation [14,15]. The effects of DRSP on platelet activation were measured as follows: human washed platelets were stimulated by low levels of each agonist (collagen, ADP, thrombin, U46619 and adrenaline) in the presence of DRSP or its vehicle control, DMSO. DRSP alone did

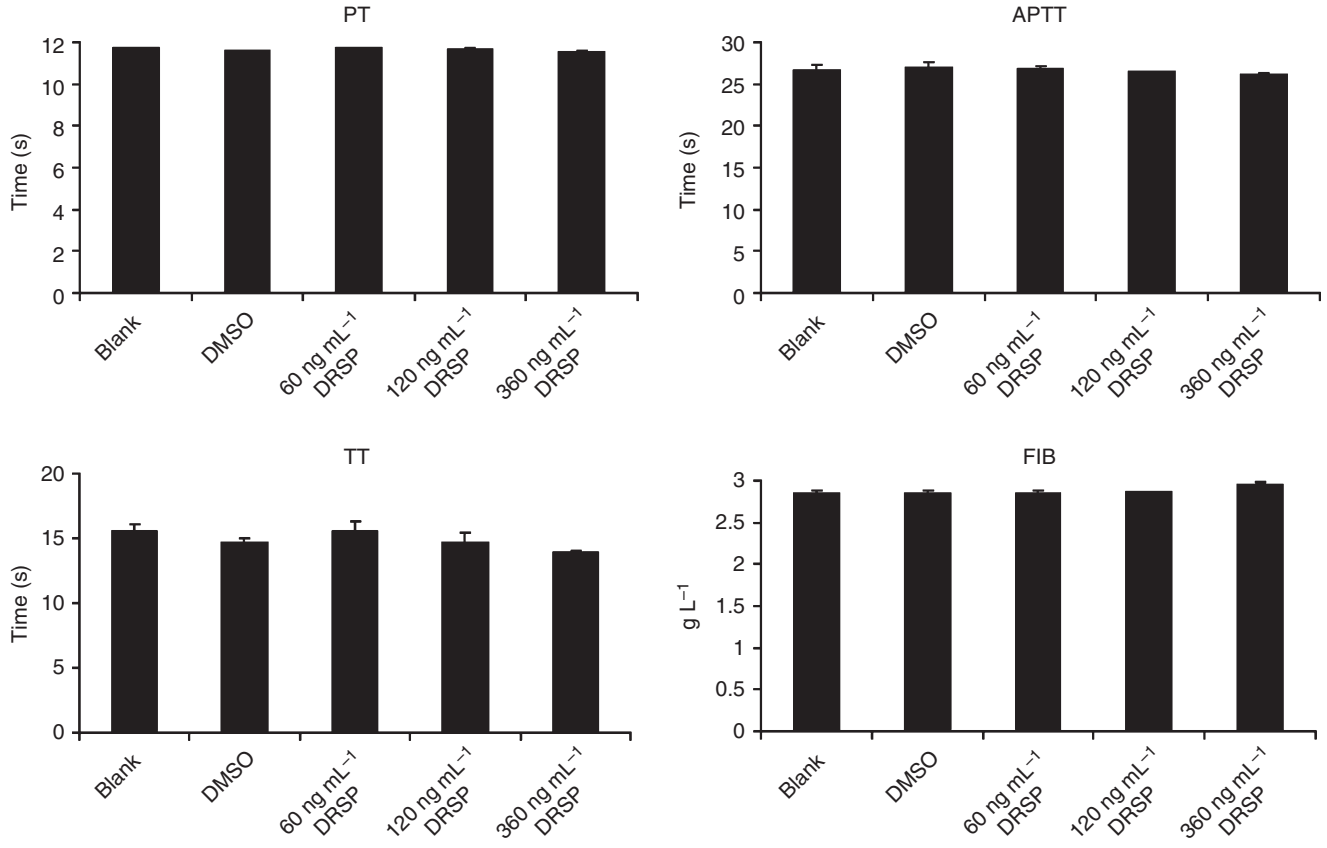

Fig. 1. Drospirenone (DRSP) does not affect prothrombin time (PT), activated partial thromboplastin time (APTT), thrombin time (TT) and fibrinogen (FIB). The effects of different concentrations of DRSP on PT, APTT, TT and FIB are shown in the bar charts. Bars represent the mean values of three independent experiments. Statistical significance was calculated using Student's *t*-test.

not induce platelet aggregation. Compared with the DMSO controls, DRSP had no effect on human platelet aggregation in response to low levels of all the platelet agonists tested (Fig. 2). These results demonstrate that DRSP did not affect platelet activation initiated by GPVI or the G-protein-coupled receptors.

