Altered expression of platelet proteins and calpain activity mediate hypoxia induced prothrombotic phenotype

Tarun Tyagi,1 Shadab Ahmad,2 Neha Gupta,1 Anita Sahu,1 Yasmin Ahmad,1 Velu Nair,3 Tathagat Chatterjee,4 Nitin Bajaj,5 Shantanu Sengupta,2 Lilly Ganju,1 Shashi Bala Singh1 and Mohammad Z Ashraf1*

1Defense Institute of Physiology & Allied Sciences, Timarpur, Delhi, India; 2Institute of Genomics and Integrative Biology, Delhi, India; 3Armed Forces Medical College, Pune, India; 4Army Hospital (Research & Referral), New Delhi, India; and 5Western Command Hospital Chandimandir, Chandigarh, India

* Correspondence: Group Head, Genomics Division, Defense Institute of Physiology & Allied Sciences, Timarpur, Delhi, 110054 India; e-mail: mohammadzashraf@gmail.com

Running Title: Role of calpain in hypoxia induced thrombosis

Key words: Platelet, platelet proteome, hypoxia, calpain, hypoxia induced thrombosis, high altitude, thrombosis, platelet tissue factor

STATEMENT OF PRIOR PRESENTATION: A part of this study was presented as a poster at ‘22nd International Congress on Thrombosis,’ 6-9 Oct, 2012, Nice, France.
Key Points:

- Hypoxia induces altered platelet proteome/reactivity which correlates with a prothrombotic phenotype.
- CAPNS1 dependent calpain activity in platelet activation cascade is associated with hypoxia induced thrombogenesis.

Abstract

The oxygen compromised environments such as high altitude, air travel, sports and solid tumors have been suggested to be prothrombotic. Despite the indispensable role of platelets in thrombus formation, the studies linking hypoxia, platelet reactivity and thrombus formation are limited. In the present study, platelet proteome/reactivity was analyzed to elucidate the acute hypoxia induced prothrombotic phenotype. Rats exposed to acute simulated hypoxia (282 torr/8% oxygen) demonstrated a decreased bleeding propensity and increased platelet reactivity. Proteomic analysis of hypoxic platelets revealed 27 differentially expressed proteins including those involved in coagulation. Among these proteins, calpain small subunit-1 (CAPNS1), a 28 kDa regulatory component for calpain function was significantly upregulated under hypoxic conditions. Moreover, intraplatelet Ca\(^{2+}\) level and platelet calpain activity were also found to be in accordance with CAPNS1 expression. The inhibition of calpain activity demonstrated reversal of the hypoxia induced platelet hyperreactivity. The prothrombotic role for calpain was further confirmed by an in vivo model of hypoxia-induced thrombosis. Interestingly, patients who developed thrombosis while placed at extreme altitude had elevated plasma calpain activities and increased sP-selectin level. In summary, this study for the first time suggests that augmented calpain activity is associated with increased incidence of thrombosis under hypoxic environments.
Introduction

Hypoxia, experienced either during physical activities such as ascent to mountains, air travel, sports activities or with pathological conditions like solid tumors has been suggested to be associated with thrombotic episodes.\(^1\text{-}^5\) In the case of high altitude hypoxic exposure, both venous as well as arterial thrombotic events can occur, which include pulmonary thromboembolism, cerebral venous thrombosis, portal vein thrombosis, aortic thrombosis, stroke, and transient ischemic attack.\(^6\text{-}^9\) At extreme altitude, adverse environmental conditions including hypobaric hypoxia and cold may facilitate the development of the corresponding prothrombotic phenotype.

