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The regulator of G-protein signaling 18 regulates platelet aggregation, hemostasis and thrombosis

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ABSTRACT

Regulators of G protein signaling (RGS) proteins are known to interact with and negatively regulate/turn-off G protein activation. RGS18 is identified as an R4 subfamily member of this family with specific expression in hematopoietic progenitors, myeloerythroid cells, megakaryocytes and platelets. Studies focused on understanding its function in platelet biology have been limited, in part, due to lack of pharmacological inhibitors. Thus, the present study investigated the function of RGS18 in platelets, using the RGS18 knockout mouse model ($RGS18^{-/-}$). We identified phenotypic differences between $RGS18^{-/-}$ and wild-type (WT) mice, and show that RGS18 plays a significant role in hemostasis and thrombosis. Hence, RGS18 deficiency markedly shortened bleeding as well as occlusion times (*in vivo*). Furthermore, $RGS18^{-/-}$ platelets displayed hyper-responsiveness with regards to agonist induced aggregation (*in vitro*). This gain of function phenotype may serve as the mechanism or explain, at least in part, the enhanced hemostasis and thrombosis phenotype observed in the RGS18 deletion mice. Collectively, our findings provide valuable insight and highlight a critical and direct role for RGS18 in modulating platelet function.

1. Introduction

Platelets, one of the blood cells, are essential for normal hemostasis, but "tight" regulation is required to avoid the destructive effects of either inappropriate activation or excessive responses to injury. Platelets, just like many other cellular systems, utilize G-Protein Coupled Receptors (GPCRs) for regulation of or mediating their signal transduction. For instance, responses for most platelet agonists are mediated by GPCRs, giving rise to the intracellular events that trigger platelet aggregation and secretion [1]. Thus, physiological agonists activate their respective GPCRs on platelet surface, which will in turn engage their specific G protein (GP) to induce a host of cellular responses: calcium release (i.e., G_q), inhibition of adenylate cyclase (i.e., G_i), and activation of Rho (i.e., G_{13}), amongst others. On the other hand, prostaglandin I_2 (PGI_2)-mediated activation of adenylate cyclase (i.e., Gs) leads to cAMP elevation, and subsequently inhibition of platelet activation. Taken together, it is clear that GP-dependent signaling is heavily involved in modulating platelet activation. Moreover, despite the fact that a good deal of information is known about extrinsic regulators of platelet function like nitric oxide and PGI_2, considerably much less is known regarding the intrinsic mechanisms designed to avert "excessively" robust platelet activation after vascular injury. On this basis, (intrinsic) mechanisms regulating GP signaling has been under investigation. Of these mechanisms, regulators of G protein signaling (RGS proteins), which are known to interact with and negatively regulate (turn-off) GP activation/function by acting as GTPase activating proteins, and driving them into their inactive GDP form, (interestingly) seem to have received the least amount of attention. Nonetheless, of the RGS family members found to be present in platelets [2–4], studies [2,5] have shown that the expression rate is highest for RGS18. Furthermore, studies in megakaryocytes/platelets revealed that RGS18 binds both G_q and G_i proteins [2,6]; suggesting that it controls the duration of GPCR responses in these cells [2,7,8]. RGS18 was also found to be regulated by phosphorylation and dephosphorylation at specific serine residues under agonist activating conditions [9,10]. Interestingly, platelet inhibitory agents were also found to modulate RGS18 phosphorylation [7,9,10]. And more recently, evidence was

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obtained in support of the notion that RGS18 serves as a context dependent modulator of platelet function [11]. Despite this apparent progress in understanding the role of RGS18 in platelet function, whether it plays a "direct" role in their activation (e.g., aggregation) or contributes to the genesis of thrombosis is still not known. For the most part, this has been attributed to lack of proper pharmacological inhibitors for RGS18.

Based on the aforementioned considerations, we undertook a genetic approach, namely, employed the RGS18 knockout ($RGS18^{-/-}$) mice and investigated its role in platelet function. Thus, in the present manuscript, we first demonstrated the successful deletion of RGS18 in platelets. Next, we found that, relative to WT littermates, $RGS18^{-/-}$ mice exhibited shortened time for occlusion in an injury-based thrombosis model, as well as shortened tail bleeding time. Finally, we observed that RGS18 deletion resulted in a significantly enhanced platelet aggregation response, relative to the WT control. Together, our findings support the notion that RGS18 does indeed play a critical role in thrombogenesis and hemostasis, by regulating platelet activation/aggregation.

