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Research Paper

Tanshinone IIA, a major component of *Salvia milthorriza* Bunge, inhibits platelet activation *via* Erk-2 signaling pathway

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ABSTRACT

Ethnopharmacological relevance: The roots of Salvia milthorriza Bunge (Lamiaceae) known as "Danshen", are used in Traditional Chinese Medicine as a remedy for activating blood and eliminating stasis. TIIA, a diterpenoid of Salvia milthorriza, is one of active components in Danshen that exhibits a significant improvement of the blood flow in the coronary circulatory system and a reduction of myocardial infarction. However, its effect on platelet and underlying mechanism remains largely unknown. On this basis, this compound could be a promising agent to improve blood viscosity and microcirculation and to prevent CVD.

Materials and methods: In order to investigate the effects of TIIA on platelet functionality and its interaction with various platelet activation pathways, rat PRP were incubated with TIIA for 1 min at 37 °C prior the addition of the stimuli (ADP or collagen). Aggregation was monitored in a light transmission aggregometer measuring changes in turbidity with continuous observation up to 10 min after the addition of the stimuli. MAPK signaling pathway and tubulin acetylation were analyzed by a Western blot technique. The effect of the TIIA was also studied *in vivo* on bleeding time in mice.

Results: TIIA selectively inhibited rat platelet aggregation induced by reversible ADP stimuli (3 μM) in a concentration-dependent manner (0.5–50 μM). Nevertheless, TIIA was less active against the irreversible stimuli induced by ADP (10 μM) and collagen (10 μg/mL). Moreover, experiments performed on platelet lysates collected at different time-point after the addition of the stimuli shown that TIIA modulated tubulin acetylation and inhibited Erk-2 phosphorylation. Concomitantly, TIIA administrated i.p. at 10 mg/kg significantly amplified the mice bleeding time with an increase of 58% compared to its control (2.06 ± 0.29 min *vs* 1.30 ± 0.07). ASA was used as reference drug for *in vitro* and *in vivo* experiments.

Conclusions: This study clarifies the intracellular signaling pathway involved in antiplatelet action of TIIA and also gives preliminary evidences for its anticoagulant activity. On this basis, this compound could be a promising agent to improve blood viscosity and microcirculation and to prevent CVD.

1. Introduction

CVD and its main underlying cause, atherothrombosis, are the major causes of morbidity and mortality worldwide (Yusuf et al., 2001). Platelet aggregation is a crucial step in normal hemostasis and in the development and progression of atherothrombosis (Kalra et al., 2013). For these reasons, pharmacological therapies with agents that inhibit platelet reactivity have proven to be effective in the treatment and/or in the prevention of CVD (Binazon et al., 2013; Silva et al., 2013). Moreover, advances in the knowledge of both platelet biology and biological functions of natural products can provide new avenues to develop pharmacological strategies aimed to promote cardiovascular health (Bonito et al., 2011; Vilahur and Badimon, 2013; Maione et al., 2013).

Danshen is a crude herbal drug isolated from dried root of *Salvia miltiorrhiza* Bunge (Lamiaceae). This plant is widely used in Asia, United States of America and European countries for the treatment of cardiovascular and cerebrovascular diseases (Wang et al., 2007). The beneficial actions are attributable to improve microcirculatory, vasodilatory, anti-coagulant, anti-thrombotic, anti-inflammatory, free

Abbreviations: ADP, adenosine-diphosphate; ASA, acetylsalicylic acid; AUC, area under the curve; CVD, cardiovascular diseases; CMC, carboxymethylcellulose; CTRL, control; DMSO, dimethylsulfoxide; ERK, extracellular-signal-regulated kinase; MAPK, mitogen-activated protein kinase; PBS, phosphate buffered saline; PRP, platelet rich plasma; PPP, platelet poor plasma; TIIA, tanshinone IIA

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radical scavenging, and mitochondria-protective effects. Although Danshen is officially listed in the Chinese Pharmacopoeia, the pharmacology of its active constituents is not yet fully described (Cheng, 2006; Zhou et al., 2005; Han et al., 2008).

TIIA is one of the most pharmacological lipophilic active components isolated from Danshen. Many experimental and clinical investigations have reported that this diterpenoid can prevent or slow the progression of a wide spectrum of diseases, including CVD, cancer, neonatal hypoxic ischemic encephalopathy as well as neurodegenerative diseases (Takahashi et al., 2002; Wu et al., 1993). Moreover, an increasing number of evidences show that TIIA is involved in pathological organ injury induced by ischemia and reperfusion (Han et al., 2008), in immune vasculitis (Li et al., 2009) and in inhibition of platelet aggregation and thrombus formation (Liu et al., 2011; Li et al., 1984; Ji et al., 2008). However, its effect on platelet and underlying mechanism remain largely unknown and considerable efforts are still being devoted to clarify these aspects.

