

Original Paper

Skepinone-L, a Novel Potent and Highly Selective Inhibitor of p38 MAP Kinase, Effectively Impairs Platelet Activation and Thrombus Formation

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Key Words

Aggregation • p38 MAPK • Secretion • Skepinone-L • Thrombus formation • Thromboxane synthesis

Abstract

Background/Aims: Platelets are critically important for primary haemostasis and the major players in thrombotic vascular occlusion. Platelets are activated by agonists, such as thrombin and collagen-related peptide as well as second-wave mediators including thromboxane A₂ via different intracellular signaling pathways resulting in degranulation, aggregation and thrombus formation. Platelet activation is paralleled by phosphorylation and activation of p38 MAPK. The limited specificity of hitherto known p38 MAPK inhibitors precluded safe conclusions on the precise role of p38 MAPK in the regulation of platelet function. The present study examined the impact of Skepinone-L, a novel and highly selective inhibitor of p38 mitogen-activated protein kinase (p38 MAPK), on platelet activation and thrombus formation. **Methods:** Experiments were performed in freshly isolated human platelets. Protein phosphorylation was quantified by Western blotting, thromboxane B₂ synthesis by enzyme immunoassay, ATP release by ChronoLume luciferin assay, cytosolic Ca²⁺ concentration by Fura-2 fluorescence-measurements, platelet aggregation by a light transmissions measurement and *in vitro* thrombus formation by a flow chamber. **Results:** Skepinone-L (1 μM) virtually abrogated the phosphorylation of platelet p38 MAPK substrate Hsp27 following stimulation with CRP (1 μg/ml), thrombin (5 mU/ml) or thromboxane A₂ analogue U-46619 (1 μM). Furthermore, Skepinone-L significantly blunted activation-dependent platelet secretion and aggregation following threshold concentrations of CRP, thrombin and thromboxane A₂ analogue U-46619. Skepinone-L did not impair platelet Ca²⁺ signaling

but prevented agonist-induced thromboxane A₂ synthesis through abrogation of p38 MAPK-dependent phosphorylation of platelet cytosolic phospholipase A₂ (cPLA₂). Skepinone-L further markedly blunted thrombus formation under low (500-s) and high (1700-s) arterial shear rates. **Conclusions:** The present study discloses a powerful inhibiting effect of p38 MAPK-blocker Skepinone-L on platelet activation and thrombus formation.

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Introduction

Platelets are key players in primary haemostasis following vascular injury as well as in acute thrombotic occlusion after rupture or erosion of atherosclerotic plaques resulting in myocardial infarction or ischemic stroke [1, 2]. Platelets are activated by numerous agonists including collagen-related peptide and thrombin as well as the second wave mediators thromboxane A₂ and ADP [3]. Platelet activation following adhesion to collagen results in degranulation, aggregation and thrombus formation [4].

Platelet activation is modified by several signaling pathways and kinases including p38 mitogen-activated protein kinase (MAPK), which is expressed in platelets [5] and activated by thrombin [6, 7], thromboxane (TXA₂) [8] or collagen [8, 9]. The activation is paralleled by phosphorylation of the kinase [10-16].

p38 MAPK is a potent activator of MAPK-activated protein kinase-2, which phosphorylates the small heat shock protein 27 (Hsp27). While in the resting state platelet Hsp27 is mainly unphosphorylated and located in the cytoplasm, after platelet stimulation Hsp27 is phosphorylated and translocated to the cytoskeleton [17]. Thus, Hsp27 phosphorylation upon platelet stimulation was measured as an indicator of p38 MAPK activity [8].

It has been speculated that p38 MAPK could act as an important signaling kinase in the regulation of platelet adhesion and spreading [15, 18], which requires p38 MAPK dependent actin cytoskeleton reorganization [19]. It has already been shown that platelet p38 MAPK is required for thrombin- and collagen-dependent phosphorylation of cytosolic phospholipase A₂ (cPLA₂) [7, 20] with subsequent release of thromboxane A₂ (TXA₂) production [5, 21]. But the impact of p38 MAPK on platelet aggregation following different platelet agonists still remains a matter of debate. Furthermore, the role of p38 MAPK in platelet secretion and thrombus formation under different shear rates remains largely unclear.

By using the inhibitors VX-702 and SB203580 platelet p38 MAPK was reported not to be required for platelet calcium influx following stimulation with thrombin or thromboxane analogue U-46619 [21, 22], but appears to be essential for platelet aggregation induced by U-46619 [23] as well as low and medium concentrations of collagen [8, 22].