2. Materials and methods

2.1. Reagents and materials

Thrombin, stir bars and other disposables were from Chrono-Log (Havertown, PA). Other reagents were of analytical grade.

2.2. Animals

The RGS18 mice and Mouse Contract Services were provided by the Mary Lyon Centre at MRC Harwell (www.har.mrc.ac.uk), Oxfordshire, United Kingdom. Mice were housed in groups of 1–4 at 24 °C, under 12/12 light/dark cycles, with access to water and food ad libitum. All experiments involving animals were performed in compliance with the institutional guidelines, and were approved by the Western University of Health Sciences Institutional Animal Care and Use Committee.

2.3. Genotyping

RGS18 deletion mice were generated as a single nucleotide polymorphism (SNP) by the chemically induced (ENU) mutation, Rgs18m1H, is a premature stop codon (Y139STOP) which is a T to A (TAT to TAA) mutation in exon 4 of the mouse RGS18 gene and were genotyped using a PCR- and enzyme digestion-based method. PCR was performed using following primers: sense: 5′-TTGGTAGATGGAGTGGATGC-3′ and antisense 5′-GTCCACTTTGGTCAAAGCAG-3′ with the following PCR condition: denaturation: 95 °C, 3 min, amplification: 95°C- 30 s, 55°C- 30 s, 68°C-60 s for 34 cycle and finally extension for 72 °C for 5 min. After completion of PCR cycle, amplified DNA were digested by using restriction enzyme DdeI for 1 h at 37 °C. Digested DNA were run in 2% agarose gel and visualized in the gel documentation system. The following products were observed: WT: 243 bp, Het: 243/138/105 bp, and mutant: 138/105 bp.

2.4. Platelets preparation

Mouse blood was collected from a ventricle and the citrated (0.38%) blood was mixed with phosphate-buffered saline, pH 7.4, and was incubated with PGI_2 (10 ng/mL; 5 min), followed by centrifugation at 237× *g* for 10 min at room temperature (RT). Platelet-rich plasma (PRP) was recovered and platelets were pelleted at 483 × *g* for 10 min at RT. The pellets were resuspended in HEPES/Tyrode buffer (HT; 20 mM HEPES/KOH, pH 6.5, 128 mM NaCl, 2.8 mM KCl, 1 mM $MgCl_2$, 0.4 mM NaH_2PO_4, 12 mM $NaHCO_3$, 5 mM D-glucose) supplemented with 1 mM EGTA, 0.37 U/mL apyrase, and 10 ng/mL PGI_2. Platelets were washed and resuspended in HT (pH 7.4) without EGTA, apyrase, or PGI_2. Platelets were counted with an automated hematology analyzer (Drew Scientific Dallas, TX) and adjusted to the indicated concentrations.

2.5. Tail bleeding time

RGS18 WT and KO mice were used for the tail bleeding assay. Hemostasis was examined using the tail transection technique [12–15]. Briefly, mice were anesthetized with isoflurane and place on a 37 °C homeothermic blanket and their tails were transected 5 mm from the tip. The tail was placed in saline at 37 °C and the time to blood flow cessation was measured. Clotting was not considered complete until bleeding had stopped for 1 min. When required, measurements were terminated at 15 min.

2.6. In vitro platelet aggregation

RGS18 WT and KO PRP were stimulated with the following agonists: 0.5 μM U46619 and 0.025–0.1 U/ml thrombin. Platelet aggregation was measured by the turbidometric method using models 490 or 700 aggregometry systems (Chrono-Log Corporation, Havertown, PA). Each experiment was repeated at least 3 times and blood was pooled from at least three separate groups of eight mice.

2.7. In vivo thrombosis model

These studies were performed as described previously [12,14,15]. Briefly, RGS18 WT and KO mice 8–10 weeks old were anesthetized with isoflurane. Then, the left carotid artery was exposed and cleaned, and baseline carotid artery blood flow was measured with Transonic micro-flowprobe (0.5 mm, Transonic Systems Inc., Ithaca, NY). After stabilization of blood flow, 7.5% ferric chloride ($FeCl_3$) was applied to a filter paper disc (1-mm diameter) that was immediately placed on top of the artery for 3 min. Blood flow was continuously monitored for 45 min, or until blood flow reached stable occlusion (zero blood flow for 2 min). Data was recorded and time to vessel occlusion was calculated as the difference in time between stable occlusion and removal of the filter paper (with $FeCl_3$). An occlusion time of 45 min was considered as the cut-off time for the purpose of statistical analysis.