To provide further insights, in this study we tested the hypothesis that TIIA inhibited the platelet aggregation induced by collagen and ADP *via* the modulation of tubulin acetylation and inhibition of Erk-2 phosphorilation. The effect of the diterpenoid was also studied *in vivo* on bleeding time in mice.

2. Materials and methods

2.1. Animals

Male Wistar rats and CD1 mice (250–300 g and 25–30 g respectively; Harlan Nossan, Correzzana, Milan, Italy) were used for all the experiments. Animals were kept under standard conditions, with food and water *ad libitum* and maintained in a 12 h/12 h light/dark cycle at 22 ± 1 °C. All the *in vivo* procedures were in accordance with the Italian legislative decree (D. L.) no. 116 of January 27, 1992 and associates European Community guidelines (EEC Directive of 1986; 86/609/EEC). All efforts were made to minimize animal suffering and to reduce their number.

2.2. Reagents

Tanshinone IIA (≥ 97%, HPLC), adenosine diphosphate (ADP) and acetylsalicylic acid (ASA) were obtained from Sigma-Aldrich Co. (Milan, Italy). For Western blot, the primary antibodies mouse monoclonal anti-phospho Erk2 and anti-Erk2 with related horseradish peroxidase conjugated secondary antibodies were obtained from Santa Cruz Biotechnology (Milan, Italy). The primary antibodies mouse monoclonal anti-acetylated tubulin and anti-tubulin were obtained from Sigma-Aldrich Co. (Milan, Italy). Unless otherwise stated, all the other reagents were from Carlo Erba Reagents (Milan, Italy).

2.3. In vitro platelet aggregation assay

In vitro platelet aggregation was measured according to the turbidimetric method, using two-channel aggregometer (Chrono-Log, Corporation, Mod. 490, USA). Blood anticoagulated with 3.2% sodium citrate (1:9 citrate/blood, v/v) was withdrawn from male Wistar rats (anesthetized by enflurane) by cardiac puncture. Platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were prepared as previously described (Pearce et al., 2004; Cicala et al., 2007). Briefly, PRP was obtained by centrifugation at 800 rpm for 15 min at 25 °C. PPP was prepared from the precipitated fraction of PRP by centrifugation at 2000 rpm for 20 min at 25 °C. PRP was adjusted to 3×10^8 platelets/mL. Next, 250 μL of PRP were incubated at 37 °C for 1 min in the cuvette with 20 μL of TIIA solution at final concentration of 0.5, 5 and 50 μM. ASA (50 μM) and TIIA-vehicle (0.3% methanol in distilled water) or ASA-vehicle (3.3% DMSO in distilled water) were used as control and reference drug, respectively. After incubation, platelet aggregation was induced by the addition of 20 μL ADP (3–10 μM) or collagen (10 μg/mL). The maximum platelet aggregation rate was recorded within 10 min with continuous stirring at 37 °C. The light transmittance was calibrated with PPP. The percentage (%) of inhibition of platelet aggregation was calculated by the following formula: $[(X-Y)/X] \times 100\%$. X was the maximum aggregation rate of vehicle-treated PRP; Y was the maximum aggregation rate of sample-treated PRP and was expressed in terms of amplitude (% of aggregation at a given time interval from reagent addition) or AUC (% of total response duration from reagent addition).

2.4. Western blot analysis

Platelets (3×10^8 platelets/mL) were stimulated with ADP (3 μM) in the presence of TIIA (50 μM) or its vehicle and immediately stopped adding 250 μL of formaldehyde buffer (4% formaldehyde in saline) at different times (1,3 and 5 min). We have selected the indicated time point in order to evaluate platelets intracellular signaling during the first ascending wave (1 min), the plateau (3 min) and the descending wave (5 min) of aggregation curve. Successively, platelets suspension was collected, washed with PBS and centrifuged. The platelet pellet was then lysed in the following lysis buffer: Tris–HCl (pH:7.5, 50 mM), NaCl (150 mM), glycerophosphate (20 mM), EDTA (2 mM), PMSF (1 mM), sodium ortovanadate (1 mM), leupeptin (5 μg/ml), aprotinin (5 μg/mL), pepstatin (5 μg/ml). Protein concentration was determined by the Bio-Rad protein assay kit (Bio-Rad, Italy). Protein samples (50 μg) were subjected to electrophoresis on an SDS 12% polyacrylamide gel and transferred onto a nitrocellulose transfer membrane, using standard procedure as previously described (Pederzoli-Ribeil et al., 2010; Maione et al., 2011; Iqbal et al., 2011). The membranes were saturated by incubation with non-fat dry milk (5% wt/v) in PBS supplemented with 0.1% (v/v) Tween 20 (PBS-T) for 2 h at room temperature and then incubated with a primary antibody (mouse monoclonal anti-phospho Erk2; dilution 1:500, anti-Erk2, anti-acetylated tubulin or anti-tubulin; dilution 1:2000), overnight at 4 °C. Successively, membranes were washed 3 times with PBS-T and then incubated with the secondary antibody conjugated with horseradish peroxidase, anti-mouse IgG-HRP (dilution 1:2000), for 2 h at room temperature. Protein bands were detected using the enhanced chemiluminescence (ECL) detection kit and Image Quant 400 GE Healthcare software (GE Healthcare, Italy). Protein bands for p-Erk and acetylated-tubulin were quantified using GS 800 imaging densitometer software (Biorad, Italy) and normalized with respective Erk-2 and tubulin.

