HHS Public Access

Author manuscript

Thromb Res. Author manuscript; available in PMC 2022 June 20.

Published in final edited form as:

Thromb Res. 2022 March ; 211: 123–126. doi:10.1016/j.thromres.2022.01.029.

Calpain-1 inhibition attenuates *in vivo* thrombosis in a humanized model of sickle cell disease

Farha Mithila[a],

Christopher Schwake[a],

Chao Fang[b],

Glenn Merrill-Skoloff[b],

Lidija Covic[c],

Daniel I. Fritz[a],

Toshihiko Hanada[a],

Robert Flaumenhaft[b],

Athar H. Chishti[a,*]

[a]Department of Developmental, Molecular, and Chemical Biology, Graduate School of Biomedical Sciences, Tufts University School of Medicine, Boston, MA, USA

[b]Division of Hemostasis and Thrombosis, Department of Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, USA

[c]Division of Hematology/Oncology, Department of Medicine, Center for Hemostasis and Thrombosis Research, Tufts Medical Center, Boston, MA, USA

Keywords

Calpain-1; Platelets; Sickle cell disease; Thrombosis

Sickle cell disease (SCD) is a beta globin gene disorder characterized by misshapen erythrocytes that are susceptible to hemolysis resulting in hyperactive platelets, inflammation, and vaso-occlusive crisis. Increasing evidence suggests that activation of the cysteine protease, calpain-1 (CAPN1), regulates SCD pathophysiology. However, there is

*Corresponding author at: Department of Developmental, Molecular & Chemical Biology, Tufts University School of Medicine, 150 Harrison Avenue, Boston, MA, USA. athar.chishti@tufts.edu (A.H. Chishti).

CRediT authorship contribution statement

Contribution: A.C. conceived and designed the study; and contributed to the development of manuscript and figures. F.M. maintained multiple genotypes of mouse models, performed platelet aggregation, and calcium mobilization studies. C.F. and G.M.S. performed intravital thrombosis experiments. C.S., F.M., L.C., and D.F. participated in the assembly of manuscript and figures. R.F. supervised the intravital thrombosis studies and contributed to writing and editing of the manuscript. L.C. provided expert advice to F.M. and D.F. for calcium measurements. T.H. provided technical advice for genotyping of mice and troubling shooting of multiple assays during the course of this study. All authors read and approved the manuscript.

Declaration of competing interest

The authors declare no competing financial interests.

Appendix A. Supplementary data

The online version of this article contains a data supplement (Methods). Supplementary data to this article can be found online at https://doi.org/10.1016/j.thromres.2022.01.029.

no direct evidence indicating a functional role of CAPN1 in thrombotic lesions associated with SCD. Previously, we generated a CAPN1 null Townes SCD mouse model (SSCKO) revealing attenuated chronic pain and platelet activation phenotypes [1]. Here, we provide the first evidence that SSCKO mice exhibit a substantial reduction in platelet accumulation at the vascular injury site following laser ablation *in vivo*, a phenotype mechanistically consistent with reduced calcium mobilization in CAPN1 null platelets.

Sickle cell disease (SCD) is characterized by sickled red blood cells (sRBCs) and hyperactive platelets. Frequent hemolysis of sRBCs is associated with anemia, increased oxidative stress, activation of cell adhesion, and inflammatory responses, thus resulting in vaso-occlusion, strokes, multi-organ damage, and pain episodes. Elevated calcium is a hallmark of RBCs in SCD [2]. Calpains are calcium-activated cysteine proteases, and their aberrant activation contributes to cardiovascular diseases. Calpain 1 knockout (CKO) platelets show reduced platelet activity without exhibiting a bleeding phenotype [3]. We generated a CKO Townes SCD mouse model (SSCKO) to investigate the specific role of CAPN1 in SCD pathophysiology [1]. The SSCKO mice exhibited reduced whole blood platelet aggregation and clot retraction [4]. To further investigate the specific role of CAPN1 in SCD, we evaluated platelet aggregation and calcium mobilization of washed platelets in SSCKO. Given that calcium plays a central role in platelet signaling and sRBCs physiology, calcium mobilization was measured in platelets *via* release from intracellular channels as well as entry through the plasma membrane. In addition, laser-induced *in vivo* thrombosis was performed to visualize platelet accumulation and fibrin accumulation at the injury site.