2.8. Statistical analysis

All experiments were performed at least three times. Analysis of the data was performed using GraphPad PRISM statistical software (San Diego, CA) and presented as mean ± SEM. The Mann–Whitney test was used for the evaluation of differences in mean occlusion and bleeding times. Analysis was also conducted using t-test, and similar results were obtained. Significance was accepted at $P < 0.05$ (two-tailed P value), unless stated otherwise.

3. Results

3.1. Confirmation of RGS18 protein deletion

To dissect the role of RGS18 in platelets, we employed RGS-specific knockout (KO) mouse line. We first sought to confirm the deletion of the RGS18 protein from these mice, by PCR and western blotting experiments. As can be seen (Fig. 1A and B), the deleted RGS gene and protein cannot be detected in the $RGS18^{-/-}$ mouse line. We have also confirmed that these deletions did not have any apparent impact on the protein expression level of other RGS proteins (RGS10 and RGS16) known to be expressed in platelets. It is

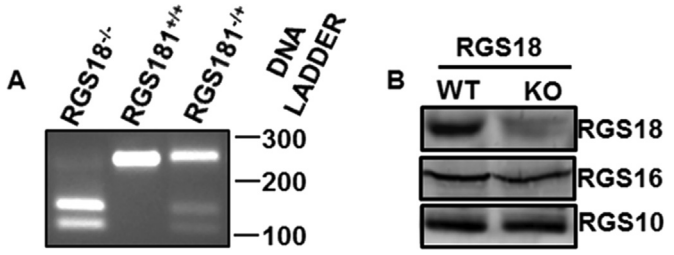

Fig. 1. Deletion of RGS18 from platelets. (A) DNA was isolated, PCR and digestion of DNA were performed as described in the "Methods" section. Digested DNA were separated in 2% agarose gel and visualized in the gel documentation system (B) Platelet extracts (2×10^8/mL) were prepared from wild type (WT) and Knockout (KO) mice and the indicated proteins were detected by Western blotting.

noteworthy that the RGS18 KO mice were viable and fertile, and appeared to be healthy, undistinguishable visibly from their WT littermates, and showed no physical abnormalities.

3.2. Effect of RGS18 deletion on physiological hemostasis

Thus far, virtually nothing is known regarding the direct role of RGS protein in platelet function *in vivo*. Nonetheless, recent evidence has shown that mice with an RGS-insensitive mutation for the Gi2α (G184S; i.e., blocks RGS-Gi2α interactions) exhibited enhanced thrombus formation [16]; suggesting that RGS proteins play an important role in modulating platelet function. However, such "guilt-by-association" experiments, which targeted interaction of all RGS proteins with Gi2α, do not necessarily provide evidence that RGS proteins play a direct role in these processes. To this end, we observed a significantly shortened bleeding time in the RGS18 KO animals, when compared to the wild-type (WT) littermates (Fig. 2). These data demonstrate that RGS proteins, namely RGS18 is indeed involved in physiological hemostasis, and suggest a prothrombotic phenotype in these KO mice.

3.3. Effect of RGS18 deletion on agonist-induced platelet function

Since bleeding time was enhanced in these animals, we next sought to determine the underlying mechanism and the involvement of platelets, in this phenotype. Thus, we studied platelet aggregation under agonist activation conditions. Indeed, we found that RGS18 KO platelets had an enhanced 0.025 U/ml thrombin-induced aggregation response (Fig. 3), when compared to the WT littermate platelets. Furthermore, this enhanced aggregation response was still evident at 0.1 U/ml thrombin. These data suggest a leftward shift in the dose–response for this agonist, in the absence of the RGS18 proteins. We also observed enhanced aggregation in response to the thromboxane receptor agonist U46619 (0.5 μM; Fig. 3). Together, these data clearly indicate that RGS proteins regulate platelet activation (i.e., stimulatory signaling pathways), and explain the enhanced hemostasis responses observed in the RGS18 KO mice.