2.5. In vivo assessment of tail bleeding time.

The bleeding time was assayed according to the described method with slight changes (Bowie and Owen, 1974). Mice were anaesthetized intraperitoneally (i.p.) with a mixture of ketamine (75 mg/kg) and xylazine (10 mg/kg) and the tail was pre-warmed for 3 min in a 0.9% saline solution at 37 °C 1 h after the i.p. injection of TIIA (10 mg/kg), ASA (10 mg/kg) and their vehicles (8% methanol in saline and 1% CMC in saline respectively for TIIA and ASA). The bleeding was induced by precise transaction of the mouse tail at 5 mm from the tip. The distal portion of the tail (3 cm) was immersed vertically into the 0.9% saline solution at 37 °C. Blood flowing from the incision was carefully monitored and

the time to cessation of bleeding was recorded as the bleeding time. Bleeding cessation was considered to be the time when the flow of blood stops.

2.6. Statistical analysis

All assays were repeated at least in triplicate and the results were expressed as mean ± standard error of mean (SEM). Results were analyzed with one way analysis of variance (ANOVA), followed by Bonferroni's test for multiple comparisons, or by unpaired two tailed Student's *t*-test when appropriate. In some cases, One Sample *t*-test was used to evaluate significance against the hypothetical zero value. The analysis was performed using GraphPad Prism Software version 4.0. *P* values less than 0.05 were considered significant.

3. Results

3.1. Effect of TIIA on rat platelet aggregation

Fig. 1 shows typical records of concentration-dependent effect of TIIA (0.5, 5 and 50 μM) and the effect of ASA (50μM) on ADP-induced rat platelet aggregation.

Fig. 2 shows a concentration-dependent inhibition of reversible platelet aggregation expressed as % of inhibition of AUC (Fig.1A) or amplitude (Fig.1B) induced by TIIA (0.5, 5 and 50 μM) and ASA (50 μM) added 1 min before the addition of ADP (3 μM). TIIA and ASA at a concentration of 50 μM displayed the maximum inhibitory activity in terms of inhibition of AUC (63.3 ± 11.3%; $P < 0.001$ and 86.0 ± 3.78%; $P < 0.001$) and amplitude (25.67 ± 5.77%; $P < 0.01$ and 45.0 ± 2.88%). TIIA at a concentration of 100 μM displayed a similar effect compared to TIIA 50 μM (data not shown). The diterpenoid at a concentration of 50 μM was also investigated for its effect on irreversible aggregation (Fig. 3). TIIA only partially inhibited the platelet aggregation induced by ADP (10 μM) or by collagen (10 μg/mL) in terms of AUC (16.10 ± 5.6% and 9.25 ± 0.75% for ADP and collagen respectively; $P < 0.05$) and amplitude (9.61 ± 0.92% and 5.52 ± 1.50% for ADP and collagen respectively; $P < 0.05$) (Figs. 3A and 3B).

The lowest concentration of TIIA (0.5 and 5 μM) did not affect platelet aggregation, whereas TIIA at a concentration of 100 μM displayed a similar effect compared to TIIA 50 μM (data not shown).