Knowledge on the functional significance of p38 MAPK in platelets is in large part dependent on pharmacological inhibition of the kinase by SB203580 and SB202190 [19] or VX-702 [21]. The majority of p38 MAPK inhibitors are only moderately potent in physiologically relevant whole blood studies [24] and require high dosing *in vivo* to achieve effective plasma concentrations [25]. Most p38 inhibitors including SB203580 and SB202190 are effective via their ATP-competitive binding mode and display poor selectivity because the ATP-binding site is highly conserved among a variety of proteins [25, 26]. Recently, Skepinone-L, a highly selective inhibitor of p38 MAPK has been developed. Skepinone-L is, to our knowledge, the first ATP-competitive p38 MAPK inhibitor with excellent *in vivo* efficacy and selectivity [25, 27]. At a concentration of 1 μM, Skepinone-L did not bind any of the tested kinases in two different selectivity screens except p38 MAPK [25]. Following that knowledge, p38 MAPK dependent pathways in platelet activation can be explored exactly.

The present study thus examined the effect of the novel p38 MAPK inhibitor Skepinone-L on platelet function, especially with focus on secretion, Ca²⁺ influx, thromboxane synthesis and aggregation following threshold and maximal concentrations of different platelet agonists speculated to be effective through platelet p38 MAPK signaling. Furthermore, the present study examined for the first time the impact of selective and potent inhibitor of p38 MAPK by Skepinone-L on thrombus formation under different arterial shear rates.

Materials and Methods

Chemicals

Platelets were activated using collagen-related peptide (CRP, provided by R. Farndale, University of Cambridge, United Kingdom), thrombin (Roche) or the thromboxane A_2 analogue U-46619 (Enzo). Skepinone-L was generated by S. Laufer (University of Tübingen, Germany) as described previously [25].

Preparation of human platelets

Human platelets were isolated as described previously [28]. Blood from healthy volunteers was collected in ACD buffer and centrifuged at 200 g for 20 minutes. The obtained platelet-rich plasma was added to modified Tyrode-HEPES (N-2-hydroxyethyl-piperazine-N'2-ethanesulfonic acid) buffer (137 mM NaCl, 2.8 mM KCL, 12 mM NaHCO_3 , 5 mM glucose, 0.4 mM Na_2HPO_4 , 10 mM HEPES, 0.1% bovine serum albumin, pH 6.5). After centrifugation at 900 g for 10 minutes and removal of the supernatant, the resulting platelet pellet was resuspended in Tyrode-HEPES buffer (pH 7.4, supplemented with 1 mM CaCl_2).

Western blot analysis

To examine phosphorylation of MAPK p38 protein kinase effector Hsp27 as well as phosphorylation of cPLA_2 , isolated platelets were lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton-X, 0.5 % Na_2HPO_4 , 0.4 % β -mercaptoethanol) containing protease inhibitor cocktail (Roche). Proteins (40 μg) were solubilized in Laemmli sample buffer at 95°C for 5 minutes and resolved by 10 % SDS-PAGE. For immunoblotting proteins were electro-transferred onto a nitrocellulose membrane and blocked with 10 % nonfat milk in TBS-0.1% Tween 20 (TBST) at room temperature for 1 hour. Then, the membrane was incubated with affinity purified rabbit anti-pHsp27 (1:1000; Cell Signaling) or anti-phospho (Ser⁵⁰⁵) cPLA_2 (1:1000, Cell Signaling) and actin antibody (1:1000, Cell Signaling) as loading control at 4°C overnight. After washing 3 times with TBST (10 minutes each) the blots were incubated with horseradish peroxidase conjugated secondary anti-rabbit antibody (1:3000; Cell Signaling) for 1 hour at room temperature. After washing antibody binding was detected with the ECL detection reagent (Amersham). Antibody-binding was quantified densitometrically with Quantity One Software (Biorad, München, Germany).

Thromboxane synthesis

Because thromboxane A_2 (TXA_2) is very unstable and rapidly hydrolyzed into stable metabolite thromboxane B_2 (TXB_2), we measured the latter instead of TXA_2 . After the challenge of platelet agonists for 5 minutes in the presence or absence of Skepinone-L, EDTA (2 mmol/L) and indomethacin (50 $\mu\text{mol/L}$) were added to stop reactions as described previously [29]. The platelet suspensions were centrifuged for 3 minutes at 13,000 g, and TXB_2 in the supernatants were assayed using an enzyme immunoassay (EIA) kit according to the manufacturer's ELISA instructions (Cayman Chemical).

ATP release

ATP release was determined to study degranulation of platelet dense granules. To this end, isolated human platelets were pretreated either with 1 μM Skepinone-L or DMSO as solvent control for 15 minutes at 37°C and activated by different agonist concentrations. For determination of ATP release, the isolated platelets were adjusted to a concentration of 250×10^6 platelets per μl . After calibration of one sample with ATP standard (ChronoLog, Havertown, USA), the ATP concentration was determined utilizing the ChronoLume luciferin assay (ChronoLog) on a luminoaggregometer (Modell 700, ChronoLog) according to the manufacturer's protocol.

