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Translational regulation of the serum- and glucocorticoid-inducible kinase-1 (SGK1) in platelets

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ABSTRACT

Activation of platelets by thrombin opens pore forming channel protein Orai1 with subsequent store operated Ca^{2+} entry (SOCE) and Ca^{2+} dependent platelet granule release, integrin $\alpha_{IIb}\beta_3$ activation, adhesion, aggregation and thrombus formation. Orai1 and thus SOCE as well as platelet activation are upregulated by the serum- and glucocorticoid-inducible kinase-1 (SGK1), which transcriptionally regulates Orai1 expression in megakaryocytes and thus determines Orai1 protein abundance in mature, circulating platelets. As platelets are devoid of nuclei, they are unable to modify protein abundance by regulation of transcription. However, they contain mRNA and thus could express novel protein by stimulation of protein translation. Translation is sensitive to actin polymerization and phosphoinositide-3-kinase (PI3K). Translational regulation of SGK1 expression has never been described before. The present study thus explored whether thrombin regulates SGK1 expression in platelets. As a result, according to RT-PCR mRNA encoding SGK1 is present in circulating platelets and significantly decreased by activation of platelets with thrombin (1 U/ml). The protein abundance of SGK1 is significantly enhanced by thrombin treatment, an effect significantly decreased by inhibition of translation with puromycin (100 nM) but not by inhibition of transcription with actinomycin (4 μg/ml). The increase of SGK1 protein abundance is blunted by inhibition of PI3K with wortmannin (100 nM) or LY294002 (25 μM), and by disruption of the cytoskeleton with cytochalasin B (1 μM). In conclusion, activation of platelets with thrombin stimulates the translation of SGK1.

1. Introduction

Platelets play a pivotal role in primary hemostasis following vascular injury and by the same token are main players in the development of acute thrombotic occlusion during myocardial infarction and ischemic stroke [1].

One of the most potent activators of platelets is thrombin, which triggers platelet granule release, integrin $\alpha_{IIb}\beta_3$ activation, adhesion, aggregation and thrombus formation [2]. All those consequences of platelet activation require an increase of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) [3,4], which is accomplished by inositol-1,4,5-trisphosphate (IP_3)-mediated Ca^{2+} release from intracellular stores, intracellular store depletion and subsequent stimulation of store operated calcium entry (SOCE) across the plasma membrane [5]. SOCE is accomplished by Orai1, a pore forming cell membrane protein [6,7] and its regulator stromal interaction molecule 1 (STIM1) [8], which senses the Ca^{2+} content of the intracellular stores in platelet sarcoplasmatic reticulum [9]. Both, Orai1 and STIM1 are essential for the function of platelets [9–11].

Most recently, Orai1 protein abundance and activity have been shown to be under powerful regulation of the serum- and glucocorticoid-inducible kinase 1 (SGK1) [12,13], which has originally been cloned as a glucocorticoid sensitive gene [14], but later shown to be regulated by a variety of hormones and other triggers [15], including thrombin [16] and TGF-β [17]. SGK1 is activated by phosphorylation at ^{422}Ser and ^{256}Thr through the PI3K signaling cascade [18], which is a powerful regulator of platelet function [19–21]. SGK1 regulates a wide variety of carriers and further ion channels including the epithelial Ca^{2+} channels TRPV5 and TRPV6 [15,22].

Circulating platelets lost their nuclei and are unable to transcribe novel protein [23]. On the other hand, platelets still harbour pre-mRNA and mRNA and are thus able to translate mRNA into proteins [24–26]. The translation is sensitive to phosphatidylinositide-3-kinase (PI3K) [26] and cytoskeletal organization [27].

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The present study explored whether activation of platelets by thrombin modifies the expression of SGK1 protein and elucidated the involvement of PI3K and cytoskeleton. To this end, mRNA abundance has been determined by RT-PCR and protein abundance by Western blotting in platelets prior to and following stimulation with thrombin. Experiments were performed in the absence or presence of PI3K inhibitors wortmannin, LY294002 or of cytochalasin B, an inhibitor of actin polymerization [28].

2. Materials and methods

2.1. Preparation of human platelets

Human platelets were isolated as described previously [29]. Blood from healthy volunteers was collected in ACD-buffer and centrifuged at 200 g for 20 min. The obtained platelet-rich plasma was added to modified Tyrode-HEPES (*N*-2-hydroxyethyl-piperazone-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 min and removal of the supernatant, the resulting platelet pellet was resuspended in Tyrode-HEPES buffer (pH 7.4, supplemented with 1 mM $CaCl_2$). Platelets were activated using 1 U/ml thrombin (Roche, Basel Switzerland).