DRSP enhanced GPIb-IX-V-mediated platelet activation

GPIb-IX-V is an abundant membrane receptor complex [16] that plays important roles in arterial [17] and venous thrombosis [18]. It is well known that botrocetin enhances the binding of VWF to its platelet receptor, the GPIb-IX-

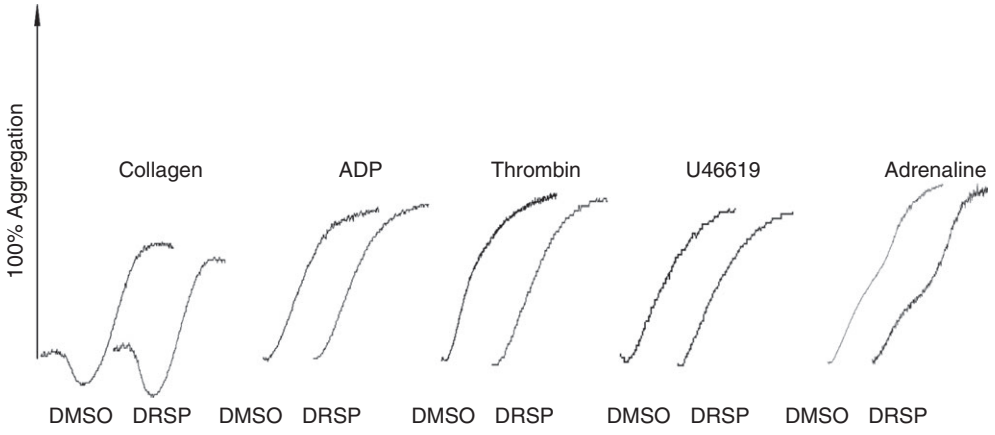

Fig. 2. Drospirenone (DRSP) has no effect on platelet activation induced by collagen, adenosine 5′-diphosphate (ADP), thrombin, U46619 and adrenaline. The aggregation of washed human platelets did not differ between dimethylsulfoxide (DMSO) and DRSP (60 ng mL^{-1}) treatments in response to 1 μg mL^{-1} collagen, 10 μM ADP, 0.03 U mL^{-1} thrombin, 1 μM U46619 and 0.3 μg mL^{-1} adrenaline. Traces are representative of three independent experiments with platelets from different donors.

V complex. The binding of VWF to the GPIb-IX-V complex initially mediates agglutination, and if the conditions are appropriate platelet aggregation occurs. [19]. DRSP was incubated with human washed platelets in the presence and absence of botrocetin/VWF to test the effect of DRSP on platelet aggregation mediated by the GPIb-IX-V complex. Extensive enhancement of human platelet activation, as reflected by aggregation, was observed at a threshold concentration of botrocetin/VWF in the presence of DRSP (60 ng mL^{-1} and 300 ng mL^{-1}) (Fig. 3A). As a consequence of VWF binding, GPIbα cytoplasmic domains can mediate signaling that causes platelet integrin αIIbβ3 activation and platelet aggregation [20,21]. The VWF/GPIb-IX-V interaction induces the activation of molecules in the PI3K/Akt and PKG/MAPK signaling pathways [22,23]. Western blot analyses revealed that DRSP (60 ng mL^{-1}) significantly up-regulated the levels of phospho-Akt, phospho-p38 and phospho-ERK (Fig. 3B). Apparently, DRSP-induced activation of PI3K/Akt and PKG/MAPK signaling increased botrocetin/VWF-induced platelet aggregation.

DRSP enhanced GPIb-IX-V-mediated platelet activation via PGRMC1

Progesterone has been shown to elicit its effects via either the 'classical' progesterone receptor (PR), which has been described as a nuclear transcription factor, or 'non-classical' mechanisms mediated by membrane-associated receptors and described as non-nuclear signaling mechanisms [24,25]. Because platelets have no nucleus, the classic nuclear progesterone receptors should not play a role in platelet activation. So far, two types of membrane-associated receptors, progesterone membrane receptors (mPRs) and progesterone receptor membrane component 1 (PGRMC1), a member of the b5-like heme/steroid-binding protein family, have been reported to mediate these non-classical progesterone actions [25,26]. In contrast to the mPRs, PGRMC1 displays moderately high binding affinity for progesterone and can act as an adaptor protein for multiple classes of steroid receptors, including mPRs [27–29]. Furthermore, PGRMC1 expression was detected in human platelet lysates, indicating that PGRMC1 does exist