Platelet aggregometry

Aggregation of isolated human platelets was studied at a concentration of 250×10^6 platelets per μl in Tyrode buffer pH 7.4 and was estimated from light transmission determined with a luminoaggregometer model 700 (ChronoLog). After adjusting the measurement according to the manufacturer's protocol platelets were activated for 10 minutes, 37°C and a stirr speed of 1000 rpm with the indicated concentrations of agonists. Analysis was performed with the aggrolink8 software (ChronoLog).

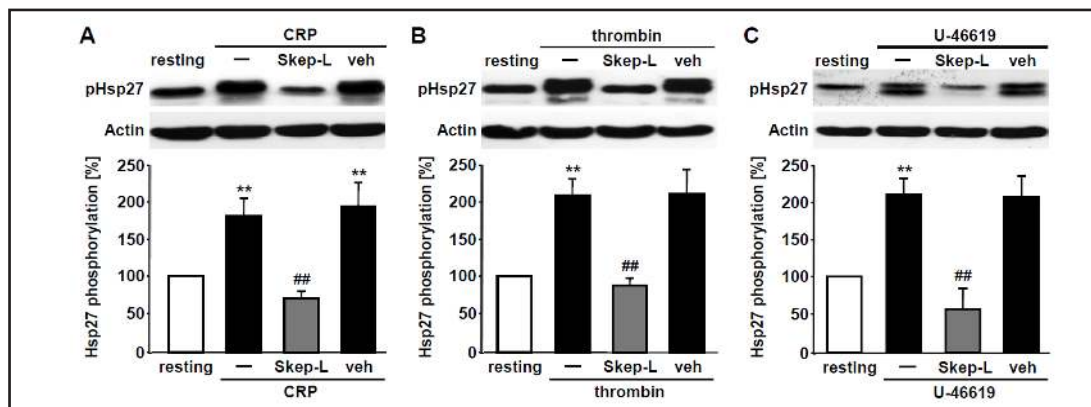


Fig. 1. Skepinone-L abrogates activation-dependent Hsp27 phosphorylation in platelets. Representative Western blots and arithmetic means \pm SEM ($n = 4-6$) demonstrating the expression of phosphorylated Hsp27 and of the respective actin protein abundance in isolated human platelets prior to (resting) or following stimulation with either CRP (1 $\mu\text{g}/\text{ml}$, A), thrombin (5 mU/ml, B) or thromboxane A_2 analogue U-46619 (1 μM , C) in the presence of Skepinone-L (1 μM) or vehicle (DMSO). **($p < 0.01$) indicates significant difference from value prior to activation, ##($p < 0.01$) indicates significant difference between pretreatment with Skepinone-L or vehicle (DMSO).

Calcium measurements

Washed platelets were suspended in Tyrode buffer without calcium and loaded with 5 μM Fura-2 acetoxymethylester (Invitrogen) in the presence of 0.2 $\mu\text{g}/\text{ml}$ Pluronic F-127 (Biotium) at 37°C for 30 minutes. Loaded platelets, washed once and resuspended in Tyrode buffer containing 0.5 mM EGTA (Roth) or 1 mM Ca^{2+} , were activated with indicated agonists. Calcium responses were measured under stirring with a spectrofluorimeter (LS 55, PerkinElmer), at alternate excitation wavelength of 340 and 380 nm (37°C). The 340/380 nm ratio values were converted into nanomolar concentrations of $[\text{Ca}^{2+}]$ by lysis with Triton X-100 (Sigma-Aldrich) and a surplus of EGTA.

In vitro thrombus formation

Heparinized whole mouse blood was diluted 1:3 in modified Tyrode buffer (pH 7.4) and perfused through a transparent flow chamber (slit depth 50 μm) over a collagen-coated surface (200 $\mu\text{g}/\text{ml}$) with a wall shear rate of 500 $^{\circ}$ (low shear rate) or 1700 $^{\circ}$ (high shear rate) for 5 minutes. After perfusion the chamber was rinsed for 5 minutes by perfusion with Tyrode buffer (pH 7.4) and pictures were taken from 5 to 6 different microscopic areas (20 \times , Carl Zeiss). Analysis was done with AxioVision (Carl Zeiss) and the mean percentage value of the covered area was determined.

Statistical analysis

Data are provided as means \pm SD or SEM, n represents the number of experiments. All data were tested for significance using paired or unpaired Student t-test and one-way ANOVA with Dunnett's post-hoc test. Results with $p < 0.05$ or $p < 0.01$ were considered statistically significant.

Results

Skepinone-L decreased p38 MAPK-dependent phosphorylation of Hsp27 upon platelet stimulation.

In a first series of experiments Western blot analysis was employed to determine the cellular potency of Skepinone-L. Platelets were treated with different agonists potentially activating platelet p38 MAPK. The level of stimulation-dependent phosphorylation of heat shock protein 27 (Hsp27) through the p38 MAPK pathway was determined as a marker of platelet p38 MAPK activity.