2.2. RT-PCR analysis

To determine SGK1 mRNA abundance in human platelets RNA was extracted using Trizol reagent (Invitrogen) according to manufacturer's instructions. Subsequently 500 ng of total RNA was reverse transcribed to cDNA using random hexamer primers (0.1 mM, Roche), 1st strand buffer (Invitrogen), DTT (10 mM, Invitrogen) and SuperScript II Reverse Transcriptase (200 U, Invitrogen) for 1 h at 42 °C. Then RNase H (Roche) was added and the samples were incubated for additional 20 min at 37 °C. Quantitative real-time PCR was applied on the CFX96 Real-Time System® C1000 Thermal Cycler (Biorad) using the following primer pairs (5′-3′ orientation): SGK1 forward ATGAGATGCTGTATGGCCTG; SGK1 reverse AGCTGGAGAGGCTTGTTCA. For comparison the transcript levels of the house-keeping gene *gapdh* were determined for each sample using the following primers (5′–3′ orientation): forward ATGAC AACTTTGGCATCGTG and reverse GAATGGGAGTTGCTGTTGAAG.

2.3. Western blot analysis

Fresh isolated human platelets were stimulated and incubated at 37 °C and centrifuged for 5 min at 900 g. The pellet was resuspended in lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Trion-X, 0.5% Na_2HPO_4, 0.4% β-Mercaptoethanol) containing protease inhibitor cocktail (Roche) for 30 min on ice. After centrifugation for 15 min at 15,000*g* and 4 °C the supernatant was taken for western blot analysis.

The protein concentration was measured with Bradford (Biorad) and 50–100 μg of protein was taken for each sample. Roti®-Load1 (Roth) was added and the samples were boiled for 10 min at 95 °C. For immunoblotting proteins were electrotransferred onto a PVDF membrane and blocked with 5% milk in TBS-0.1% Tween 20 (TBST) at room temperature for 1 h. The membrane was incubated with the primary antibody against β-actin (1:1000, Cell Signaling), SGK1 (1:200, Pineda) at 4 °C overnight. After washing with TBST the blots were incubated with secondary antibody conjugated with horse radish peroxidase (HRP) (1:1000, Cell Signaling) for 1 h at room temperature. After washing antibody binding was detected with the ECL detection reagent (Amersham). Bands were quantified with Quantity One Software (Biorad).

2.4. Statistical analysis

As indicated, data are provided as means ± SEM; *n* represents the number of independent experiments. All data were tested for significance using ANOVA Dunnett's test. Only results with $^{*}p < 0.05$, $^{**}p < 0.01$ or $^{***}p < 0.001$ were considered statistically significant.

3. Results

Reverse transcription polymerase chain reaction (RT-PCR) was employed to determine the abundance of mRNA encoding the serum- and glucocorticoid-inducible kinase 1 (SGK1). As illustrated in Fig. 1A, platelets expressed mRNA encoding SGK1. Activation of platelets with thrombin (1 U/ml) was followed by a rapid decrease of SGK1 transcript levels within 15–60 min.

Western blotting revealed that platelets express SGK1 protein. Activation of platelets with thrombin (1 U/ml) was followed by a significant increase of SGK1 protein abundance (Fig. 1B and C) within the same time as the transcipt levels, shown in Fig. 1A,