We propose calpain 1 (CAPN1) as a potential therapeutic target for SCD that can modulate platelet pathology given that this cysteine protease serves an essential role in the platelet activation cascade [3,5]. Previously, in whole blood SSCKO platelets, we reported reduced aggregation initiated by TRAP4, an agonist specific to the PAR4 receptor, as compared to SS and humanized control (AA) mouse models [1]. To further evaluate the SSCKO platelet phenotype without contamination from hemolysis, platelet aggregation of washed platelets was measured in the presence of human γ-thrombin (0.5 U/mL). Consistent with our previous findings [1], SSCKO platelets rescued the hyperactivity defect observed in the SS model and showed aggregation similar to AA platelets (Fig. 1A). This finding indicates that the attenuated platelet aggregation phenotype of CKO is conserved in SSCKO, even in the presence of high inflammation SCD pathology. Since calpain deficiency is known to suppress mast cell activation and leukocyte-mediated inflammation, targeting of CAPN1 signaling may be a useful strategy for reducing platelet hyperactivity as well as inflammation in SCD.

To investigate the mechanism of reduced platelet activity of SSCKO, we measured calcium mobilization in washed platelets. It is known that elevated calcium flux acts as a secondary messenger for integrin activation regulating platelet aggregation and the clot retraction cascade [6]. Calcium mobilization was quantified using a calcium-sensitive dye in WT, CKO, AA, SS and SSCKO platelets [6]. The calcium measurements were performed both in the presence and absence of extracellular calcium to distinguish the relative contributions of internal calcium release and external calcium influx to total calcium mobilization. Calcium mobilization was reduced in SSCKO platelets as compared to both SS and AA (Fig. 1B).

Interestingly, the CKO platelets also showed an overall decrease in calcium flux upon thrombin activation as compared to WT mice (Fig. 1C, D), implying that similar calcium storage deficit mechanisms may operate in SSCKO platelets. Given a functional role of elevated calcium in SCD [2] and calpain in the adhesion of platelets to endothelium [7], our findings suggest a plausible calcium-dependent mechanism for reduced platelet activity in both CKO and SSCKO platelets. Although the precise molecular mechanism of impaired calcium mobilization is not yet known, our previous findings indicate a differential regulation of erythrocyte membrane calcium pump (PMCA) by calmodulin in CKO mice, suggesting a broad range of calcium pump regulation by CAPN1 [8]. Whether similar mechanisms operate in SSCKO platelets remain to be investigated.

To assess the effect of CAPN1 deficiency on thrombus formation in the setting of SCD, we evaluated mice using a laser-induced injury model of thrombus formation in which the accumulation of platelets and formation of fibrin are monitored following laser ablation of arterioles within the cremaster muscle microcirculation. These studies showed a significant decrease in platelet accumulation at the injury site in $CAPN1^{-/-}$ mice compared with $CAPN1^{+/+}$ mice (26.0% reduction compared to $CAPN1^{+/+}$ mice, $P = 0.04$) (Fig. 2A, B). In contrast to the protective effect of CAPN1 deficiency on platelet accumulation, no significant difference in fibrin formation was observed between $CAPN1^{+/+}$ and $CAPN1^{-/-}$ mice (Fig. 2C, D). The SS mice showed approximately equivalent platelet accumulation compared with AA mice (Fig. 2E, F). However, SS mice lacking CAPN1 demonstrated significantly less platelet accumulation (63.4% reduction compared to SS, $P < 0.05$, Fig. 2E, F). Fibrin formation was markedly elevated in SS mice compared to controls (Fig. 2G, H). In contrast to its protective effect in reducing platelet accumulation, CAPN1 deficiency did not protect mice from enhanced fibrin accumulation observed in SS mice (Fig. 2G, H). Consistent with its effects on platelet calcium flux and platelet aggregation, the loss of CAPN1 results in reduced platelet accumulation at sites of injury, thus raising the possibility that calpain-1 activity may serve as a therapeutic target for the prevention of thrombosis in SCD. It is interesting to note that SS-WT and SS-CKO mice differ with regard to platelet accumulation (Fig. 2E–F) but not fibrin formation (Fig. 2G–H). That inhibition of platelet accumulation can occur in the absence of defects in fibrin formation has been previously documented in the laser-induced injury model. Platelet accumulation in this model results from a balance of the attachment and detachment of individual platelets from the thrombus. Impaired calcium mobilization in the SS-CKO platelets could impair the ability of individual platelets to remain attached to the thrombus. Alternatively, platelets incorporated into the thrombus could embolize en masse. Further studies will be required to distinguish between these possibilities.