As illustrated in Fig. 1, activation of platelets with CRP (1 $\mu\text{g}/\text{ml}$, A), thrombin (5 mU/ml, B) or the thromboxane A_2 analogue U-46619 (1 μM , C) was followed by a marked

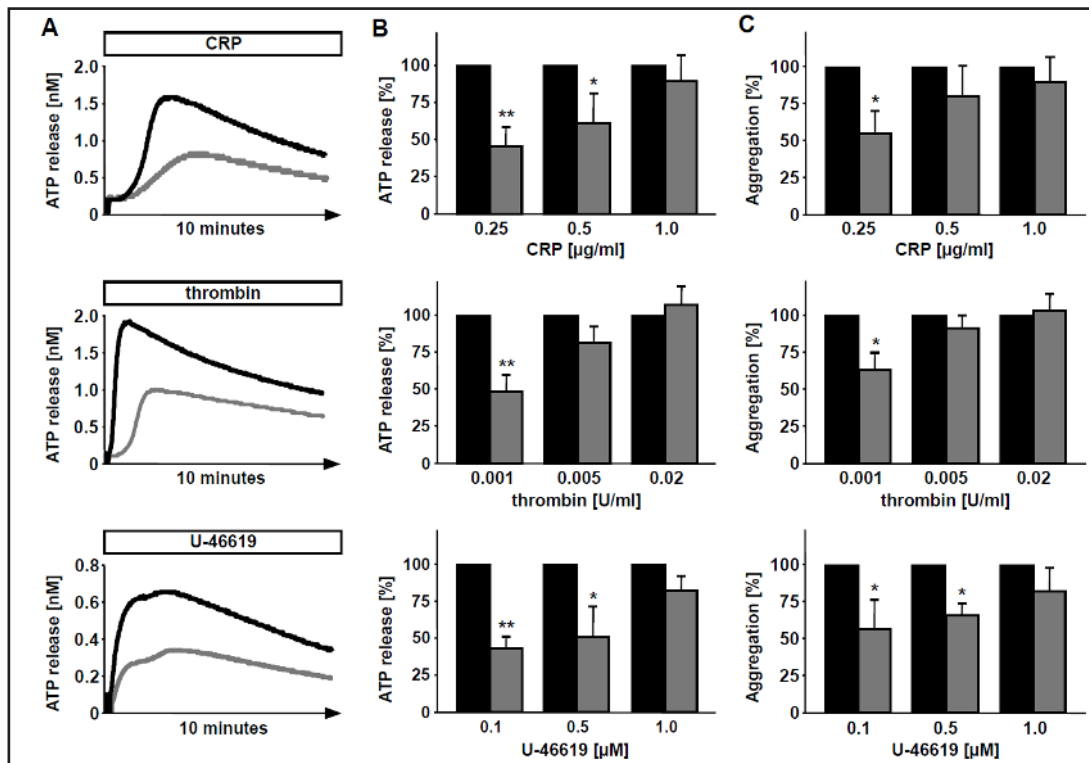


Fig. 2. Skepinone-L significantly affects activation-dependent platelet secretion and aggregation. A. Representative tracings illustrating the increase of ATP concentration in platelet supernatant following stimulation with either 0.25 $\mu\text{g/ml}$ collagen-related peptide (CRP), 1 mU/ml thrombin or 0.1 μM thromboxane A_2 analogue U-46619 in the absence (black tracings) and presence (grey tracings) of Skepinone-L (1 μM). B. Arithmetic means \pm SEM (n = 6) of ATP concentration in platelet supernatant following stimulation of platelets with collagen-related peptide (CRP, 0.25-1.0 $\mu\text{g/ml}$), thrombin (1-20 mU/ml) or U-46619 (0.1-1.0 μM) in the absence (black bars) or presence (grey bars) of Skepinone-L (1 μM). *(p<0.05) and ** (p<0.01) indicate significant difference between pretreatment with Skepinone-L or vehicle (DMSO). C. Arithmetic means \pm SEM (n = 6) of light transmission aggregometry following stimulation of platelets with collagen-related peptide (CRP, 0.25-1.0 $\mu\text{g/ml}$), thrombin (1-20 mU/ml) or the thromboxane A_2 analogue U-46619 (0.1-1.0 μM) in the absence (black bars) and presence (grey bars) of Skepinone-L (1 μM). *(p<0.05) indicates significant difference between pretreatment with Skepinone-L or vehicle (DMSO).