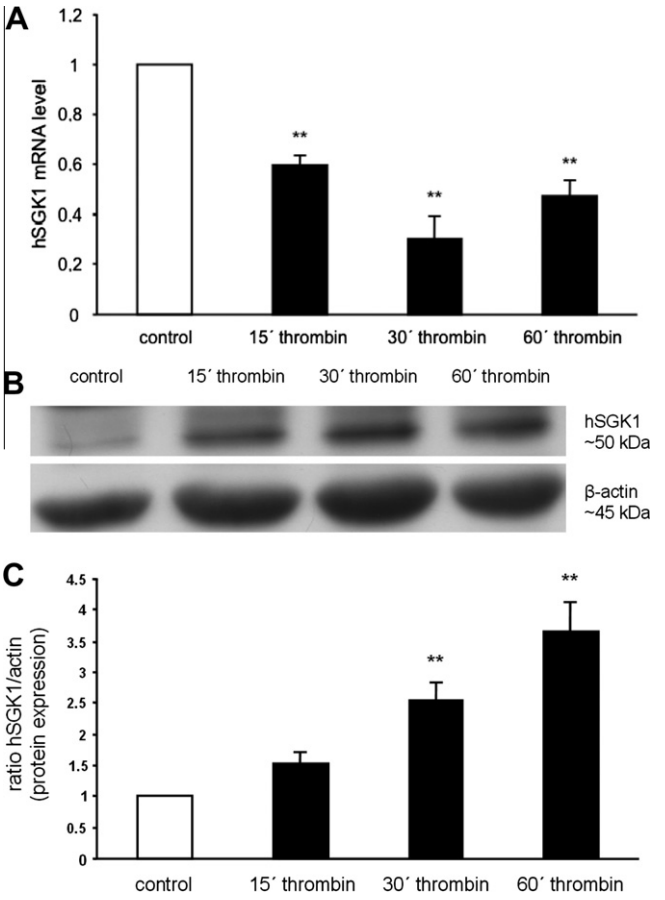

Fig. 1. Effect of thrombin on SGK1 mRNA and SGK1 protein abundance in platelets. (A) Arithmetic mean ± SEM ($n = 4–9$) of SGK1 mRNA abundance in human platelets prior to (white bar) or following administration of 1 U/ml thrombin (black bars) for the indicated time periods. Data are expressed in % of the value prior to thrombin treatment. $^{**}p < 0.01$ indicates statistically significant difference to value prior to thrombin administration. (B) Original Western blot of SGK1 protein abundance in human platelets prior to (control) and following (15′–60′ thrombin) administration of thrombin (1 U/ml) for the indicated time periods. (C) Arithmetic mean ± SEM ($n = 7–10$) of SGK1 protein abundance in human platelets prior to (white bar) and following (black bars) administration of thrombin (1 U/ml) for the indicated time periods. Data are expressed in fractions of the value prior to thrombin treatment. $^{**}p < 0.01$ indicates statistically significant difference to value prior to thrombin administration.

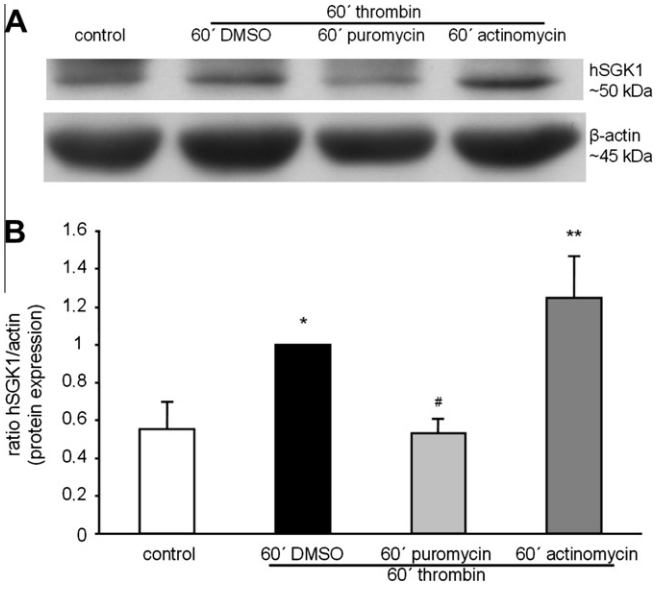

Fig. 2. Influence of inhibition of transcription or translation on SGK1 protein abundance in platelets. (A) Original Western blot of SGK1 protein abundance in human platelets prior to (control) and 60 min following administration of thrombin (1 U/ml) in the presence of puromycin (100 nM), actinomycin (4 μg/ml) or DMSO as solvent control for the indicated time periods. (B) Arithmetic mean ± SEM (n = 6–8) of SGK1 protein abundance in human platelets prior to (white bar) and 60 min following (black and gray bars) administration of thrombin (1 U/ml) in presence of puromycin (100 nM, light gray bar), actinomycin (4 μg/ml, dark gray bar) or DMSO as solvent control (black bar) for the indicated time periods. Data are expressed in fractions of the value in the presence of thrombin with DMSO alone. $^{*}p < 0.05$ and $^{**}p < 0.01$ indicate statistically significant difference to value prior to thrombin administration, $^{\#}p < 0.05$ indicates statistically significant difference to value in the presence of thrombin (1 U/ml) compared to DMSO solvent control.