Recent clinical findings have demonstrated enhanced coagulation, thrombosis, and strokes in patients afflicted with severe COVID-19 [9]. Moreover, patients with SCD and COVID-19 are prone to higher incidence of infection and pain [10]. Interestingly, the SARS-CoV-2 genome encodes two essential cysteine proteases raising the possibility that CAPN1 and its shared substrates may constitute druggable therapeutic targets against thrombotic lesions in both SCD and COVID-19. Given the reduced chronic pain phenotype observed in SSCKO [1], CAPN1 inhibition may serve as a compelling therapeutic target by reducing episodes of vaso-occlusion and pain crises in SCD with or without COVID-19.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported in part by grants from the National Heart, Lung, and Blood Institute (NHLBI) RO1-HL060961, RO1-HL089517 (AC), Tufts Collaborates Seed Grant Program (AC); R35HL135775 (RF); American Heart Association Predoctoral Fellowship (DF); and R25GM066567 under the Post-Baccalaureate Research Education Program (PREP) (FM). We are grateful to Jennifer O. Nwankwo, former graduate student, for the generation of CAPN1 null Townes SCD (SSCKO) mice [1]. We are also grateful to Donna-Marie Mironchuk for her contributions in the administrative organization of the project, proofreading, and improvements of figures.

References

[1]. Nwankwo JO, Lei J, Xu J, Rivera A, Gupta K, Chishti AH, Genetic inactivation of calpain-1 attenuates pain sensitivity in a humanized mouse model of sickle cell disease, Haematologica 101 (10) (2016) e397–e400. [PubMed: 27418647]

[2]. Eaton JW, Skelton TD, Swofford HS, Kolpin CE, Jacob HS, Elevated erythrocyte calcium in sickle cell disease, Nature 246 (5428) (1973) 105–106. [PubMed: 4585849]

[3]. Azam M, Andrabi SS, Sahr KE, Kamath L, Kuliopulos A, Chishti AH, Disruption of the mouse mu-calpain gene reveals an essential role in platelet function, Mol. Cell. Biol 21 (6) (2001) 2213–2220. [PubMed: 11238954]

[4]. Nwankwo JO, Gremmel T, Gerrits AJ, Mithila FJ, Warburton RR, Hill NS, Lu Y, Richey LJ, Jakubowski JA, Frelinger AL 3rd, Chishti AH, Calpain-1 regulates platelet function in a humanized mouse model of sickle cell disease, Thromb. Res 160 (2017) 58–65. [PubMed: 29101791]

[5]. Kuchay SM, Kim N, Grunz EA, Fay WP, Chishti AH, Double knockouts reveal that protein tyrosine phosphatase 1B is a physiological target of calpain-1 in platelets, Mol. Cell. Biol 27 (17) (2007) 6038–6052. [PubMed: 17576811]

[6]. Varga-Szabo D, Braun A, Nieswandt B, Calcium signaling in platelets, J.Thromb. Haemost 7 (7) (2009) 1057–1066. [PubMed: 19422456]

[7]. Yuan Y, Dopheide SM, Ivanidis C, Salem HH, Jackson SP, Calpain regulation of cytoskeletal signaling complexes in von Willebrand factor-stimulated platelets. Distinct roles for glycoprotein Ib-V-IX and glycoprotein IIb-IIIa (integrin alphaIIbbeta3) in von Willebrand factor-induced signal transduction, J. Biol. Chem 272 (35) (1997) 21847–21854. [PubMed: 9268316]