In the past, various studies have been reported with the focus on hematological factors, and proteins involved in thrombin generation and fibrinolysis to understand the altitude-induced thrombotic events.\(^10\text{-}^13\) Although platelets play an indispensable role in thrombogenesis, the involvement of platelets in hypoxia induced thrombotic events has not been adequately explored. Most of the studies were focused on changes in platelet numbers with only a few reports on platelet reactivity at high altitude.\(^14\text{-}^17\) In fact, in chronic obstructive pulmonary disease and sleep apnea, hypoxia has been associated with increased platelet reactivity.\(^18\text{-}^19\) Platelet hyperreactivity reflected by enhanced platelet adhesion, activation and aggregation, is a sum of finely coordinated cell signaling events involving a shift in platelet proteome/secretome and structural proteins. In conjunction with this, the tightly controlled cytosolic Ca\(^{2+}\) also act as an important secondary messenger to regulate the fundamental platelet reactivity via the key downstream signaling cascades.\(^20\)

The proteome analysis, which is utilized for identifying novel proteins and pathways, has become an ideal tool to study anucleated cells like platelets. Previously, proteomic analysis
revealed differential regulation of proteins in diseases like acute coronary syndrome. The cellular functions in platelets are regulated primarily by changes in protein expression and their modifications. In the present study, exposure of Sprague Dawley rats to acute high altitude hypoxia resulted in platelet hyperreactivity leading to a prothrombotic phenotype. The proteome analysis of these phenotypically altered platelets revealed differential expression of proteins, which are involved in coagulation, calcium homeostasis, signal transduction, acute phase response, and cytoskeletal reorganization. The disturbed calcium homeostasis was further explored by addressing cytosolic Ca\(^{2+}\) levels and calpain activity in platelets of hypoxic animals. Calpain small 28 kDa subunit-1(CAPNS1), which functions like chaperone for calpain proteases and controls cell spreading and migration, was found to be upregulated in hypoxia exposed animals. Taking into consideration that calpains are activated by elevated cytosolic Ca\(^{2+}\) and are involved in platelet reactivity, the prothrombotic role for calpain under hypoxia was further confirmed by in vivo model of hypoxia-induced thrombosis. To investigate the potential of these pre-clinical investigations for clinical relevance, the calpain activity and sP-selectin level were analyzed in plasma samples of patients who developed deep vein thrombosis (DVT) at high altitudes (>3648 m). The results from these studies for the first time suggest that hypoxic environment alters the platelet proteome and induces platelet hyperreactivity leading to a prothrombotic phenotype which is mainly mediated by activation of calpain.

**Materials and Methods**

**Materials**

Alpha-thrombin, adenosine diphosphate (ADP) and chronolume luciferin-luciferase reagents were purchased from Chrono-log (Havertown, PA). Anti-CD41 and anti-\(\alpha\)IIb\(\beta\)\(_3\) antibodies were
purchased from Abcam (Cambridge, MA). IPG gel strips, ampholytes and mineral oil for two-dimensional electrophoresis were products of GE Healthcare (Piscataway, NJ) while trypsin was from Promega (Madison, WI). Rat specific ELISA kits were purchased from USCN Life Sciences Inc. (Wuhan, China) and Bmassay (Beijing, China). PD150606 was from Tocris Biosciences (Bristol, UK). RT PCR kit for platelet RNA analysis and all other reagents were from Sigma-Aldrich (St Louis, MO).

Animal exposure to simulated high altitude (HA) conditions

All experiments were conducted in accordance with Committee for the Purpose of Control and Supervision of Experiments on Animals, Government of India. Male Sprague Dawley rats, weighing 150-250g were kept under standard laboratory conditions. The animals were exposed to simulated HA (hypobaric hypoxia) conditions in a specially designed animal decompression chamber, which was maintained at pressure of 282 torr (equivalent to an altitude of 7620 m with 8% oxygen) and at 10°C for various durations. Animals were exposed to simulated hypobaric hypoxia with cold surrounding since at high altitude regions the associated low temperatures may also affect the biological systems. Following optimization of the temperature range, 10°C was selected as a suitable temperature and was used for further hypoxic exposure studies. Based on initial hematological and coagulation screening data and previous studies, platelet reactivity studies were conducted after 6h exposure to simulated altitude.