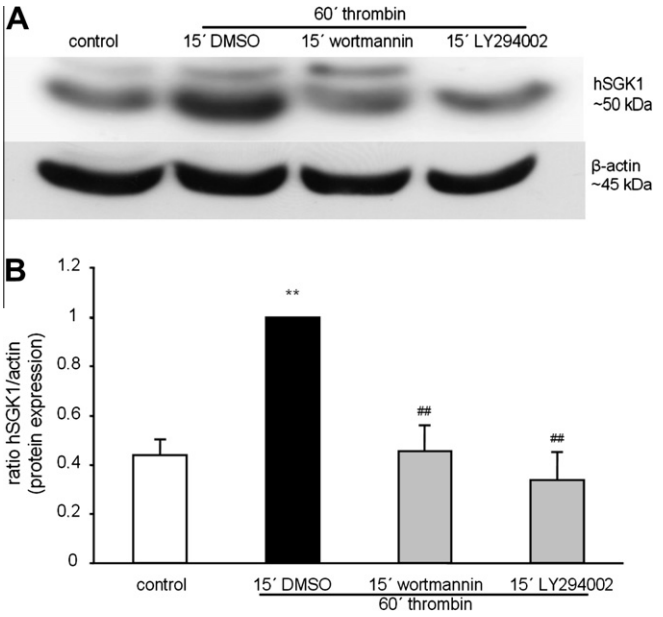

Fig. 3. Effect of PI3K inhibition on SGK1 protein abundance in platelets. (A) Original Western blot of SGK1 protein abundance in human platelets prior to (control) and 60 min following administration of thrombin (1 U/ml) in the presence of wortmannin (100 nM), LY294002 (25 μM) or DMSO as solvent control for the indicated time periods. (B) Arithmetic mean ± SEM (n = 6–7) of SGK1 protein abundance in human platelets prior to (white bar) and following (black and gray bars) administration of thrombin (1 U/ml) for the indicated time periods in the presence of wortmannin (100 nM, gray bar), LY294002 (25 μM, gray bar) or DMSO as solvent control (black bar). Data are expressed in fractions of the value in the presence of thrombin with DMSO alone. $^{**}p < 0.01$ indicates statistically significant difference to value prior to thrombin administration, $^{\#\#}p < 0.01$ indicates statistically significant difference to value in the presence of thrombin (1 U/ml) compared to DMSO solvent control.

decreased. The formation of SGK1 protein occurred despite a decline of transcript levels and was thus not the result of enhanced SGK1 transcription. Accordingly, disruption of transcription with actinomycin (4 μg/ml) was without effect on the increase of SGK1 protein abundance (Fig. 2). In contrast, inhibition of translation by puromycin (100 nM) significantly blunted the increase of SGK1 protein abundance following thrombin treatment (Fig. 2).

Additional experiments were performed to elucidate the involvement of phosphoinositide-3-kinase (PI3K) in the regulation of SGK1 translation. As illustrated in Fig. 3, inhibition of PI3K with wortmannin (100 nM) or LY294002 (25 μM) abrogated the stimulation of SGK1 protein expression by thrombin (60 min, 1 U/ml).

A further series of experiments elucidated the role of cytoskeleton. As shown in Fig. 4, pretreatment of platelets with cytochalasin B (1 μM) again prevented the up-regulation of SGK1 protein abundance by thrombin (60 min, 1 U/ml).

4. Discussion

The present study unravels the translational up-regulation of the serum- and glucocorticoid-inducible kinase 1 (SGK1) following platelet activation by thrombin. The up-regulation results from stimulation of protein translation, which apparently depends on cytoskeletal rearrangement and activation of the phosphoinositide-3-kinase (PI3K) cascade. PI3K is critically important for platelet activation [30].

Most recent experiments revealed that SGK1 is a powerful stimulator of Orai1 protein abundance [13] and up-regulates expression of the channel in several cell types [31] including megakaryocytes [12]. Accordingly, Orai1 protein abundance is significantly decreased and Orai1 dependent platelet function is significantly impaired in gene targeted mice lacking functional SGK1 ($sgk1^{-/-}$) [12]. SGK1 is effective by both, inhibition of channel protein degradation [13] and up-regulation of Orai1 transcription [31]. The inhibition of Orai1 protein degradation results from inhibition of the ubiquitin ligase Nedd4-2 [13], the stimulation of Orai1 protein expression from activation of the transcription factor NF-κB [31].

In large part due to decreased Orai-1 transcription in $sgk1^{-/-}$ megakaryocytes [12], platelet degranulation and integrin $\alpha_{IIb}\beta_3$ activation in response to low doses of collagen-related peptide (CRP), convulxin or G-protein-coupled receptor (GPCR) agonists thrombin and ADP are impaired in circulating $sgk1^{-/-}$ platelets [12]. Moreover, SGK1 deficiency impaired platelet tethering, adhesion and thrombus formation [12].