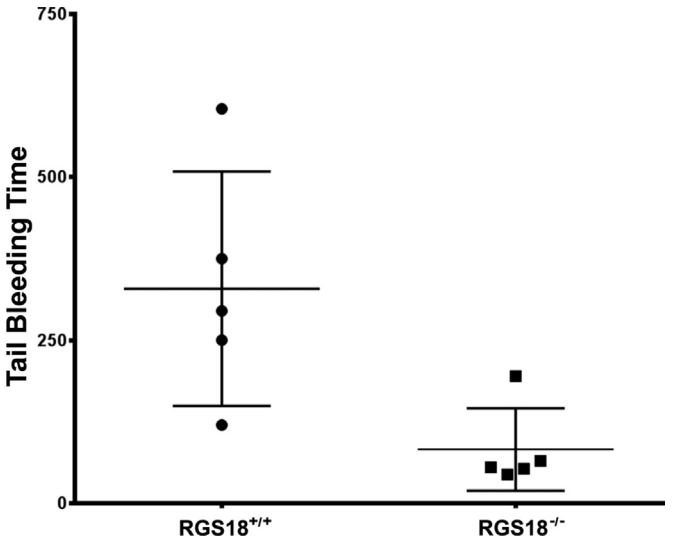

Fig. 2. Effect of RGS18 on bleeding time. RGS18 (WT and KO) mice were studied for tail bleeding time assay and conducted as described in the "Methods" section (*P < 0.01, Mann–Whitney test). Each point represents the bleeding time of a single animal (n = 5).

3.4. Effect of RGS18 deletion on thrombosis development

Whereas platelet activation is an integral part of hemostasis, increased aggregability of platelets is also a major causative factor in occlusive arterial thrombosis formation. Moreover, given the well-established role of platelet GPCR signaling in the pathogenesis of thromboembolic disorders, it is possible that RGS proteins may also contribute to such disease states. To this end, previous studies

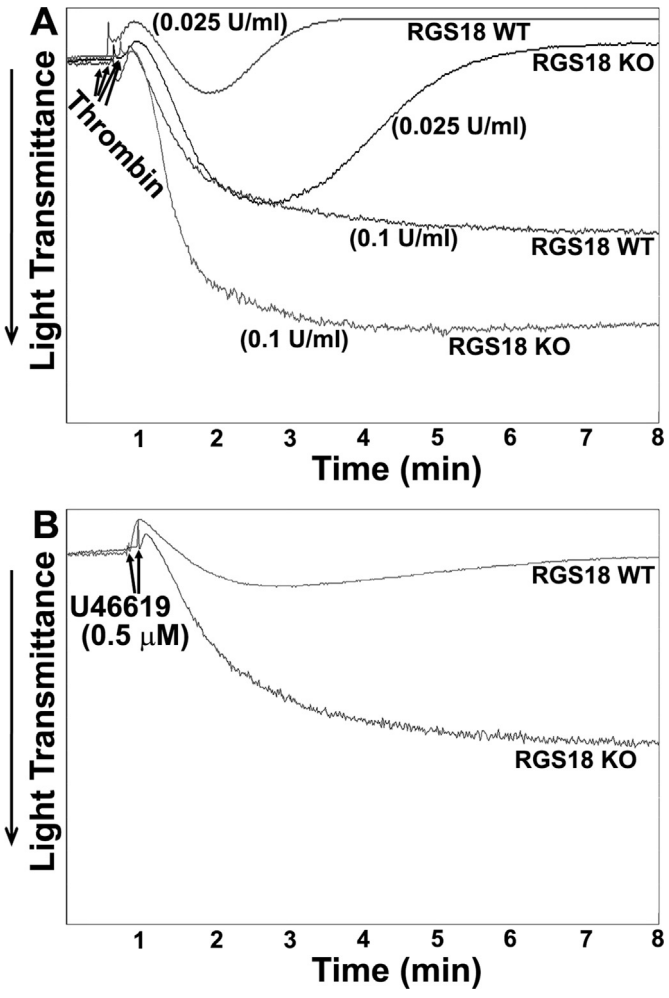

Fig. 3. Effect of RGS18 deletion on agonist-induced platelet aggregation. WT and KO platelets were prepared (2.5×10^8/ml), stimulated with (A) thrombin (0.025–0.1 U/mL) and (B) U46619 (0.5 μM). Platelet aggregation was measured with constant stirring. Each experiment was repeated at least 3 times and blood was pooled from at least three separate groups of eight mice.

with the RGS-insensitive Gi2α mutation did suggest that RGS proteins may be "associated" with thrombus formation. Nonetheless, these studies did not provide conclusive evidence that RGS proteins directly regulate thrombogenesis, nor did they identify which RGS protein(s) is involved. Thus, we addressed this issue by using our RGS18$^{-/-}$ animals, and observed a significantly shortened occlusion time in the $FeCl_3$ carotid artery thrombosis model, when compared to the wild-type (WT) littermates (Fig. 4). This finding demonstrates that RGS18 contributes to the genesis of thrombosis.