Flow restriction animal model

To model the localized hypoxia induced thrombosis, thrombus was induced in rats by proximal ligation of the inferior vena cava (IVC) just below the renal veins and ligation of lateral tributaries as previously described\textsuperscript{27} with some modifications (see supplementary methods for details).
Human studies

Young (<40 yr) male patients with lower limb DVT evacuated to either Western Command Hospital Chandimandir, Chandigarh, or Army Hospital (R&R), Delhi, India (tertiary care hospitals) were approached for consent to participate in the study. All patients (n = 10) had onset of DVT while placed at high altitudes (>3648 m). Patients with pre-existing systemic diseases, malignancy, any prior surgery or vasculitis were excluded. All patients had their diagnosis confirmed by objective imaging methods. The complete thrombophilia screening assessment comprised of Protein C and S deficiency, Antithrombin III deficiency, Factor V Leiden and prothrombin 20210G/A polymorphisms was also performed and laboratory investigations details are provided in supplemental methods. Equal numbers of healthy, age matched male subjects were taken as controls, with no prior history for risk factors. Informed consent was obtained according to the Declaration of Helsinki.

Statistical Analysis

Values are expressed as mean ± standard error in mean (SEM). The statistical significance between two groups was tested by nonparametric unpaired two tailed t test using Prism5 software (Graphpad) and for multiple group comparison one way ANOVA was applied followed by Dunnett’s test. A p value less than .05 was considered as significant.

Results

Hypoxic exposure results in hypercoagulative state

In order to investigate whether hypoxic exposure results in prothrombotic phenotype in rats, bleeding time (by tail immersion and filter paper methods) and coagulation assays (PT, aPTT) were performed after hypoxic exposure. A significant shortening of bleeding times (202 ± 29)
was observed in exposed animals as compared to controls (440 ± 54; \( P < .05, n = 10 \)) (Figure 1A). The visual inspection of filter paper indicates the thickening of blood in exposed animals (Figure 1B). Similarly, coagulation assays demonstrated a hypercoagulative state under hypoxic environment as reflected by a significantly decreased PT value and a similar trend in aPTT (Figure 1C-D). No significant change was observed in hemoglobin, hematocrit levels or platelet count in hypoxia exposed animals except for decrease in WBC count (Supplementary Table S1). As hypoxia exposure to rats also involved cold surroundings (chamber temperature of 10°C), bleeding time assay was also performed in rats exposed to hypoxia under normothermic surrounding (chamber temperature of 28°C) to ascertain the possible role (if any) of moderate cold surrounding on hypercoagulative tendency. The exposure under normothermic conditions resulted in significant reduction in bleeding time as under hypothermic conditions (Supplementary Figure S1A). Collectively, these results support the view that altitude hypoxic conditions induce a prothrombotic phenotype.