The present observations illustrate that activation of platelets up-regulates the expression of SGK1, thus boosting the influence of this powerful regulator on platelet function. Translation in platelets is regulated by the translation initiation factors eIF-4E and eIF-2alpha [32,33], which are under control of cytoskeleton and attachment to the inhibitory 4E binding protein (4E-BP1) [32]. In unstimulated platelets mRNA is primarily associated with the cytoskeletal core, whereas eIF-4E is localized in the membrane skeleton and soluble fraction of platelets separate from most mRNAs [32]. Upon activation of the platelets, the translation initiation factors are redistributed to mRNA-rich areas leading to subsequent stimulation of protein synthesis [32]. In resting platelets, eIF-4E is bound to 4E-BP1 and thus prevented to initiate translation [26]. The binding is disrupted and thus translation fostered by rapamycin sensitive and phosphatidylinositide-3-kinase (PI3K) dependent phosphorylation of 4E-BP1 [26]. Upon activation PI3K associates with the cell membrane skeleton [27].

Despite the importance of platelets for hemostasis [34] and despite the powerful up-regulation of Orai1 protein abundance, Ca^{2+}

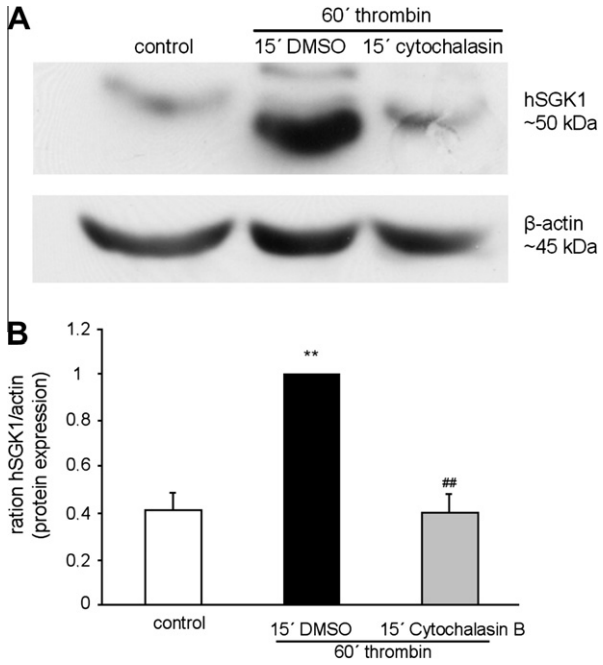

Fig. 4. Influence of actin depolymerization on SGK1 protein abundance in platelets. (A) Original Western blot of SGK1 protein abundance in human platelets prior to (control) and 60 min following administration of thrombin (1 U/ml) in the presence of cytochalasin B (1 μM) or DMSO as solvent control added 15 min prior to thrombin. (B) Arithmetic mean ± SEM ($n = 5$) of SGK1 protein abundance in human platelets prior to (white bar) and following (black and gray bars) administration of thrombin (1 U/ml) for 60 min with prior (15 min) treatment with cytochalasin B (1 μM, gray bar) or DMSO as solvent control (black bar). Data are expressed in fractions of the value in the presence of thrombin with DMSO alone. $^{**}p < 0.01$ indicates statistically significant difference to value prior to thrombin administration, $^{\#\#}p < 0.01$ indicates statistically significant difference to value in the presence of thrombin (1 U/ml) compared to DMSO solvent control.

entry and platelet activation by SGK1 [12], SGK1 deficient mice do not suffer from striking bleeding abnormalities contrasting the severe phenotype of the Orai deficient mice [10]. Thus, SGK1 insensitive residual Orai1 abundance is in SGK1 deficient mice sufficient to maintain basic platelet function.

Since SGK1 is mainly effective through activating NF-κB with subsequent upregulation of Orai-1 transcription in megakaryocytes [12], the stimulation of SGK1 translation in circulating platelets is presumably too late to significantly affect Orai1 protein abundance during platelet activation. Nevertheless, SGK1 phosphorylates and thus regulates a wide variety of proteins [15] and SGK1 may regulate platelet function not only by modifying Orai1 protein abundance. In any case, the present observations reveal a novel mechanism of SGK1 protein regulation, i.e. the stimulation of SGK1 translation. To the best of our knowledge, regulation of SGK1 translation has never been shown before. According to the present observations, SGK1 translation is sensitive to both PI3K signaling and cytoskeletal architecture. Presumably, SGK1 translation is similarly regulated in nucleated cells, where, however, the regulation of translation is masked by simultaneous regulation of transcription.

In conclusion, activation of platelets by thrombin leads to upregulation of SGK1 and Orai1 protein expression, an effect expected to further enhance activation-dependent Ca^{2+} entry into platelets with subsequent Ca^{2+}-dependent degranulation, adhesion, aggregation and thrombus formation.

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