3.2. Effect of TIIA on platelet tubulin acetylation and p-erk signaling

Figs. 4A and 5A show a representative Western blot and related cumulative densitometric analysis (Fig. 4B and Fig. 5B)

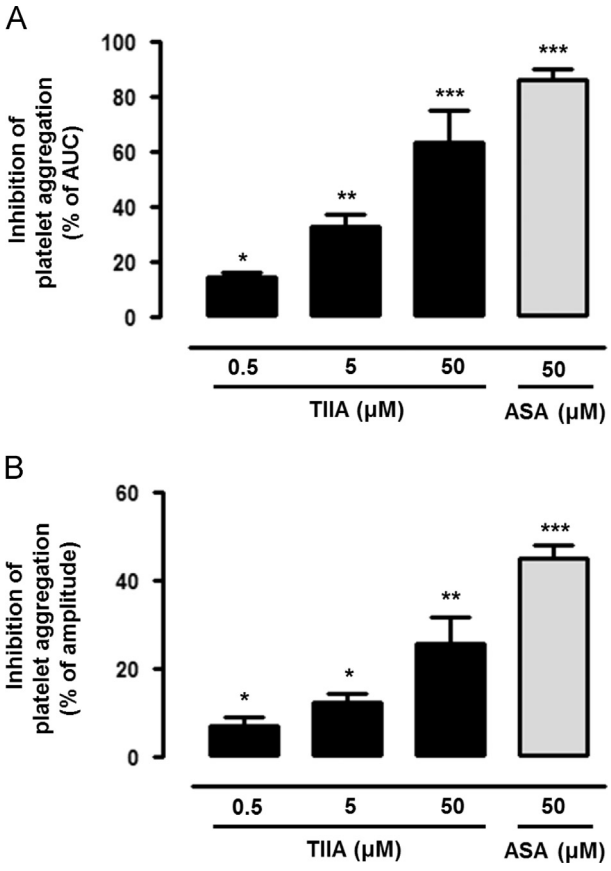

Fig. 2. Concentration dependent effect of TIIA on ADP-induced platelet aggregation. Rat PRP were incubated with TIIA (0.5–50 μM) or ASA (50 μM) for 1 min, and then exposed to ADP (3 μM) to induce platelet aggregation. Percent (%) inhibition of aggregation was expressed in terms of AUC **(A)** or amplitude **(B)** calculated as the difference between the maximum value of aggregation in presence of ADP plus vehicle and the value obtained in the presence of ADP plus TIIA or ASA. Data are expressed as mean ± SEM. $^{*}P < 0.05$ *vs* vehicle, $^{**}P < 0.01$ *vs* vehicle, $^{***}P < 0.001$ *vs* vehicle (one way ANOVA; $n = 7–10$).

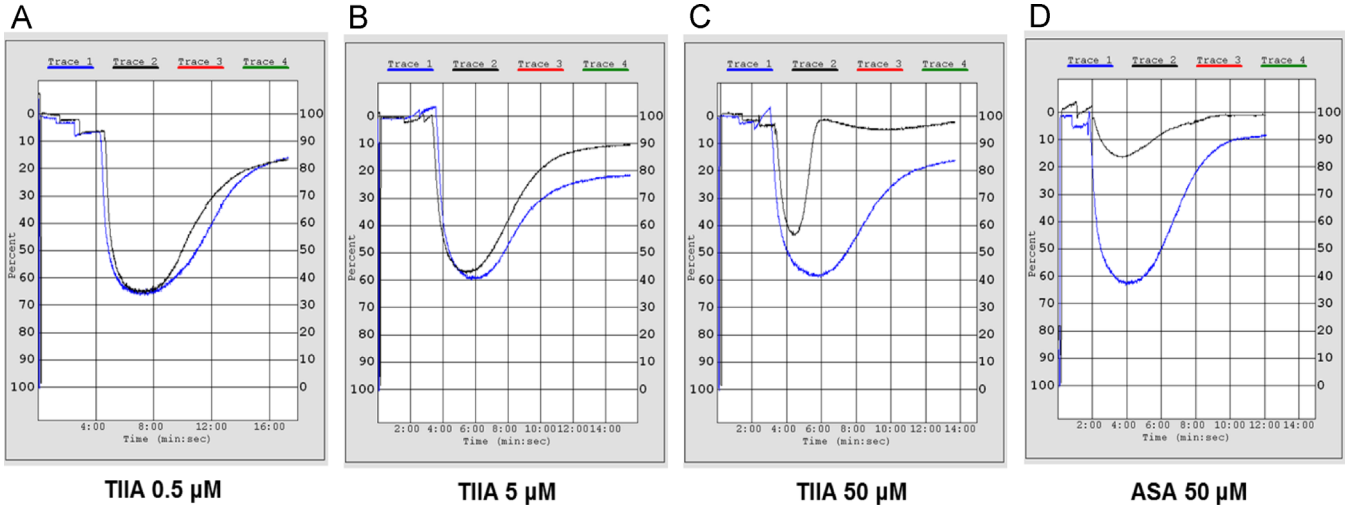

Fig. 1. Typical records of platelet aggregation. Figures present aggregation curves following addition of 0.5 **(A)**, 5 **(B)** and 50 μM **(C)** of TIIA or 50 μM **(D)** of ASA compared to TIIA/ASA-vehicle, after addition of ADP 3 μM.