4. Discussion

Regulators of G protein Signaling (RGS) play essential regulatory roles in the signaling of G protein-coupled receptors (GPCRs) and display remarkable specificity and selectivity in their regulation of GPCR-mediated physiological events [3,17,18]. To this end, RGS18 is a GTPase-activating protein for the G-αq and G-αi subunits of GPs, thereby it turns off signaling by GPCRs known to couple to these G proteins [7,8]. RGS18 was first shown time to be expressed in megakaryocytes and platelets by Nagata et al. (2001), but not in lymphocytes or erythrocytes [6,19,20]. Moreover, it was previously shown [5] that the RGS18 protein is the most abundant member of the RGS family in platelets. However, thus far, studies regarding RGS18 function in platelets have been limited, and "associative" in nature. In fact, progress in this area has been hampered largely due to lack of specific pharmacological inhibitors. Nonetheless, recent studies have shown that RGS18 integrates activating and inhibitory signaling in platelets [20]. However, given the limitations of, and/or the nature of such association studies, whether RGS18 plays a direct role in platelet aggregation (*in vitro*) and thrombosis and hemostasis (*in vivo*) is still unknown. Based on these considerations, we undertook a genetic approach, by employing RGS18 deletion mice, and investigated the biology of RGS18 in the context of platelets. Our results revealed a gain of function phenotype in platelets, as a result of RGS18 deficiency. Thus, the dose–response curve for *in vitro* platelet aggregation induced by thrombin and U46619 was found to be shifted to the left. Of note, this notion is consistent with a report that single nucleotide polymorphisms in RGS18 is associated with enhanced platelet aggregation [21,22]. We also observed an *in vivo* phenotype in RGS18 deletion mice, namely enhanced occlusion time at sites of vascular injury as well as enhanced bleeding time. Collectively, our aforementioned observations underscore, at least in part, a direct functional relevance for RGS18 in platelets, and suggest that its physiological role involves limiting platelet activation and accumulation during thrombus formation.

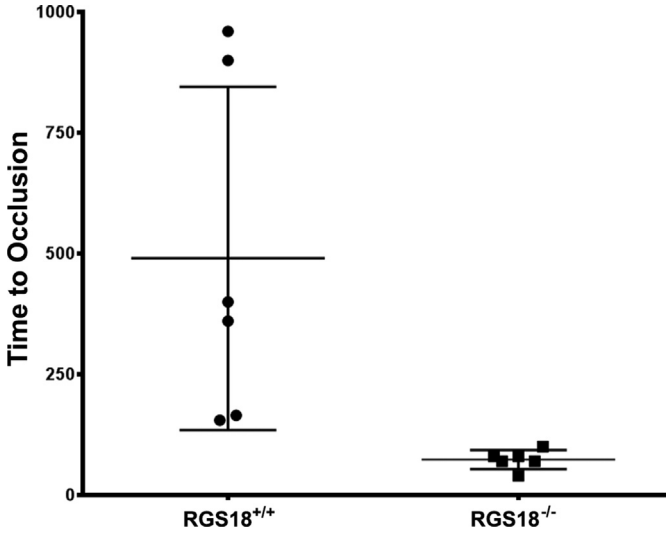

Fig. 4. Effect of RGS18 on thrombosis. RGS18 (WT and KO) mice were studied for $FeCl_3$ carotid artery thrombosis assay, which was conducted as described in the "Methods" section (***P < 0.002, Mann–Whitney test). Each point represents the occlusion time of a single animal (n = 6).

The present studies advance our understanding of the role of RGS proteins in platelet function. This understanding is important as for many G protein-coupled receptors, intense efforts to develop orthosteric drugs have failed to yield highly selective ligands. And in recent years, major advances have been made and established allosteric GPCR modulators as a novel approach to modulate the activity of this important class of drug targets [23–25]. In particular, these allosteric modulators have provided novel tools and drug leads for multiple receptors for which efforts aimed at discovery of orthosteric ligands had been unsuccessful. In essence, there is accumulating evidence highlighting RGS proteins as attractive targets for the development of potential future therapeutics that would control GPCR signaling in a tissue- or pathway-specific manner. As for RGS18's clinical perspective, while it is tempting to propose that enhancing its function could protect from cardiovascular or hematological diseases, what can be said at this point with some degree of assurance is that enhancers rather than inhibitors may be more useful in RGS18 drug development. Finally, it is important to note that there is ample data showing reductions in RGS protein expression or function in several pathophysiological states, and strategies to increase RGS function are now emerging [26].

Conflict of interest

None.

Disclosures

None.

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