**Exposure to hypoxia induces platelet hyperreactivity**

The effect of hypoxic exposure on platelets’ tendency for adhering to extracellular matrix proteins was investigated *in vitro* by platelet adhesion assay. Platelets isolated from exposed animals demonstrated an enhanced adhesion to both collagen and fibrinogen coated surfaces. The average size of adhered platelets and percent area covered by the same were significantly higher in exposed group as compared to controls (Figure 2A-F); the increase was greater on collagen surface. Next, we tested whether platelet aggregation and dense granule release was affected with hypoxic exposure. Platelets from exposed animals showed significantly increased aggregation in response to ADP in a dose dependent manner (Figure 2G-H). Both rate and extent
of platelet aggregation were higher in exposed group in response to ADP (2.5 and 5µM; \( P < .05 \)) whereas only modest difference was observed with thrombin (data not shown). The dense granule release, as evaluated by luminescence based ATP release, was also found to be significantly higher \((P < .05)\) in platelets from exposed animals compared to that of controls (Figure 2I-J). Activated platelets bear increased numbers of various transmembrane proteins which serve as platelet activation markers. A flowcytometry based approach was used for analyzing the surface expression of CD41 and \( \alpha \)IIb\( \beta \)3. The anti-\( \alpha \)IIb\( \beta \)3 antibody that interacts with \( \alpha \)IIb\( \beta \)3 receptor complex\(^{28}\) demonstrated an enhanced surface expression of this complex which reflects greater platelet activation upon induction with ADP in exposed animals than that in controls (Figure 3A,C). CD41, another platelet activation marker also exhibited a similar response (Figure 3B,D). Since platelet aggregation requires the binding of fibrinogen to its receptor \( \alpha \)IIb\( \beta \)3 on the platelet surface,\(^{29}\) we further evaluated platelet-fibrinogen interactions using an \textit{in vitro} clot retraction assay. As shown in Figure 3E and F, hypoxic exposure resulted in higher reduction in clot size or increased the clot retraction. The clot retraction in exposed groups were 30% and 20% more than that of control groups at 30 minutes and 60 minutes respectively. These observations reflected higher platelet reactivity and greater platelet-fibrinogen interactions in exposed animals which corroborate with the other platelet assays like aggregation, activation and adhesion.

Platelet function was also tested in rats exposed to hypoxia under normothermic surrounding (chamber temperature of 28°C) to determine whether moderate hypothermic surrounding (chamber temperature of 10°C) are contributing to platelet hyperreactivity under hypoxic environment. The hypoxia exposure under normothermic conditions resulted in significant enhancement of rate and extent of platelet aggregation as under hypothermic conditions.

8
In summary, all platelet functional assays demonstrated significantly enhanced platelet reactivity in response to hypoxic exposure.

**Hypoxic exposure modulates platelet proteome**

To obtain an insight into the molecular events underlying platelet hyperreactivity in response to hypoxic exposure, the platelet proteome was analyzed using two dimensional gel electrophoresis followed by identification of differentially expressed proteins by MALDI TOF MS/MS (Figure 4A-C). Initial optimizations suggested that the pH range of 4-7 was ideal for 2D-PAGE on platelet samples as this range provided the detection of maximum number of protein features and improved resolution as compared to other narrow pI ranges. Software analysis of gel images resulted in detection of over 700 spots per gel, from which 258 spots were chosen for differential expression analysis. The MS/MS data analysis resulted in identification of 27 differentially expressed proteins in platelet samples obtained from exposed animals. From 27 identified proteins, 21 proteins had Mascot™Mowse score equal to or greater than 50 (Table 1) and 19 were up-regulated while 8 proteins were suppressed. Bioinformatic analysis of identified differentially expressed proteins revealed that all these proteins belong to different cellular locations like cell membrane, cytoskeletal, mitochondrial, endoplasmic reticulum, vesicular etc. (Figure 4D) and were found to be involved in various important biological processes including acute phase response, blood coagulation and complement activation, oxidative stress response, platelet activation, lipoprotein metabolism, lipid transport, vasodilation etc. (Table 1). Furthermore, these proteins correspond to diverse molecular functions including nucleotide binding, hormone binding, calcium channel regulation, ATPase activity, calcium binding, enzyme inhibition and response to ions like Fe^{3+}, Mg^{2+}, Ca^{2+} etc. (Figure 4E). Based on cutoff
score, fold induction and functional priorities, a protein list was prepared for analysis, validation and follow up experiments. To validate proteomic results, the levels of selective proteins which included fibrinogen gamma, calreticulin, calumenin, CAPNS1, non-neural alpha enolase, Janus kinase 3 and rho guanine exchange factor-7 (ARHGEF7) were quantitated by rat specific ELISA kits (Figure 5A-B). The platelet specific protein-protein interaction analysis using web tool ‘PlateletWeb’ (http://plateletweb.bioapps.biozentrum.uni-wuerzburg.de) suggested the involvement of these proteins in key regulatory events (Figure 5C). However, ELISA results for three of the proteins including fibrinogen beta, fibrinogen alpha and coronin 1A were not in accordance with the proteomic findings and thus not followed further (data not shown). Proteins with prothrombotic nature which includes important coagulation factors, tissue factor and fibrinogen were upregulated, while those having antithrombotic activity, including calcium binding proteins calreticulin and calumenin\textsuperscript{30,31} were suppressed under hypoxic conditions. The identified proteins also included some upregulated proteins playing major roles in platelet activation process. A recent study had observed TF expression in human platelets but not in platelets from mice.\textsuperscript{32} Also, there is conflicting information regarding tissue factor (TF) expression in platelets, thus we have examined the TF mRNA to verify its expression in platelets from control and hypoxia exposed animals using RT-PCR (Supplementary Figure S2). The RT-PCR data demonstrated the presence of TF transcripts in platelets and TF expression was found to be enhanced in hypoxia exposed rats. These results strongly supported proteomic and ELISA data and suggest that differential TF expression regulation in rat platelets may probably be due to signal dependent splicing as demonstrated in human platelets earlier.\textsuperscript{33}