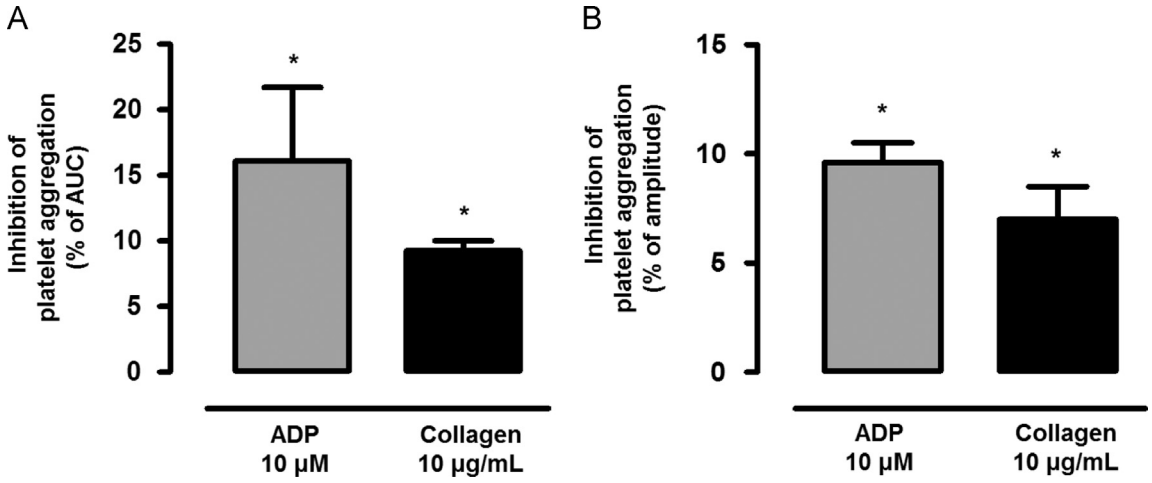

Fig. 3. Effect of TIIA on ADP (10 μM) and collagen (10 μg/mL)-induced platelet aggregation. Rat PRP were incubated with TIIA (50 μM) for 1 min, and then exposed to the pro-aggregatory stimuli (ADP or collagen). Percent (%) inhibition of aggregation expressed in terms of AUC **(A)** and amplitude **(B)** was calculated as the difference between the maximum value of aggregation in presence of the stimuli plus vehicles and the value obtained in the presence of TIIA. Data are expressed as mean ± SEM. ✻$P < 0.05$ *vs* vehicle (Student's *t*-test; $n=7$).