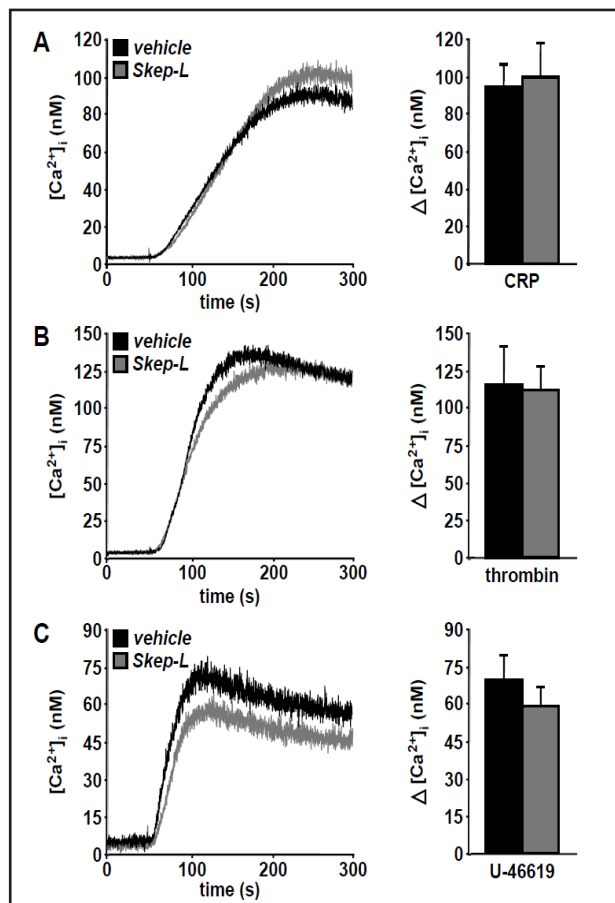
increase of Hsp27 phosphorylation. The increase of phosphorylated Hsp27 was abrogated in the presence of 1 μM Skepinone-L, but not in the presence of DMSO, used as vehicle (Fig. 1).

Further experiments explored the functional significance of Skepinone-L sensitive p38 MAP kinase activity.

Inhibition of platelet p38 MAPK activity by Skepinone-L abrogated stimulation-dependent platelet secretion and aggregation in response to threshold agonist concentrations.

To elucidate the impact of Skepinone-L on platelet secretion, ATP release (dense granules) was quantified prior to and following agonist activation with increasing concentrations of CRP (0.25, 0.5 and 1 $\mu\text{g/ml}$), thrombin (1, 5 and 20 mU/ml) and thromboxane A_2 analogue U-46619 (0.1, 0.5 and 1.0 μM) in the presence of Skepinone-L (1 μM) or DMSO (vehicle). As illustrated in Fig. 2 A and B, treatment of platelets with thrombin, CRP or thromboxane A_2 analogue U-46619 was followed by a sharp increase of ATP release (luminescence analysis). Skepinone-L significantly blunted the ATP release following stimulation with low concentrations of either thrombin (1 mU/ml) or CRP (0.25 and 0.5 $\mu\text{g/ml}$). Increasing concentrations of CRP and thrombin overcame the inhibitory effect of Skepinone-L, whereas Skepinone-L blocked

Fig. 3. Agonist-induced Ca^{2+} influx in platelets in absence and presence of Skepinone-L. A. Representative tracings and arithmetic means \pm SEM ($n = 4$) of maximal $\Delta[\text{Ca}^{2+}]_i$ of Fura-2-fluorescence reflecting cytosolic Ca^{2+} concentration $[\text{Ca}^{2+}]_i$ in platelets prior to and following stimulation with collagen-related peptide (CRP 0.5 $\mu\text{g}/\text{ml}$) in the absence (black curve and bar) and presence (grey curve and bar) of Skepinone-L (1 μM). B. Representative tracings and arithmetic means \pm SEM ($n = 4$) of maximal $\Delta[\text{Ca}^{2+}]_i$ of Fura-2-fluorescence reflecting cytosolic Ca^{2+} concentration $[\text{Ca}^{2+}]_i$ in platelets prior to and following stimulation with thrombin (5 mU/ml) in the absence (black curve and bar) and presence (grey curve and bar) of Skepinone-L (1 μM). C. Representative tracings and arithmetic means \pm SEM ($n = 4$) of maximal $\Delta[\text{Ca}^{2+}]_i$ of Fura-2-fluorescence reflecting cytosolic Ca^{2+} concentration $[\text{Ca}^{2+}]_i$ in platelets prior to and following stimulation with thromboxane A_2 analogue U-46619 (0.5 μM) in the absence (black curve and bar) and presence (grey curve and bar) of Skepinone-L (1 μM).



stimulation-dependent degranulation following submaximal concentrations of thromboxane analogue A_2 U-46619 (0.1 – 0.5 μM).