Taken together, the hypoxia induced changes in the expression pattern of platelet proteins indicate that hypoxic exposure shifts the platelet proteome towards prothrombotic state.
Calpains play a vital role in hypoxia induced prothrombotic phenotype

Analysis of hypoxia induced differentially expressed proteins directed our focus towards calcium triggered events. Calcium based regulation of intracellular events are known to be central in platelet activation. Calpain, a thiol protease, has been found to be regulated by calcium influx and oxidative stress.\textsuperscript{34,35} Upregulation of CAPNS1 in platelets from exposed animals was evident from proteomic and ELISA results (Figures 4-5). To confirm the activation of platelet calpain, platelet intracellular calcium and calpain activity were measured in PRP, which were found to be significantly higher in exposed animals as compared to controls (Figure 6A-B). Thus, differential regulation of calcium binding proteins, elevated platelet intracellular Ca\textsuperscript{2+}, and higher calpain activity in hypoxic animals suggested that a disturbed Ca\textsuperscript{2+} homeostasis and activated calpain might contribute to hyperreactive platelets and ultimately a prothrombotic phenotype under hypoxia. To confirm the role of calpain in hypoxia induced platelet hyperreactivity, a cell permeable calpain specific inhibitor 3-(4-iodophenyl)-2-mercapto-(Z)-2-propenoic acid (PD150606), which binds to CAPNS1\textsuperscript{36} was used for further animal studies. Pre-incubation of PRP with PD150606 (50µM) for 10 minutes at 37°C resulted in reversal of hypoxia induced platelet hyperreactivity (Figure 6C). Further, the preinfusion of animals with PD150606 (1mg/kg body weight) via tail vein prior to hypoxic exposure resulted in partial reversal of hypoxia induced platelet hyperreactivity (Figure 6D) and partial restoration of reduced bleeding time in exposed animals (360 ± 45 with PD150606, vs 212 ± 22 with vehicle). These in vitro and in vivo observations indicated that CAPNS1 regulated calpain might be playing an important role during hypoxia induced thrombogenesis. To further investigate the role of CAPNS1 dependent calpain regulation in hypoxia induced thrombosis in vivo, a flow restriction animal model was used that result in generation of thrombus due to ligation (Figure 6E). In this model the thrombus
formation was induced by stasis of blood flow at the site of ligation which creates a hypoxic microenvironment. Animals pre-infused with calpain inhibitor, PD150606 (1mg/kg body weight) demonstrated significantly reduced thrombus size (0.80 ± 0.33) as compared to those preinfused with vehicle (3.41 ± 0.51, \( P < .01 \)) (Figure 6E-F). Similarly, calpain activity and platelet aggregation were found to be significantly lower in animals pre-infused with PD150606 as compared to vehicle controls (Figure 6G-H). The histological investigations also suggested the reduction in thrombus formation (Figure 6I). Moreover, to demonstrate the direct effect of hypoxia on thrombus formation, the IVC ligated animals were exposed to simulated environmental hypoxia (using decompression chamber). The thrombus formation was found to be accelerated after 6h of hypoxic exposure, reflected by significantly enhanced thrombus size in these animals as compared to unexposed ligated animals (Supplementary Figure S3). Furthermore, the effect of preinfusion with PD150606 was more pronounced as indicated by thrombus size in exposed ligated animals as compared to their unexposed counterparts (Supplementary Figure S3). To exclude the off-target effect of a single calpain inhibitor (PD150606), two additional known peptidyl cell permeable inhibitors (calpeptin and MDL28170) of calpain activity were also tested. The preinfusion of animals with these inhibitors individually resulted in significant attenuation in thrombus size (Supplementary Figure S4). However, the PD150606 proved to be the most potent among these three inhibitors used.