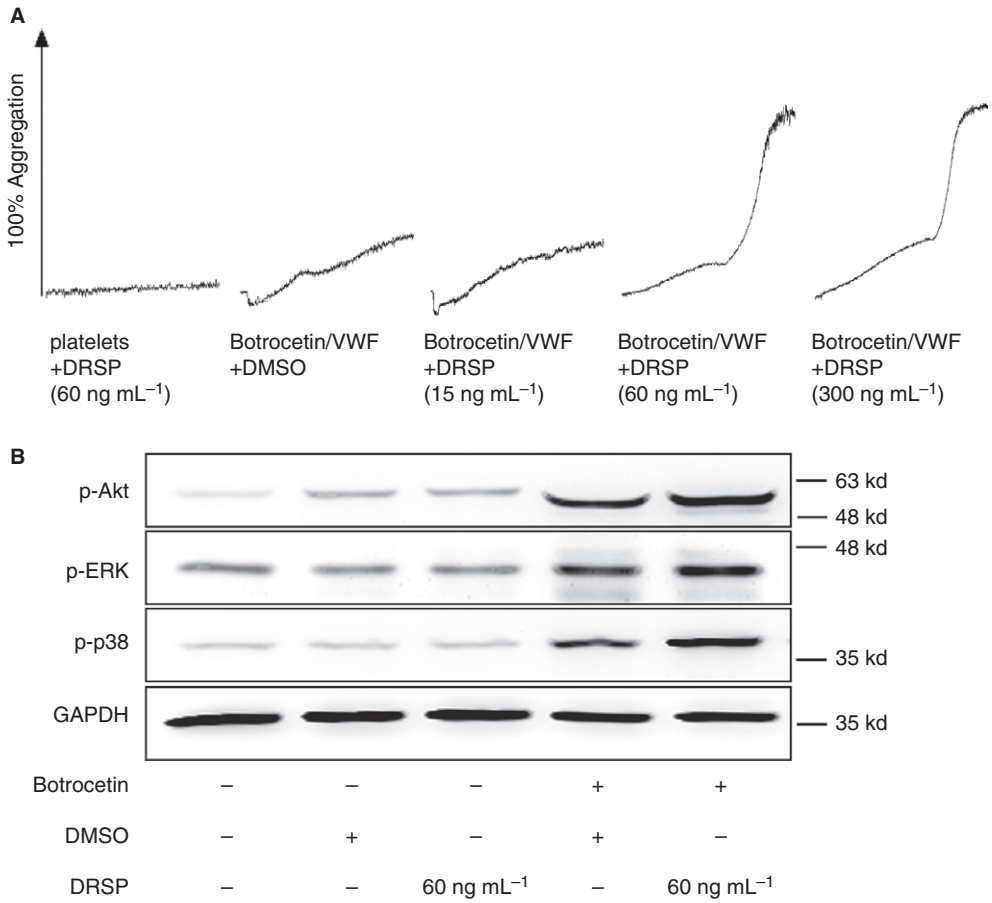

Fig. 3. Drospirenone (DRSP) enhances GPIb-IX-V-mediated platelet activation. (A) Washed human platelets were pre-incubated with vehicle (dimethylsulfoxide, DMSO) or different concentrations of DRSP. The aggregation of washed human platelets was increased in the DRSP (60 ng mL^{-1} and 300 ng mL^{-1}) treatment group in response to threshold concentrations of botrocetin (0.3 μg mL^{-1}) and von Willebrand factor (VWF) (10 μg mL^{-1}). (B) The platelets in (A) were solubilized (60 ng mL^{-1} DRSP-treated platelets were used) and immunoblotted with anti-p-Akt (Ser473), anti-p-p38 and anti-p-ERK1/2 antibodies. An anti-GAPDH antibody indicated protein loading levels. Traces are representative of three independent experiments with platelets from different donors.

