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DREAM as a novel therapeutic target for anti-thrombotic agents

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Abstract

Circulating platelets participate in the process of numerous diseases including thrombosis, inflammation, and cancer. Thus, it is of great importance to understand the underlying mechanisms mediating platelet activation under disease conditions. Emerging evidence indicates that despite the lack of a nucleus, platelets possess molecules involved in gene transcription occurring in nucleated cells. This review will summarize downstream regulatory element antagonist modulator (DREAM), a transcriptional repressor, and highlight recent findings suggesting its novel non-transcriptional role in hemostasis and thrombosis.

Graphical abstract

Keywords

DREAM; platelet activation; thrombosis and hemostasis

1. Introduction

Platelet-mediated thrombogenesis is a critical component of cardiovascular disease, which is the leading cause of death in the U.S. Following vascular injury, circulating platelets adhere

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Conflict of interest

The author declares no competing financial interests.

to subendothelial matrix proteins such as collagen and von Willebrand factor through their receptors (1, 2). Adherent platelets release the contents of α - and dense-granules and produce thromboxane A₂ to recruit other platelets to the injury sites. Intracellular signaling induced by the interactions between ligands and receptors enhances the activity of a series of lipid and protein kinases, increases the cytosolic Ca²⁺ level, and activates α IIB β 3 integrin (3). Finally, binding of plasma fibrinogen to activated α IIB β 3 integrin mediates platelet aggregation and thrombus formation. In many cases, thrombotic and bleeding disorders result from abnormalities in signal transduction pathways during platelet activation. Thus, a better understanding of the molecular mechanisms mediating platelet activation will lead to the development of a novel therapy for the prevention and treatment of life-threatening bleeding and thrombosis.

Unlike nucleated cells, platelets have neither a nucleus nor genomic DNA. However, the anucleate cells have a variety of transcription factors including nuclear factor- κ B (NF- κ B) (4), signal transducer and activator of transcription 3 (STAT3) (5), steroid hormone receptors (6), retinoid X receptors (7), peroxisome proliferator-activated receptors (PPARs) (8), and farnesoid X receptor (9), which play non-genomic roles in regulating platelet function. For example, studies with pharmacological inhibitors suggested the importance of NF- κ B signaling in platelet functions including granule secretion, α IIB β 3 integrin activation, clot retraction, and thrombus formation (4, 10). Another study showed that platelet STAT3 acts as a scaffold protein for the interaction between Syk and phospholipase γ 2 after stimulation with collagen and that inhibition or deletion of STAT3 hampers platelet P-selectin exposure and aggregation induced by a low concentration of collagen or collagen-related peptides (CRP), a ligand for glycoprotein VI (GPVI), but not thrombin or ADP, a ligand for G-protein-coupled receptors (5). It was recently reported that farnesoid X receptor is present in human platelets and binding of its ligands to the receptor regulates platelet activation and α IIB β 3 integrin function (9). Moreover, a previous report that platelet-derived microparticles containing the PPAR γ -retinoid X receptor complex induce PPAR γ -mediated signaling in a monocytic cell line after internalization (11) suggests that transcription factors in platelets may have a paracrine effect on gene transcription in other nucleated cells. We have recently demonstrated that downstream regulatory element antagonist modulator (DREAM), a transcriptional repressor, is expressed in human and mouse platelets and a human megakaryocytic cell line (12). This review will summarize the known functions of DREAM in nucleated cells and discuss the novel regulatory mechanism of DREAM in platelet activation and its contributions to hemostasis and thrombosis.

2. DREAM

DREAM was identified as a neuronal Ca²⁺-sensing protein that binds to the DRE motif on DNA as a tetramer (Figure 1). DREAM is also named Kv channel interacting protein 3 (KChIP3) or calsenilin as they are encoded at the same gene locus (13, 14). Among four EF-hand domains in DREAM, the third and fourth EF-hand domains bind Ca²⁺ with a K_d of 0.2–1 μ M (15, 16), whereas the second EF-hand preferentially binds Mg²⁺ under physiological conditions (15). The first EF-hand domain was functionally dead due to the presence of a CPXG motif that prevents Ca²⁺ binding (15). Upon an increase in intracellular Ca²⁺, the DREAM tetramer binds Ca²⁺ (15, 16), is dissociated from DNA without disrupting

the tetrameric structure (17), and is exported out of the nucleus to the cytoplasm as dimers, thereby allowing gene transcription (Figure 1) (18). The DRE sequence is observed in multiple genes, including *PDYN* (prodynorphin), *SLC8A3* (Na^+ - Ca^{2+} exchanger), *c-fos*, *HRK*, and *TNFAIP3* (TNF- α -induced protein 3, A20) (17, 19–22). Thus, DREAM blocks the transcription of those genes. It was reported that DREAM also localizes in the endoplasmic reticulum (ER) (13), in the cytoplasm and around perinuclear regions of the ER and Golgi apparatus (23), and to the plasma membrane (24). However, the function of extra-nuclear DREAM remains unclear.