In a next series of experiments, light transmission measurements of platelet aggregation were performed prior to and following activation with increasing concentrations of CRP (0.25, 0.5 and 1 $\mu\text{g}/\text{ml}$), thrombin (1, 5 and 20 mU/ml) or thromboxane A_2 analogue U-46619 (0.1, 0.5 and 1 μM). As illustrated in Fig. 2 C, platelet aggregation following stimulation with the indicated agonist concentrations was significantly affected following threshold concentrations of CRP (0.25 $\mu\text{g}/\text{ml}$), thrombin (1 mU/ml) or thromboxane A_2 analogue U-46619 (0.1 μM). The defect in platelet aggregation was overcome by increasing concentrations of CRP and thrombin whereas thromboxane-induced aggregation was significantly decreased following increasing concentrations of thromboxane A_2 analogue U-46619 (0.5 μM) in the presence of p38 MAPK inhibitor Skepinone-L (1 μM).

Skepinone-L did not affect stimulation-dependent increase of cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) in platelets.

Since platelets are reactivated by an increase of cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) we examined the impact of p38 MAPK on platelet cytosolic Ca^{2+} activity $[\text{Ca}^{2+}]_i$ by using p38 MAPK inhibitor Skepinone-L. As shown in Fig. 3, a series of spectrofluorimetric calcium measurements prior to and following CRP (0.5 $\mu\text{g}/\text{ml}$, A), thrombin (5 mU/ml, B) or thromboxane A_2 analogue U-46619 (0.5 μM , C) stimulation revealed that activation-dependent increase of platelet cytosolic Ca^{2+} activity $[\text{Ca}^{2+}]_i$ was similar in the absence and presence of 1 μM Skepinone-L.

Inhibition of p38 MAPK by Skepinone-L impaired activation-dependent of platelet cytosolic phospholipase A_2 phosphorylation and thromboxane A_2 synthesis.

In a further series of experiments the effect of Skepinone-L on p38 MAPK-dependent phosphorylation of platelet cytosolic phospholipase A_2 (cPLA $_2$) and thromboxane A_2 synthesis

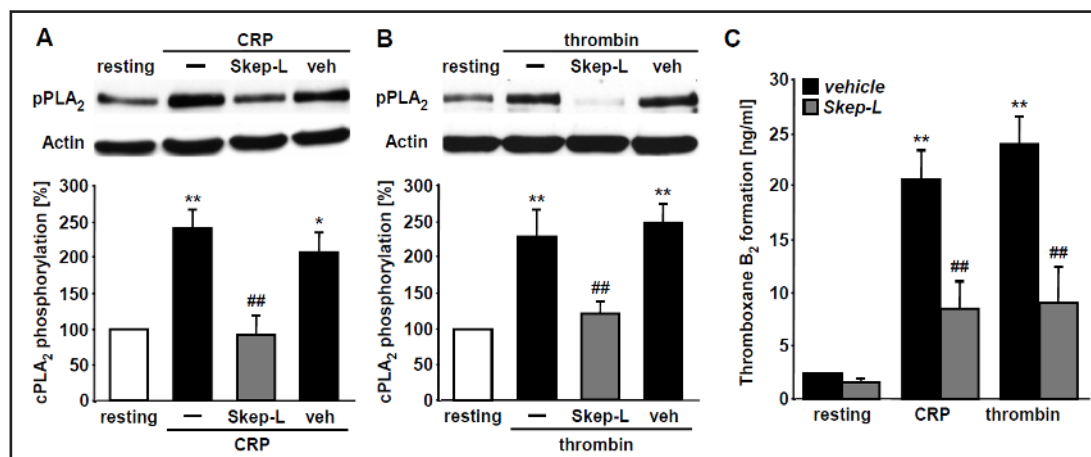


Fig. 4. Skepinone-L impairs phosphorylation/activation of platelet cytosolic phospholipase A₂ (cPLA₂) and platelet thromboxane synthesis. A. Representative Western blot and arithmetic means \pm SEM (n = 6) of activation-dependent phosphorylation of platelet cytosolic phospholipase A₂ at Ser⁵⁰⁵ following stimulation with CRP (1.0 μ g/ml). ** (p < 0.01) or * (p < 0.05) indicate significant difference from value prior to activation, ## (p < 0.01) indicates significant difference between pretreatment with Skepinone-L or vehicle (DMSO). B. Representative Western blot and arithmetic means \pm SEM (n = 6) of activation-dependent phosphorylation of platelet cytosolic phospholipase A₂ at Ser⁵⁰⁵ following stimulation with thrombin (5 mU/ml). ** (p < 0.01) indicates significant difference from value prior to activation, ## (p < 0.01) indicates significant difference between pretreatment with Skepinone-L or vehicle (DMSO). C. Arithmetic means \pm SEM (n = 8) of activation-dependent platelet thromboxane B₂ formation following stimulation with CRP (1.0 μ g/ml) or thrombin (5 mU/ml). ** (p < 0.01) indicates significant difference from value prior to activation, ## (p < 0.05) indicates significant difference between pretreatment with Skepinone-L or vehicle (DMSO).

upon platelet activation was evaluated. Platelet activation with CRP (1 μ g/ml, Fig. 4 A) or thrombin (5 mU/ml, Fig. 4 B) was followed by a significant increase of PLA₂ phosphorylation (Ser⁵⁰⁵) as well as a significant stimulation of thromboxane formation (Fig. 4 C). Activation-dependent cPLA₂ phosphorylation at Ser⁵⁰⁵ and thromboxane synthesis were significantly blunted in the presence of Skepinone-L (1 μ M, grey bars).