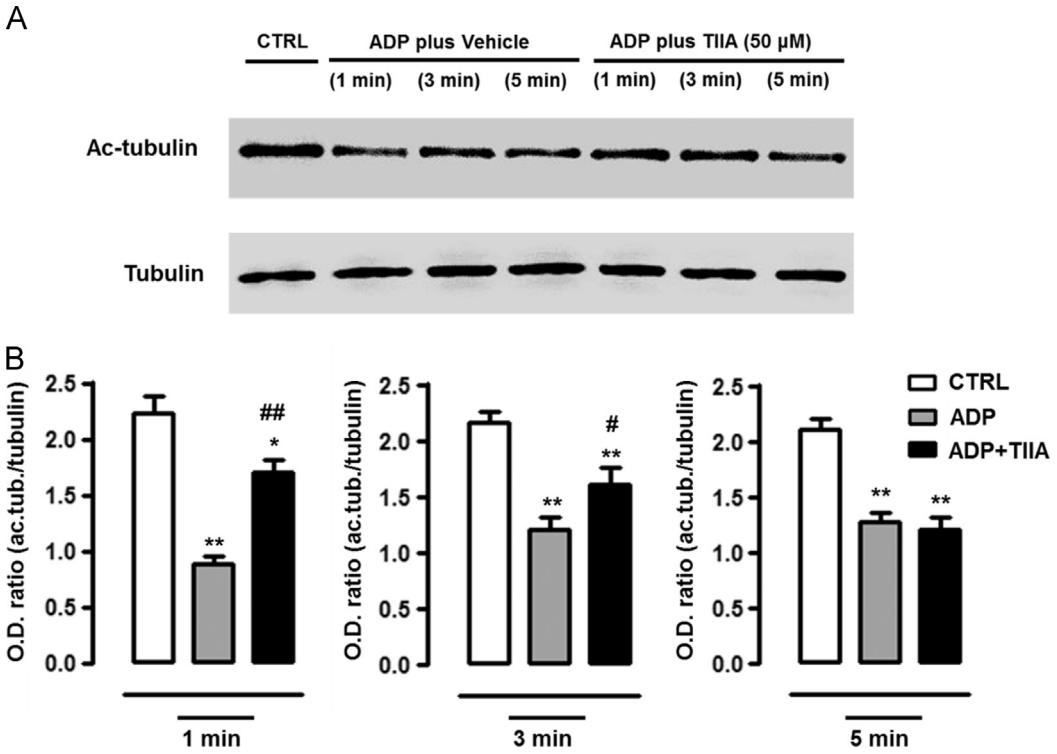

Fig. 4. Representative Western blotting **(A)** and related cumulative densytometric analyses **(B)** of acetylated tubulin (ac-tubulin) and tubulin in rat PRP stimulated with ADP (3 μM) plus TIIA-vehicle or with ADP (3 μM) plus TIIA (50 μM) at indicated time point. Data were expressed as mean ± SEM. O.D. (optical density) normalized against tubulin.$^{*}P < 0.05$ *vs* ctrl,$^{**}P < 0.01$ *vs* ctrl, $^{\#}P < 0.05$ *vs* ADP plus TIIA-vehicle, $^{\#\#}P < 0.01$ *vs* ADP plus TIIA-vehicle (one way ANOVA followed by Student's *t*-test, $n=3$).

of rat PRP lysates non-stimulated (ctrl) or collected 1, 3 and 5 min after the stimulation with ADP (3 μM) or ADP plus TIIA (50 μM) and successively blotted for acetylated tubulin and p-Erk-2 antibodies.

Fig. 4B shows that platelets of the ctrl group had elevated levels of acetylated tubulin at all time points. These levels were significantly reduced in the presence of ADP and were reverted 1 and 3 min after incubation with TIIA.

Fig. 5B shows that the platelet of the ctrl group did not shown any increase in intracellular signaling of p-Erk-2 for all time points. Stimulation with ADP induced a time-dependent increase in Erk-2 phosphorylation that peaked between 3 and 5 min. Pre-incubation with TIIA significantly reduced p-Erk signaling at 3 min.

3.3. *Effect of TIIA on in vivo bleeding time*

As shown in Fig. 6, the pretreatment of mice with TIIA (10 mg/kg, i.p.) significantly increased mice bleeding time (2.06 ± 0.29 min; $P < 0.01$) compared to the control group (1.29 ± 0.07 min). Similar results were observed after ASA administration (2.28 ± 0.34 *vs* 0.75 ± 0.20 min; $P < 0.01$).