In neuroblastoma cell lines, protein kinase A-mediated phosphorylation of α -cAMP-response element modulator (α CREM) facilitates complex formation with DREAM, releasing DREAM from the DRE site and enabling transcription of the prodynorphin gene (Figure 1) (25). The binding of DREAM to the Hrk gene is decreased when cells are treated with a phosphatidylinositol 3-kinase (PI3K) inhibitor (19). Although this result implicates the importance of PI3K-mediated DREAM phosphorylation for its transcriptional repressor activity, the authors did not identify the responsible residues on DREAM and determine whether downstream molecules of PI3K participate in DREAM phosphorylation. Another study showed that DREAM function may be regulated by phosphorylation of several Ser residues including Ser11, Ser25, Ser60, Ser62, Ser63, and Ser65, the last of which was reported as a cleavage site for caspase-3 (26). Casein kinase 1 is responsible for Ser63 phosphorylation, and protein phosphatase 1 and 2A are the associated phosphatases (26). Also, DREAM binds to G protein-coupled receptor kinase 2 near the plasma membrane in a Ca^{2+} -dependent manner (27). The authors also found that Ser95 in DREAM is phosphorylated by the kinase and that this phosphorylation is important for the membrane expression of the Kv4.2 potassium channel. In addition to Ser residues, there are several Thr and Tyr residues in DREAM. Further studies will be required to determine whether these residues are phosphorylated during cell activation, which kinases and phosphatases participate in the regulation, and how each phosphorylation event affects DREAM function.

3. Function of DREAM

It is known that DREAM regulates numerous cellular functions. In neuronal cells, DREAM acts as a transcriptional repressor involved in protein folding (28), presenilin-induced β -amyloid formation (13), nitric oxide-induced inflammatory pain (29), apoptosis (30–33), and synaptic plasticity (34). Earlier work demonstrated that DREAM represses transcription of the *PDYN* (prodynorphin) gene in neuroblastoma cells (17). Dynorphin interacts with opioid receptors and modulates acute and chronic pain states (35). Consistently, DREAM knockout (KO) mice exhibit increased levels of prodynorphin mRNA and dynorphin A peptides in the spinal cord, and have a hypoalgesic phenotype, corroborating its critical role in pain modulation (36). Studies using an inducible expression system in neuroglioma cells suggested that DREAM enhances apoptosis by altering the cytosolic Ca^{2+} level and increasing caspase and calpain activities (31). Nonetheless, the role of DREAM in apoptosis may be cell specific since knockdown of DREAM attenuates apoptosis induced by increased cytosolic Ca^{2+} levels in HeLa cells and a T-lymphocyte cell line (32), whereas down-regulation of DREAM in retinal ganglion cells promotes apoptosis (33).

Recent studies have shown that DREAM is able to interact with calmodulin, a Ca^{2+} binding protein in the presence of Ca^{2+} (37, 38). The binding of DREAM to calmodulin reduces association of DREAM with the DRE sites on DNA (37) and increases activation of calcineurin, a Ser/Thr phosphatase regulated by Ca^{2+} -calmodulin (38). Studies using DREAM KO and Ca^{2+} -insensitive/CRE binding(CREB)-independent dominant-active DREAM transgenic mice demonstrated that DREAM is involved in L-DOPA-induced dyskinesias and DREAM decreases L-DOPA-induced expression of FosB, dynorphin-B, and phosphoacetylated histone H3 in the striatum (39). Another study using the same transgenic mice suggested that the expression of dominant-active DREAM in the forebrain results in downregulation of Npas4, thereby reducing GABAergic inhibitory transmission and impairing learning and memory (18). Also, reduced DREAM expression in a transgenic mouse model of Huntington disease results in neuroprotection (40). The authors found that inhibiting DREAM activity with repaglinide, a DREAM-binding anti-diabetic drug, blocks the interaction between DREAM and the unfolded protein response sensor activating transcription factor 6, thereby delaying neurodegeneration and prolonging life span in the transgenic mouse. It was recently reported that DREAM is expressed in endothelial cells and induces NF- κ B signaling by repressing the expression of A20, an inhibitor of transforming growth factor β -activated kinase 1, resulting in transcription of pro-inflammatory genes in endothelial cells during acute lung injury (21).