Next, for translational implications, we conducted a human study to investigate the calpain activity in patients who had developed DVT at high altitude. Consistent with in vivo animal studies, the calpain activity was found to be significantly higher in human patients’ plasma samples as compared to the controls (1.213 ± 0.121 vs. 0.70 ± 0.062, \( P = .0014, n = 10 \)) (Figure 6K). All ten patients selected for this study were negative for two common thrombophilia traits.
i.e. Factor V Leiden (1691G/A, rs6025) and prothrombin (202210G/A, rs1799963) mutations. Moreover, we did not find any association between thrombophilia factors and enhanced calpain activity in these patients (see Supplementary Table S2 for details). There was no significant difference in the mean age (35 yr) of patients and controls (32 yr) and were healthy with normal range of BMI (<29.9 kg/m²). We also analyzed the levels of soluble P-selectin (sP-selectin), a soluble marker of platelet activation in the plasma samples of patients and control subjects. Plasma sP-selectin was significantly higher in patients as compared to control subjects suggestive of increased platelet activation in patients (Figure 6J). These results emphasize the translational implication of pre-clinical data and strongly support a prothrombotic role for calpain under hypoxic conditions.

Discussion

This study is the first attempt to analyze the platelet proteome under hypoxic conditions as well as to demonstrate the hypoxia induced differential expression of platelet proteins. We found that the enhanced calpain activity regulated by CAPNS1 plays a major role in platelet hyperreactivity and thrombogenesis under hypoxic environment. Although in many studies hypoxia associated with high altitude has been suggested to be prothrombotic10-12, certain studies have challenged these observations.38,39 Hence, in the present study we tried to investigate and understand the effect of acute hypoxic exposure on coagulation and platelet reactivity.

We observed the prothrombotic phenotype in rats after hypoxia exposure, which was reflected by decreased bleeding and prothrombin times. The platelets from these experimental hypoxic rats showed higher reactivity, which was demonstrated by measuring multiple parameters including platelet adhesion, aggregation and activation. The hyperreactivity of platelets was further supported by the enhanced surface expression of CD41 and αIIbβ3, and increased clot retraction
in exposed animals. Earlier studies on platelet reactivity under hypoxic environment had produced conflicting results.\textsuperscript{16,39} Rats were selected as the ideal animal model to perform this study because of two factors. First, rats are preferred over mice to conduct pathophysiological studies and secondly, there has been significant similarities in rat and human platelet proteins which encourages the use of rat models to study platelets and related prothrombotic events.\textsuperscript{40} The current animal model involves hypobaric hypoxic exposure at surrounding temperature of 10°C to reproduce the hypoxic conditions at high altitude regions which have much lower temperatures than sea level regions. To evaluate the effect of cold surrounding upon hypoxia induced prothrombotic tendency, animals were exposed to hypoxia under both normothermic (chamber temperature of 28°C) and hypothermic (chamber temperature of 10°C) conditions and bleeding time as well as platelet aggregation assays were performed. The results were similar in both the groups i.e. significantly decreased bleeding time and increased platelet aggregation as compared to control (Supplementary Figure S1). These preliminary observations emphasize that hypoxia is the key factor affecting platelets at high altitude.