Skepinone-L inhibited thrombus formation at low and high shear rates

To elucidate the relevance of Skepinone-L in pathologic thrombus formation, we examined thrombus formation to collagen-coated surfaces under low shear (500^s) and high shear (1700^s) conditions. As illustrated in Fig. 5 A, collagen-triggered platelet adhesion and thrombus formation under high arterial shear rates of 1700 s⁻¹ was significantly impaired after treatment with Skepinone-L (1 μ M). Under low shear rates of 500^s untreated platelets formed massive and dense thrombi after 5 minutes perfusion, whereas Skepinone-L-treated platelets formed only smaller single thrombi with a significantly reduced thrombus surface coverage (Fig. 5 B).

Discussion

Platelet adhesion and activation are critically important for the development of acute thrombotic occlusion at regions of atherosclerotic plaque rupture, the major pathophysiological mechanism underlying ischemic diseases, such as myocardial infarction or stroke [4, 30, 31]. Several intracellular signaling pathways are involved in platelet activation upon stimulation by contact to subendothelial collagen, thrombin, thromboxane A₂ and ADP released by activated platelets. Recent studies have provided evidence that p38 MAPK dependent signaling could be critically involved in haemostasis and thrombosis [6-9]. However, lack of high potency and

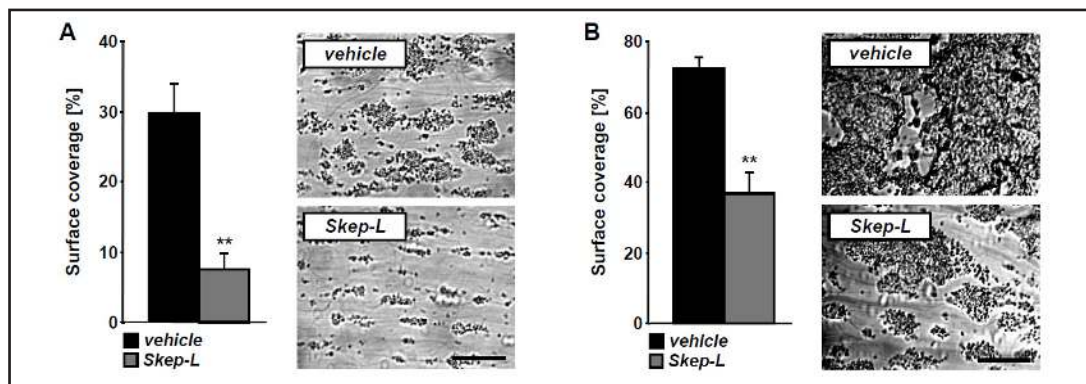


Fig. 5. Activation-dependent *in vitro* thrombus formation in absence and presence of Skepinone-L. A. Original phase contrast images (left) and arithmetic means \pm SEM (n = 6, right) of surface covering thrombi formed by adherent platelets following perfusion of whole blood over a collagen-coated surface for 5 minutes at high arterial shear rates (1700 s^{-1}) in absence (black bar) and presence (grey bar) of Skepinone-L ($1 \mu\text{M}$). Scale bar equals $50 \mu\text{m}$. **($p < 0.01$) indicates significant difference between pretreatment with Skepinone-L or vehicle (DMSO). B. Original phase contrast images (left) and arithmetic means \pm SEM (n = 6, right) of surface covering thrombi formed by adherent platelets following perfusion of whole blood over a collagen-coated surface for 5 minutes at low arterial shear rates (500 s^{-1}) in absence (black bar) and presence (grey bar) of Skepinone-L ($1 \mu\text{M}$). Scale bar equals $50 \mu\text{m}$. **($p < 0.01$) indicates significant difference between pretreatment with Skepinone-L or vehicle (DMSO).

selectivity inhibitors *in vitro* and especially *in vivo* has hampered investigation of p38 MAPK signaling pathways in platelet activation and pathological thrombus formation. The novel dibenzosuberone-type p38 MAPK inhibitor Skepinone-L shows outstanding selectivity and high *in vivo* potency [25].

The observations of the present study using Skepinone-L reveal a decisive role of p38 MAPK-dependent signaling in platelet secretion, aggregation as well as adhesion and thrombus formation under different shear rates. Moreover, p38 MAPK-dependent platelet cytosolic phospholipase A₂ (cPLA₂) activation (by phosphorylation at Ser⁵⁰⁵) as well as thromboxane synthesis were found to be abrogated in the presence of Skepinone-L, whereas our studies with Skepinone-L indicate that p38 MAPK signaling is not involved in activation-dependent increases of platelet cytosolic Ca²⁺ activity.