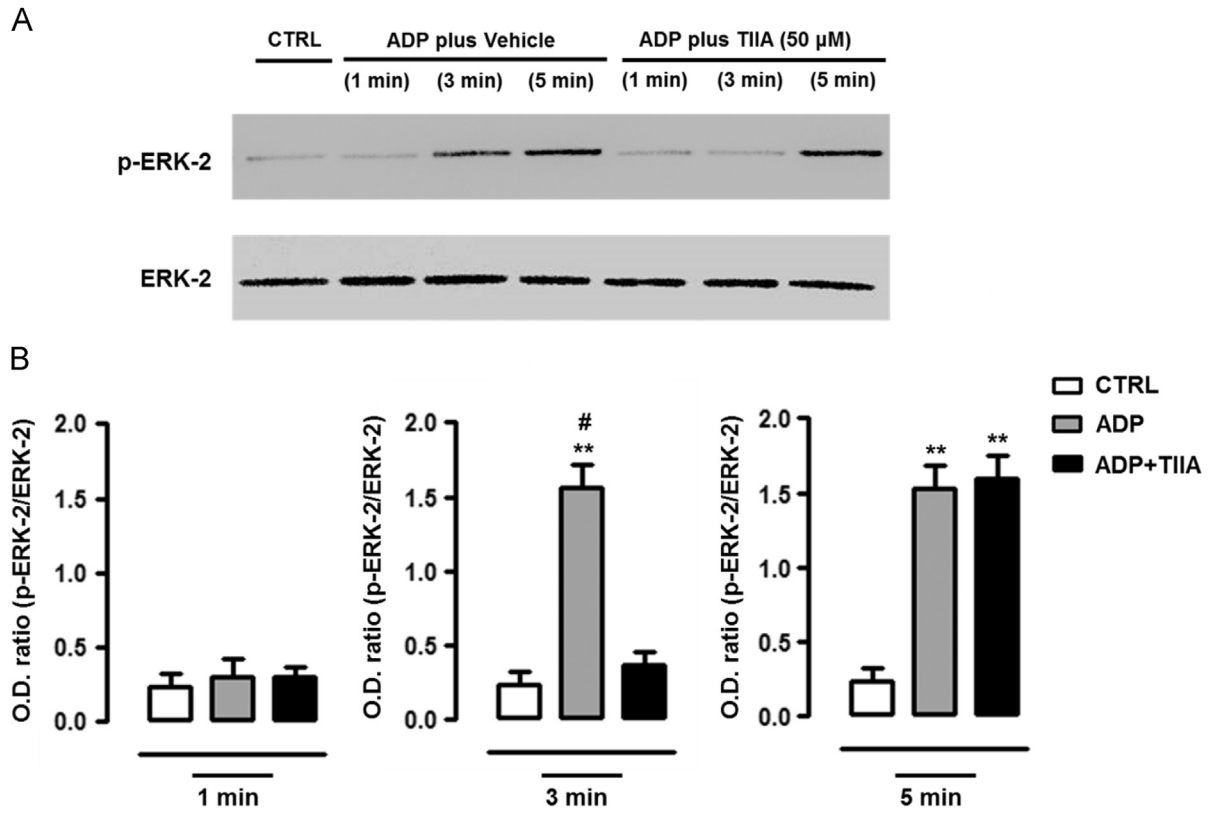

Fig. 5. Representative Western blotting **(A)** and related cumulative densytometric analyses **(B)** of p-Erk2 and total Erk-2 in rat PRP stimulated with ADP (3 μM) plus TIIA-vehicle or with ADP (3 μM) plus TIIA (50 μM) at indicated time point. Data were expressed as mean ± SEM. O.D. (optical density) normalized against Erk-2.$^{**}P < 0.01$ *vs* ctrl, $^{\#}P < 0.05$ *vs* ADP plus TIIA (one way ANOVA followed by Student's *t*-test, $n = 3$).

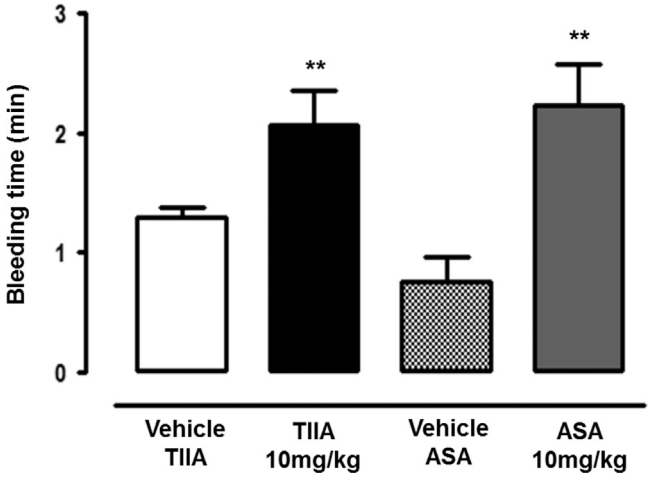

Fig. 6. Effect of TIIA on mouse tail bleeding time. TIIA (10 mg/kg) or ASA (10 mg/kg) were administered i.p. to CD1 mice 1 h before the experiments. Data were expressed as the mean ± S.E.M.$^{**}P < 0.01$ *vs* respective vehicles. (Student's *t*-test, $n = 8–10$).

However the fold increase of mice bleeding time was higher in ASA (204%, ASA *vs* vehicle group) compared to TIIA treated mice (58%, TIIA *vs* vehicle group).

4. Discussion

Danshen has long been used in the treatment of cardiovascular-related disorders. Interest in its versatile protective effects in cardiovascular and neurodegenerative diseases has been growing over the last decade (Zeng et al., 2012; Wu and Wang, 2012; Xu and Liu, 2013; Sheng et al., 2014).

Tanshinones, the major lipophilic components extracted from Danshen, are known to exhibit potent cardiovascular effects (Gao et al., 2012). In the early observations Li et al. (1984) demonstrated that tanshinone IIA sulfonate affected thrombus formation and blood coagulation in rats and mice and successively Lee et al. (1987) reported that isotanshinone IIB inhibits ADP- and collagen-induced platelet aggregation *in vitro*. A most recent paper published by Liu et al. (2011) also demonstrated the effect of TIIA on platelet aggregation in healthy newborn piglets. Other bioactives components extracted from Danshen, as such as oleoylneocryptotanshinone II and oleoyl danshenxinkun A were also reported to inhibit platelet aggregation induced by arachidonic acid in rabbits (Lin et al., 2001).