Until 1990s, it was believed that platelets carry proteins synthesized from their parent cell megakaryocytes. But with the evidences of \textit{de novo} protein synthesis and discovery of protein synthesis machinery in platelets, they were found to be active protein synthesizing anucleated cells. The subsequent discovery of platelet spliceosome and alternative splicing revealed a post transcriptional mode of regulation of protein expression in platelets making the platelet biology more complex. Therefore, we analysed the platelet proteome to study the effect of hypoxic exposure on platelet proteins and thus made an attempt to understand the events underlying hypoxia induced platelet hyperreactivity. The proteomic analysis of hypoxic platelets exhibited differential expression of many platelet proteins. These observations were comprised of
important coagulation cascade proteins like fibrinogen and tissue factor, some key signalling proteins involved in calcium regulation and platelet activation, and CAPNS1, which is responsible for regulating calpain activity. The bioinformatic analysis of the data revealed that the altered proteins belonged to different cell locations with diverse molecular functions, therefore suggestive of the whole platelet proteome alteration in the hypoxic environment. The increased levels of platelet tissue factor in hypoxia supports our coagulation assay data where PT was found to be decreased after hypoxia. Hypoxia not only resulted in up-regulation of prothrombotic proteins, but also conferred a decreased antithrombotic tendency by suppressing endoplasmic reticulum resident proteins calreticulin and calumenin, which have been reported to be antithrombotic in nature\textsuperscript{30,31} and maintains calcium homeostasis.\textsuperscript{20} The enhanced CAPNS1 regulated calpain activity along with elevated Ca\textsuperscript{2+} may play an important role in hypoxia induced thrombogenesis as demonstrated by \textit{in vivo} animal results. The \textit{in vivo} hypoxic setting for thrombus formation was created by inferior vena cava ligation approach, that resulted in a hypoxic microenvironment due to stasis.\textsuperscript{27,37} The up-regulation of ‘hypoxia inducible factor 1α’ in both exposed (but non-ligated) and thrombotic animals strongly supported our approach (data not shown). The pre-treatment with a highly selective and potent calpain inhibitor PD150606 resulted in reversal of platelet hyperreactivity and reduced the thrombus formation in these animals. The effect of calpain inhibition on thrombus formation was also analyzed with two additional calpain inhibitors i.e. MDL28170 and calpeptin to rule out possible off target effects.

With the current observations presented in this manuscript, few points about probable mode(s) of action of calpain can be drawn. Firstly, the increased proteolytic activity of calpain in platelets, which was observed without agonist induced aggregation (Figure 6A) demonstrates that the
calpain activation by hypoxia precedes agonist induced platelet aggregation. Secondly, platelet hyperreactivity induced by hypoxia depends largely on calpain activity as preinfusion of calpain inhibitor limits the hypoxia induced platelet aggregation (Figure 6D). Thirdly, calpain activity affects platelet hyperreactivity induced by hypoxia more than that of normoxic platelets (Figure 6D). It is known that calpain gets fully activated (and translocates to membrane) upon agonist induced platelet activation. Therefore, from the current observations it appears that hypoxic exposure accelerates the rate of transformation of calpain from its resting state to fully activated state and positively regulates hypoxia induced platelet reactivity. However, it remains to be explored as to whether hypoxia alters the known calpain reactions in platelets or it triggers an altogether different set of biomolecular reactions. These nascent findings about platelet-calpain relationship under hypoxia may provide the course for future directions to decipher the exact mechanism by which calpain protease system mediate hypoxia induced prothrombotic effects via regulating platelet function as well as drive the hunt for novel substrates of calpain in platelets under hypoxic environment. Another interesting approach can be investigating the role of calpain in modulating platelet proteome under hypoxia citing recently reported stabilization of the platelet proteome of diabetic patients by calpain inhibition.