In addition to the transcriptional role of DREAM, there is evidence that DREAM may play a non-transcriptional role in the process of neuronal diseases. Studies using overexpression systems showed that DREAM co-localizes with presenilin 2 in the perinuclear region and may participate in the pathogenesis of Alzheimer's disease (13). Another overexpression/knockdown study suggested that DREAM directly binds to the NR1 subunit of the N-methyl-D-aspartate (NMDA) receptor near the plasma membrane and that a cell-permeable peptide mimicking the binding site of DREAM for the NMDA receptor reduces the binding of DREAM to the NR1 subunit and has a neuroprotective effect on NMDA-induced neuronal injury (41). Although these findings that DREAM regulates cellular functions in a transcription-independent manner are of great interest, because of the lack of a DREAM inhibitor, all studies were carried out using gene manipulation systems that may create non-physiological conditions and/or alter transcription of the DREAM-targeted genes.

4. Role of DREAM in platelet function

Besides numerous transcription factors, other transcriptional regulators may be expressed in anucleate platelets. Indeed, we have recently shown that the mRNA and protein of DREAM is expressed in human and mouse platelets and a human megakaryocyte cell line (12). DREAM deletion did not affect blood counts in mice, and DREAM bone marrow chimeric mice showed no difference in their blood counts, suggesting that DREAM is dispensable for hematopoiesis. Moreover, platelets isolated from DREAM KO mice had no ultrastructural defects and expressed normal levels of numerous proteins, including P-selectin, $\alpha\text{IIb}\beta 3$ integrin, PI3K p85, phospholipase $\text{C}\gamma 2$, and AKT, implying that DREAM may not have a transcriptional repressor function in megakaryocytes and platelets.

In a series of functional assays, we found that DREAM deletion significantly reduces granule secretion, cytosolic Ca^{2+} levels, $\alpha\text{IIb}\beta 3$ integrin activation, and aggregation in platelets following stimulation with a low concentration of agonists such as thrombin, CRP, U46619, A23187, and ADP (12). Interestingly, our study using isoform-specific PI3K inhibitors (42, 43) in DREAM-null platelets provided evidence that the regulation of platelet function by DREAM results from activation of PI3K class I β after agonist stimulation. We further showed direct interaction between DREAM and PI3K class I β in a purified system, and their interaction was enhanced when platelets were stimulated with CRP. In addition, knockdown of DREAM in a human megakaryoblastic cell line, MEG-01 (44) did not affect the expression of proteins such as $\alpha\text{IIb}\beta 3$, PI3K p85, and AKT. However, pretreatment of MEG-01 cells with DREAM siRNA significantly reduced cytosolic Ca^{2+} levels upon A23187 stimulation. Importantly, our finding that overexpression of siRNA-resistant wild-type DREAM, but not its EF-hand mutants (E186Q and/or E234Q), rescues the defect in DREAM knockdown cells suggests the importance of Ca^{2+} binding to the EF-3 or EF-4 hand domain for the regulatory function of DREAM. Further, the combination study with PI3K inhibitors and DREAM knockdown MEG-01 cells supported our results in mouse platelets that DREAM regulates PI3K class I β activation.

What is the physiological role of DREAM in thrombosis and hemostasis? By using *in vivo* real-time intravital microscopy with DREAM KO mice and their bone marrow chimeras, we demonstrated that both platelet and non-hematopoietic cell DREAM contribute to platelet thrombus formation at the site of laser-induced arteriolar injury (12). In a mouse model of FeCl_3 -induced thrombosis, DREAM KO and hematopoietic cell DREAM KO mice exhibited a delayed time to occlusion, but non-hematopoietic cell DREAM KO mice did not. Since FeCl_3 -mediated chemical oxidation is believed to disrupt the endothelium (45), this result implicates the potential role of endothelial cell DREAM in arterial thrombogenesis. Tail bleeding time assays showed that only DREAM KO, but not hematopoietic nor non-hematopoietic cell DREAM KO, mice significantly prolonged bleeding time, suggesting that either hematopoietic or non-hematopoietic cell DREAM is sufficient for maintaining hemostatic function in mice.