Tanshinone IIA has been introduced as the most abundant and representative principle of all tanshinones present in Danshen (Gao et al., 2012). On this basis, in this study we tested the hypothesis that TIIA inhibited the platelet aggregation induced by collagen and ADP *via* the modulation of tubulin acetylation and inhibition of Erk-2 phosphorilation. The effect of the diterpenoid was also studied *in vivo* on bleeding time in mice.

Our results show that TIIA *in vitro* inhibits, in a concentration dependent manner, rat platelet aggregation induced by ADP 3 μM (primary aggregation). The observed effect was similar to ASA in terms of percentage of inhibition of platelet aggregation. However, TIIA displayed a less extent of activity on irreversible stimuli induced by ADP 10 μM or by collagen 10 μg/mL.

Platelet activation can be part of the normal hemostatic response to injury or a pathological reaction to disease. In the complex scenario of vascular injury, platelets initially are activated by collagen from the vessel wall. This led activated platelets to release mainly ADP and successively other mediators which

reinforce the platelet activation response and plug (Kunapuli, 1998; Pitchford, 2007). Platelet plug formation can proceed beyond thrombus growth. This might occur in case an excessive local presence of molecules that exert an autocrine and paracrine aggregatory effects, such as nitride oxide (NO) and matrix metalloproteinase (MMP). NO, released under basal conditions and in response to platelet receptor agonists such as ADP, interacts directly with circulating platelets and exerts its effects by activating soluble guanylyl cyclase in vascular smooth muscle, which in turn leads to the formation of cyclic guanosine monophosphate (cGMP) and to relaxation. On the other hand, independent of their matrix degrading activity, MMPs also regulate some cell functions relevant to atherothrombosis and thrombus stability, such as platelet activation, neutrophil activation, and vascular reactivity (Tschudi and Lüscher, 1996; Santos-Martínez et al., 2008).

In platelets, tubulin acetylation has an important role in the control of microtubule structure and microtubule-based cellular functions. However, upon activation, platelets undergo a dramatic change in shape as a result of microtubules reorganizations suggesting that their deacetylation is associated with cell activation (Aslan et al., 2013; Sadoul et al., 2012). To confirm the effects of TIIA on platelets, we next measured the effect of TIIA on tubulin acetylation signaling in rat pre-activated platelets. Intriguely and consistently with results obtained in the aggregation assay, platelets treated with TIIA (50 μM) displayed faster kinetics of tubulin acetylation compared to platelets stimulated with ADP alone, up to a period of 3 min, indicating a specific effect of TIIA on the kinetics of platelet activation.

In platelets, MAPKs mainly contributed to platelet activation and aggregation induced by various stimuli. Moreover, recent works show that phosphorylation of predominantly Erk-2, but not Erk-1, occurs in platelets stimulated with P2Y receptor agonists, such as ADP (Adam et al., 2008; Garcia et al., 2007). To gain insight into the mechanism of TIIA, we investigated the effect of the tanshinone on MAPKs signaling on rat platelets. Experiments performed on PRP lysates collected at different time-point after the addition of the stimuli showed that TIIA (50 μM) selectively inhibited at 3 min MAPK signaling pathways such as Erk-2 phosphorylation compared to the ADP-stimulated platelet. These results demonstrate that the effects of TIIA on tubulin acetylation could be related to a concomitantly inhibition of Erk-2 signaling.

Recent evidences demonstrated that Danshen may have effects on blood clotting and that potentiates the effects of the common anticoagulation drugs such as warfarin (Chan, 2001), suggesting an implication of tanshinones on blood stasis. As shown in our *in vivo* experiments, TIIA (10 mg/kg) significantly prolonged the bleeding time in mice compared with the control group ($P < 0.01$), confirming the effect of the diterpenoid on *in vitro* platelet aggregation. *In vitro*, we observed that TIIA inhibited primary aggregation through inhibition of ADP and collagen-induced platelet aggregation. This effect is also reflected *in vivo* in an increased mice bleeding time. Probably, the *in vivo* effect of TIIA is due to a reduced platelet adhesion to the vascular wall and the following reduced response to endogenous ADP.

5. Conclusion

In the present study, we have examined the effect of TIIA on platelet aggregation induced by different stimuli and we have investigated the mechanism underlying its action. Our paper clarifies the intracellular signaling pathway involved in this process and also gives preliminary evidences for its anticoagulant activity. For these reasons, this compound could be a promising agent to improve blood viscosity and microcirculation and to prevent CVD.

Author contribution

Maione Francesco, Caiazzo Elisabetta and De Martino Laura carried out experiments. Mascolo Nicola, De Feo Vincenzo and Maione Francesco conducted experiments, performed data analysis and wrote the paper. Cicala Carla helped with data analysis and with revision of the paper. All authors read and approved the final version of the paper before submission.

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