Skepinone-L significantly decreased phosphorylation of Hsp27 in platelets after stimulation with different agonists (CRP, thrombin and U-46619). p38 MAPK can influence platelet degranulation and shape change since Hsp27 acts as a potent modulator of actin cytoskeleton in platelets [32, 33]. Indeed, platelet adhesion and spreading on collagen has already been shown to be critically regulated via p38-dependent signaling [18].

Furthermore activation-dependent release of platelet dense granules (ATP) was significantly diminished after pretreatment with Skepinone-L, an effect which was overcome by increasing concentrations of CRP and thrombin (Fig. 2 A and B). In contrast, degranulation following activation with thromboxane A₂ analogue U-46619 was still significantly impaired by Skepinone-L even in submaximal agonist concentrations (Fig. 2 A and B).

According to the present observations, Skepinone-L does not appreciably alter the stimulation-dependent increase of [Ca²⁺]_i following addition of threshold CRP, thrombin or TXA₂ concentrations indicating that activation-dependent Ca²⁺ influx in platelets is not critically dependent on p38 MAPK-activity, a finding comparable to the results found with more unselective p38 MAPK inhibitors [15, 21].

According to our studies with Skepinone-L and in agreement with previous findings [8, 21] p38 MAPK-dependent signaling seems to be involved in platelet aggregation induced by threshold concentrations of CRP and thrombin (Fig. 2 C). While Skepinone-L-dependent reduction of platelet aggregation following treatment with threshold concentrations of CRP and thrombin was overcome by increasing agonist concentrations, thromboxane A₂ (TXA₂)-

triggered platelet aggregation studies show a substantial defect in the presence of Skepinone-L, even at increasing agonist concentrations of the second wave platelet activator TXA₂ up to submaximal concentrations of 0.5 μM (Fig. 2 C). These results indicate that high doses of CRP or thrombin induce further intracellular signaling that bypasses p38 MAPK dependent signaling similar to the results found for platelet degranulation. TXA₂ is known to be required for collagen- and thrombin-induced platelet activation in response to threshold concentrations [3, 34]. p38 MAPK stimulates TXA₂ synthesis upon exposure to threshold concentrations of thrombin or collagen amplifying the initially low platelet response. Moreover, TXA₂ induces further activation of p38 MAPK resulting in a feedback mechanism of platelet activation. High concentrations of collagen (CRP) or thrombin do not require the amplifying mechanism of p38-driven secondary TXA₂ formation for sufficient platelet aggregation.

In addition to its influence on platelet secretion and aggregation, p38 MAPK has been shown to participate in the triggering of platelet phosphatidylserine exposure [35] and agonist-induced bleb formation [33]. The effect on bleb formation but not on phosphatidylserine exposure involves activation of the protease calpain [33]. According to an earlier study von Willebrand factor (VWF)-dependent platelet phosphatidylserine exposure and microparticle formation was paralleled by p38 MAPK activation and bleb formation, but not phosphatidylserine exposure, was abrogated by pharmacological p38 MAPK inhibition with SB203580 [36]. Thus, the experimental evidence addressing the role of p38 MAPK in cell membrane scrambling and membrane blebbing of platelets is conflicting. Notably, p38 MAPK is involved in the triggering of cell membrane scrambling and suicidal death of other cell types, such as erythrocytes [37, 38] and diverse nucleated cells [39-48].

Rupture of an atherosclerotic lesion with endothelial denudation exposes circulating platelets to thrombogenic subendothelial collagen and thus recruits platelets to the injured vessel wall [49]. Inhibition of p38 MAPK by Skepinone-L (1 μM) may decrease the risk of collagen-triggered thrombus formation under low (500^{-s}) and high (1700^{-s}) arterial shear stress indicating that p38 MAPK is critically involved in different pathways initiating platelet induced thrombus formation. Thus, inhibition of p38 MAPK may be a therapeutic option in the prevention of thrombotic complications. Former *in vivo* studies in p38 MAPK heterozygous mice already provided first evidence that p38 MAPK seems to be involved in thrombus formation since Fe₃Cl-induced vascular injury of carotid artery results in delayed occlusion time whereas bleeding time of those mice was unaffected [23].

In conclusion, the present observations reveal that the highly potent and selective p38 MAP kinase inhibitor Skepinone-L blunts platelet secretion, aggregation and thrombus formation. Thus, Skepinone-L may prove useful in the treatment of thrombosis. Furthermore this study reports for the first time that selective and potent inhibition of p38 MAPK by Skepinone-L can reduce thrombus formation under different shear rates. Further studies are now necessary to examine the *in vivo* potency of Skepinone-L in the protection against arterial thrombosis *in vivo*. At this stage Skepinone-L could be considered a promising drug in future antiplatelet therapy.

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