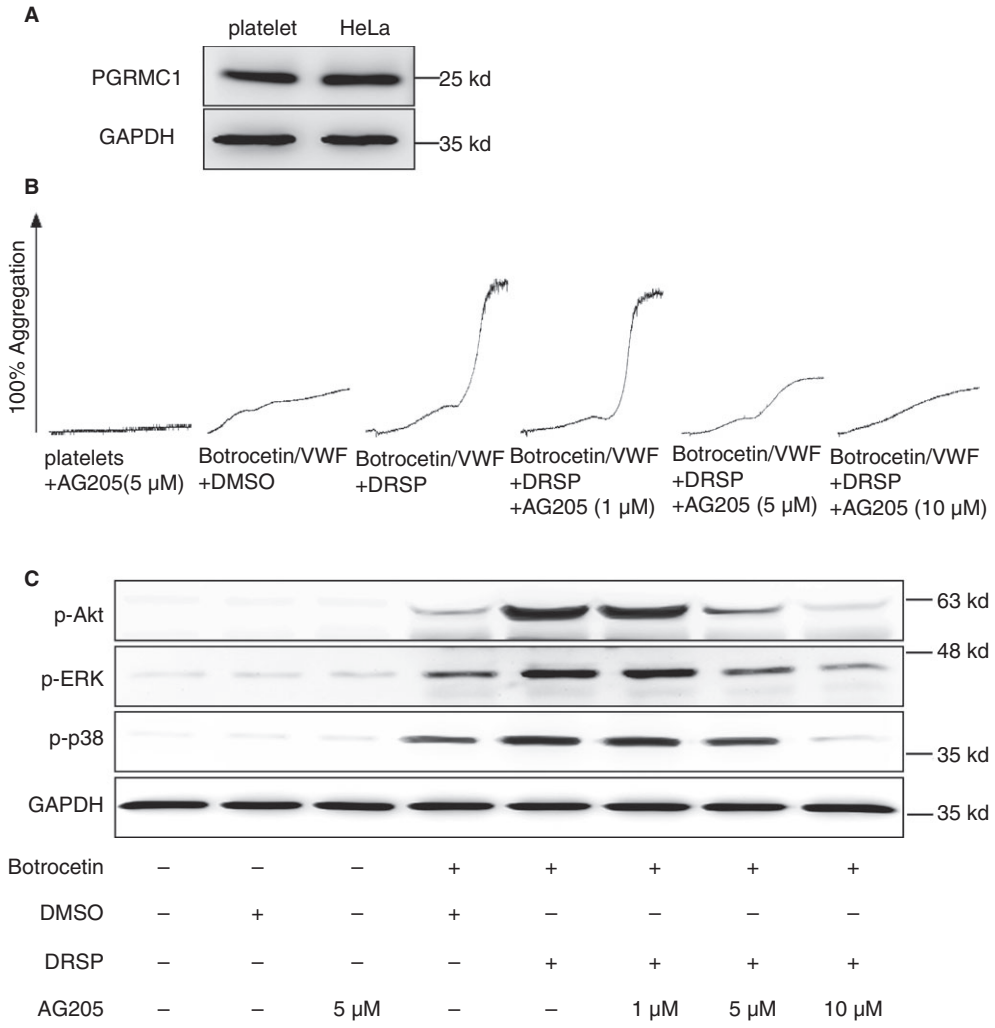

Fig. 4. The enhancement of GPIb-IX-V-mediated platelet activation in response to drospirenone (DRSP) can be blocked by the PGMRC1 inhibitor AG205. (A) Detection of PGRMC1 expression in human platelet lysates. HeLa cell protein was used as a positive control. (B) Washed human platelets were pre-incubated with dimethylsulfoxide (DMSO) or DRSP (60 ng mL^{-1}) with different concentrations of AG205 or without AG205, and were then stimulated with threshold concentrations of botrocetin (0.3 μg mL^{-1}) and VWF (10 μg mL^{-1}). (C) The platelet proteins from (B) were immunoblotted with anti-p-Akt (Ser473), anti-p-p38 and anti-p-ERK1/2 antibodies. Data are representative of three separate experiments with platelets from different donors.

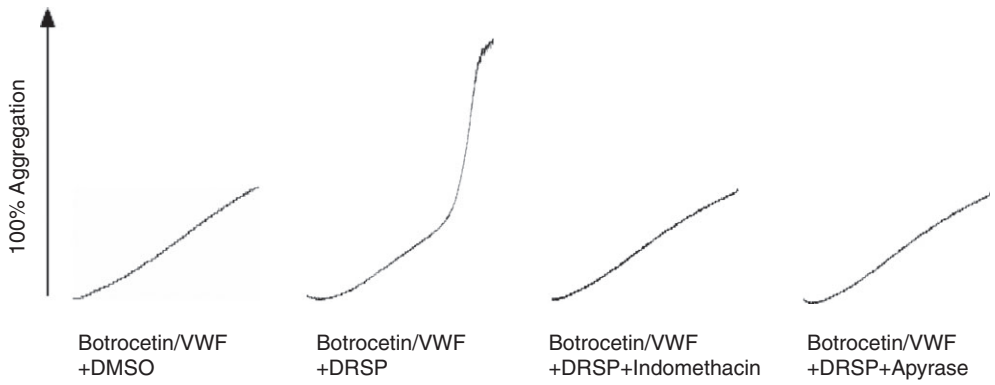

Fig. 5. Drospirenone (DRSP)-enhanced GPIb-IX-V-mediated platelet activation was eliminated by indomethacin or apyrase. Washed human platelets were pre-incubated with dimethylsulfoxide (DMSO) or DRSP (60 ng mL^{-1}) in the presence of indomethacin (75 μM) or apyrase (10 U mL^{-1}) at 37 °C for 3 min, then stimulated with threshold concentrations of botrocetin (0.3 μg mL^{-1}) and von Willebrand factor (VWF) (10 μg mL^{-1}). Traces shown are representative of three independent experiments.