[8]. Wieschhaus A, Khan A, Zaidi A, Rogalin H, Hanada T, Liu F, De Franceschi L, Brugnara C, Rivera A, Chishti AH, Calpain-1 knockout reveals broad effects on erythrocyte deformability and physiology, Biochem. J 448 (1) (2012) 141–152. [PubMed: 22870887]

[9]. Valchanov K, Krishnan U, Hoole SP, Davies WR, Pettit S, Jones N, Parmar J, Catarino P, Osman M, Berman M, COVID-19 patient with coronary thrombosis supported with ECMO and impella 5.0 ventricular assist device: a case report, Eur. Heart J. Case Rep 4 (6) (2020) 1–6.

[10]. Singh A, Brandow AM, Panepinto JA, COVID-19 in individuals with sickle cell disease/trait compared with other black individuals, Blood Adv. 5 (7) (2021) 1915–1921. [PubMed: 33792626]

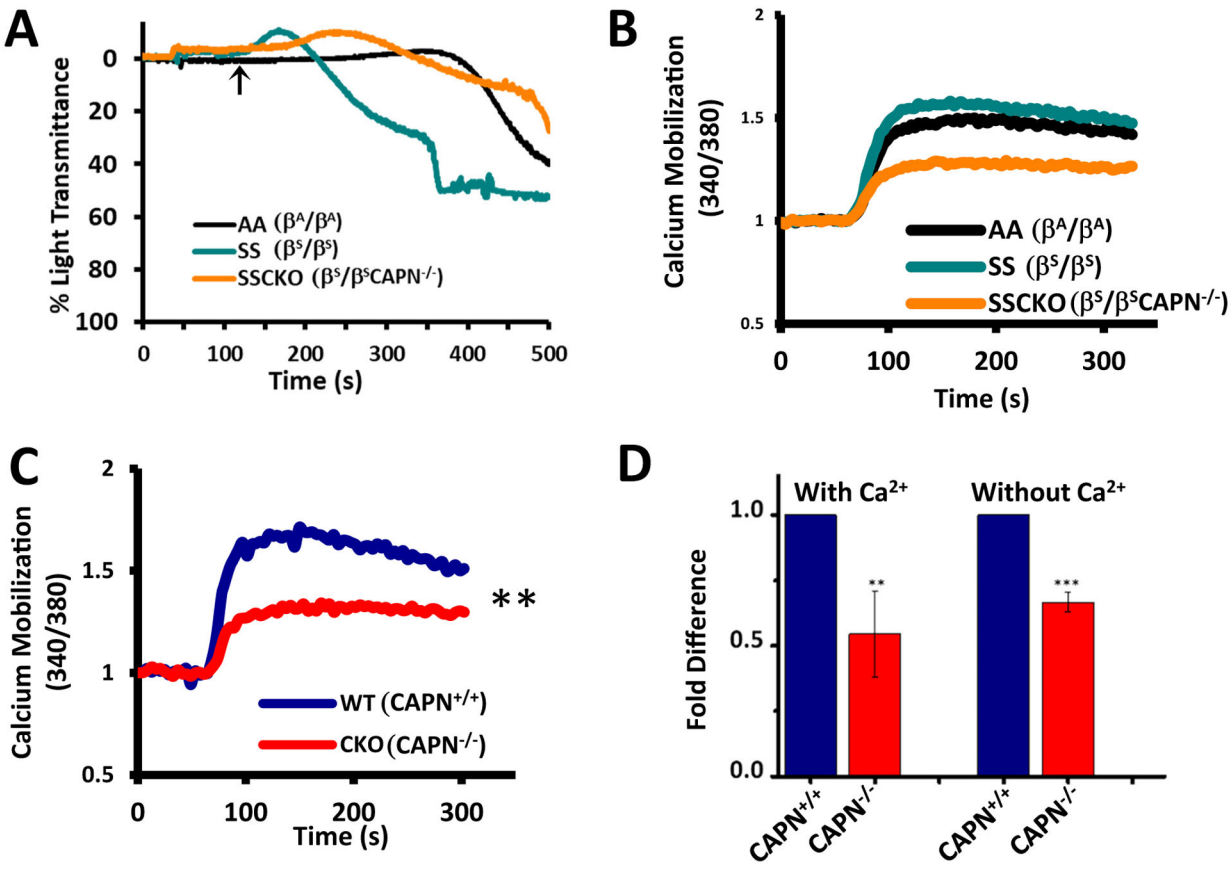

Fig. 1.