Previous studies identified PI3K as a therapeutic target to treat a variety of diseases such as cancers, thrombosis, and inflammation (46–48). In particular, PI3K class I β has been considered a novel target for anti-thrombotic therapy. Treatment of platelets with TGX221, a PI3K class I β specific inhibitor, attenuated platelet aggregation and had a minimal to moderate effect on granule secretion after stimulation with a threshold concentration of agonists (42). Moreover, administration of TGX-221, but not a PI3K p110 δ inhibitor (D-010), prevented thrombus formation in an electrolytic injury-induced arterial thrombosis model in rats without prolonging tail bleeding time, suggesting the contribution of PI3K class I β to thrombosis but not hemostasis. However, this result has been challenged by another finding that anti-thrombotic doses of TGX-221 increase bleeding times in two mouse models (49). A previous study using megakaryocyte-specific PI3K class I β conditional KO mice demonstrated that platelet PI3K class I β is important for $\alpha\text{IIb}\beta 3$ integrin-mediated platelet aggregation and fibrin clot formation and for *in vivo* thrombogenesis after FeCl_3 -induced carotid artery injury without impairing hemostatic function (50). Another study using mice expressing the kinase dead form of PI3K class I β

(K805R) also showed that the catalytic activity of PI3K class I β is crucial for GPVI-mediated signaling, AKT phosphorylation, Rap1b activation, and platelet aggregation (51). Like platelet-specific PI3K class I β -null mice, the PI3K class I β activity-null mice did not alter tail bleeding time. Although our recent report suggested the role of platelet DREAM in regulating PI3K class I β activity, it was hard to precisely compare our results with those in platelet-specific PI3K class I β -null mice because of the differences in agonists, the concentrations used, and the *in vivo* models tested. Nevertheless, it is of interest to note that global DREAM deletion prolongs bleeding time (12), whereas the platelet-specific PI3K class I β -null mice have no effect on hemostasis (42, 50). This discrepancy implicates the potential role of non-hematopoietic cell DREAM - particularly endothelial cell or leukocyte DREAM - in hemostasis, probably through a mechanism independent of PI3K class I β activation.

5. Concluding remarks

Because of the different subcellular localization, DREAM has been speculated to have non-transcriptional functions. Our recent study has revealed that DREAM is present in anucleate platelets and platelet DREAM promotes PI3K class I β activation, thereby participating in platelet activation in a transcription-independent manner (12). These results are consistent with previous findings that numerous transcription factors in platelets play non-genomic roles during platelet activation and thrombosis. Nevertheless, the recent findings have raised new questions. Which transcription factors and repressors are present in platelets and what are their precise roles during platelet activation? Does DREAM deletion affect gene transcription in megakaryocytes? How does platelet DREAM specifically regulate PI3K class I β activity during cell activation? How does deletion of DREAM from non-hematopoietic cells perturb thrombogenesis *in vivo*? More comprehensive studies such as gene arrays or RNA-seq in megakaryocytes and platelets will help answer those questions. Further, the use of tissue-specific DREAM conditional KO mice will be of great benefit.

Previous studies provide insight into DREAM as a therapeutic target to treat severe pain. Moreover, we have identified DREAM as a novel target for anti-thrombotic therapy. Since DREAM deletion prolongs bleeding time, DREAM inhibitors, like other anti-thrombotic agents, may not be completely safe from side effects such as bleeding. However, given the findings that DREAM deletion attenuates pain as well as thrombogenesis, a specific DREAM inhibitor may be an effective supplement for the treatment of patients with cancers (52, 53) and sickle cell disease (54) which can result in severe pain and thrombosis.

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Figure 1. DREAM-DRE binding in the nucleus

(A) Domain structure of DREAM. Variable region and EF hand domains (EF-1, 2, 3, and 4) are indicated. Numbers indicate the amino acid position. (B) Binding of DREAM to the DRE sequence on DNA represses gene transcription. Numerous stimuli induce intracellular signaling that increases the level of cytosolic Ca^{2+} and phosphorylates CREM by activated protein kinase A. The DREAM tetramer interacts with phosphorylated CREM (p-CREM) or Ca^{2+} in the nucleus, resulting in dissociation from the DRE sequence and allowing the transcription of numerous genes. Further, Ca^{2+} -bound DREAM is exported from the nucleus to the cytoplasm as dimers and may regulate cellular functions in a transcription-independent fashion.

Figure 2. Roles of DREAM in regulating platelet activation and thrombogenesis

Upon stimulation with an agonist such as CRP which induces GPVI-mediated signaling, DREAM binds to PI3K class I β (PI3K-I β) and facilitates the signal transduction pathway, thereby enhancing granule secretion and cytosolic Ca²⁺ levels. ADP-induced signaling pathways further activate α IIb β 3 integrin and amplify integrin outside-in signaling which also activates PI3K-I β through DREAM. Binding of fibrinogen to activated α IIb β 3 integrin results in platelet-platelet aggregation and thrombus formation. Fc γ R, Fc γ receptors; ITAM, immunoreceptor tyrosine-based activation motif; SFK, Src-family kinases.