in human platelets (Fig. 4A). The PGRMC1 inhibitor AG205, which alters the spectroscopic properties of the PGRMC1-heme complex [30], was used to investigate the role of PGRMC1 in DRSP-enhanced platelet activation. The results presented in Fig. 4(B) demonstrate that the enhancing effect of DRSP (60 ng mL^{-1}) on platelet aggregation was attenuated by 5 μM and 10 μM AG205 but not by 1 μM AG205. Additionally, phospho-Akt, phospho-p38 and phospho-ERK levels dropped accordingly (Fig. 4C). These results suggested that the effect of DRSP was mediated, at least in part, by PGRMC1.

The enhancing effects of DRSP on GPIb-IX-V-mediated platelet activation were eliminated by indomethacin or apyrase

Because the enhancement of platelet activation may increase the risk of thrombotic events, we searched for means of diminishing or eliminating the enhancement of platelet activation by DRSP. The VWF/GPIb-IX-V-mediated, agglutination-elicited aggregation of washed platelets is dependent on thromboxane A2 and ADP production [19]. When 75 μM indomethacin (an inhibitor of cyclooxygenase, which is similar in function to aspirin and inhibits thromboxane production) or 10 U mL^{-1} apyrase (adenosine diphosphatase) was incubated with platelets, the promoting effect of DRSP on platelet aggregation was reduced (Fig. 5). Therefore, indomethacin or apyrase is able to eliminate the enhancement of GPIb-IX-V-mediated platelet activation by DRSP.

Discussions

The GPIb-IX-V complex plays a critical role in platelet adhesion, activation and thrombus formation [19,22,31]. PI3K/Akt and PKG/MAPK are important signaling pathways downstream of GPIb [22,32]. It has been reported that progesterone is also able to induce PI3K/Akt and PKG/MAPK activation through 'non-nuclear' activity in cells [33–35]. Although the progesterone analog DRSP alone did not induce platelet activation or PI3K/Akt and PKG/MAPK signaling, our data shown in Figs 3 and 4 obviously demonstrate that DRSP enhances GPIb-IX-V-mediated platelet activation and the associated signaling. This conclusion is evident from the increased platelet aggregation and PI3K/Akt and MAPK activation in the presence of DRSP with botrocetin.

PGRMC1 is a progesterone binding protein, which presumably mediates 'non-nuclear' progesterone signaling [27]. Using the PGRMC1 inhibitor AG205, we found that DRSP enhanced GPIb-IX-V-mediated platelet activation signaling via PGRMC1. However, the role of PGRMC1 in GPIb-IX-V-mediated platelet activation is unclear.

In summary, we found that DRSP enhanced GPIb-IX-V-elicited platelet activation, presumably through a DRSP-dependent effect of PGRMC1. However, further studies are needed to provide more details on this effect; for example, the physiologic function of PGRMC1 in platelets remains unknown. Overall, our study provides direct evidence that DRSP is associated with an increased risk of thrombosis and predicts the clinical benefits of using a combination of antiplatelet drugs with DRSP for contraception to reduce the risks of thrombus formation.

Addendum

X. Wu and J. Zhang designed the research, interpreted the data and wrote the manuscript. J. Liu critically reviewed the manuscript. X. Fan performed the research, interpreted the data and wrote the manuscript. X. Chen, C. Wang, J. Dai, Y. Lu and K. Wang helped with the experiments. All the authors approved the final version of the manuscript. All the authors are fully responsible for the content and editorial decisions regarding this paper.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Drospirenone enhances GPIb-IX-V-mediated platelet activation.
Fig. S1. Densitometry measurements from the results in Fig. 3(B).
Fig. S2. AG205 has no effect on platelet aggregation induced by collagen, ADP, thrombin, U46619 and botrocetin.
Fig. S3. The DRSP-enhanced TxA2 and ADP release was eliminated by indomethacin or apyrase in response to ristocetin-induced platelet aggregation.

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