Calpain-1 (CAPN1) mediated regulation of platelet aggregation and calcium mobilization in AA, SS, and SSCKO mice. (A) Washed platelets from AA, SS, and SSCKO mice were analyzed for aggregation upon stimulation with 0.5 U/mL thrombin, $n \geq 3$. The arrow indicates the addition of agonist at the start of platelet aggregation. (B) Calcium mobilization was measured in washed AA, SS, and SSCKO platelets upon stimulation with 0.5 U/mL thrombin and 2.0 mM calcium **$P \leq 0.005$, $n \geq 3$. (C) Calcium mobilization of washed platelets from WT ($CAPN1^{+/+}$) and CKO ($CAPN1^{-/-}$) mice upon stimulation with 0.5 U/mL thrombin and 2.0 mM calcium. (D) Quantification of data shown in panel C. Data in this panel reflect reduced calcium mobilization in CKO ($CAPN1^{-/-}$) platelets both in the presence and absence of supplemented calcium.

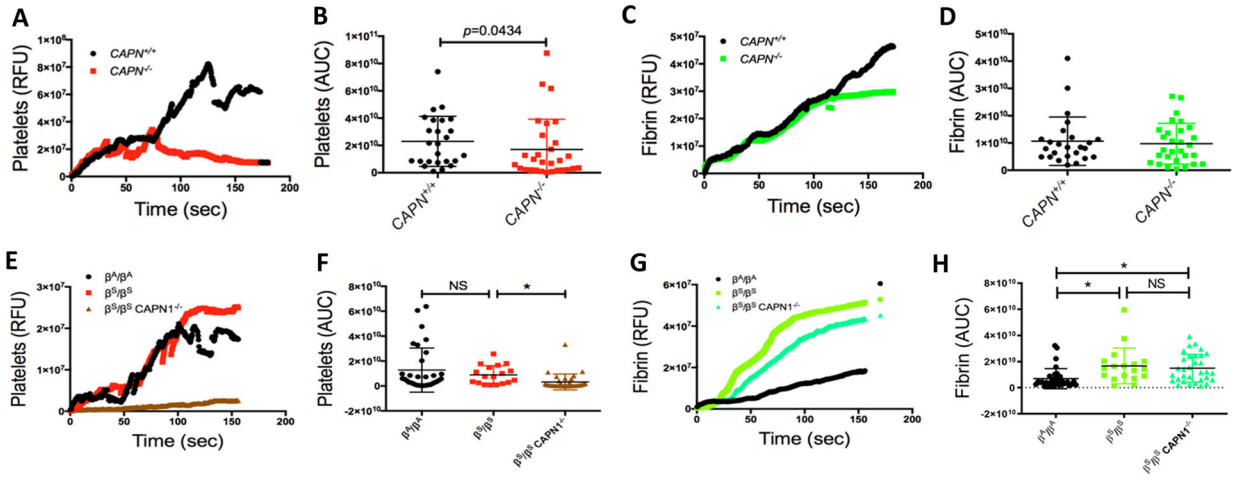

Fig. 2.

Calpain-1 (CAPN1) mediated regulation of thrombus formation. (A, B) Laser-induced thrombus formation and accumulation of platelets in arterioles within the cremaster microcirculation of CAPN1 null mice. (C, D) Formation of fibrin following laser ablation of arterioles in CAPN1 null mice. (E, F) Laser-induced thrombus formation and accumulation of platelets in AA (β^A/β^A), SS (β^S/β^S), and SSCKO (β^S/β^SCAPN1$^{-/-}$) mice. (G, H) Formation of fibrin following laser ablation of AA (β^A/β^A), SS (β^S/β^S), and SSCKO (β^S/β^SCAPN1$^{-/-}$) mice. Data in panel H show statistical difference between AA and SSCKO for fibrin AUC using the Kruskal-Wallis test.