Besides platelet activation, the prothrombotic nature of calpain can also be attributed to the diverse nature of its substrates which comprise of cytoskeletal proteins, membrane proteins, kinases, phosphatases and ATPases. More importantly, the human plasma samples of the rare type of lower limb DVT caused by hypoxic environment at high attitudes, also had elevated calpain activity and sP-selectin levels. The sample size in the study was limited due to certain constraints such as lower inhabitability and accessibility at extreme altitudes as well as
complicated logistic issues. In spite of these constraints, the important findings in the present study are suggestive of its potential translational application.

In conclusion, this study for the first time reports that a hypoxic environment results in an altered platelet proteome inducing the platelet hyperreactivity. Further, CAPNS1 mediated regulation of calpain activity appears to play a major role in hypoxia induced thrombogenesis both in animals and humans proposing a potential link between oxygen compromised status and thrombogenic index.
Acknowledgements:

The authors are extremely thankful to Directorate General Armed Forces Medical Sciences, India for approving the human study, Dr. Somnath Singh, Mrs. Minakshi Basu, Dr. Manish Sharma, Dr. R. J. Tirpude, Mr. Karan Pal, Havaldar Satish for their support during the study, Prof. Emmanuel J. Favaloro, Dr. Manojkumar Valiyaveettil, and Dr. Manju Bala for editing the manuscript.

This work was funded by Defense Research & Development Organization, Project no. SL-10/DIP-255. Tarun Tyagi is a senior research fellow of Indian Council of Medical Research.

Authorship

Contribution: T.T. performed the experiments, analyzed data and wrote manuscript; S.A., Y.A. and S.S. performed MS experiments; N.G. performed in vivo rat thrombosis model experiments; A.S. performed the platelet RNA experiments, V.N., T.C. and N.B. participated in human study; S.B.S and L.G. edited manuscript; M.Z.A. designed the study, interpreted data and drafted manuscript.

Conflicts-of-Interest disclosure: The authors declare no competing financial interests.

Correspondence: Mohammad Z. Ashraf, Head, Genomics Division, Defense Institute of Physiology & Allied Sciences, Timarpur, Delhi, 110054 India; e-mail: mohammadzashraf@gmail.com.
References


35. Ray SK, Fidan M, Nowak MW, Wilford GG, Hogan EL, Banik NL. Oxidative stress and 
Ca\textsuperscript{2+} influx upregulate calpain and induce apoptosis in PC12 cells. *Brain Res.* 

36. Todd B, Moore D, Deivanayagam CC, et al. A structural model for the inhibition of 
calpain by calpastatin: crystal structures of the native domain VI of calpain and its 
complexes with calpastatin peptide and a small molecule inhibitor. *J Mol Biol.* 
2003;328(1):131-146.

(4):S30-S34.


long-haul air travel, on coagulation, fibrinolysis, platelet function, and endothelial 

40. Yu Y, Leng T, Yun D, et al. Global analysis of the rat and human platelet proteome — the 
molecular blueprint for illustrating multi-functional platelets and cross-species function 

41. Saido, T. C., Suzuki, H., Yamazaki, H., Tanoue, K., and Suzuki, K. In situ capture of mu- 

42. Randriamboavonjy V, Isaak J, Elgheznawy A, et al. Calpain inhibition stabilizes the 

43. Randriamboavonjy V, Fleming I. All cut up! The consequences of calpain activation on 
Table 1. List of platelet proteins differentially expressed in hypoxia exposed animals

<table>
<thead>
<tr>
<th>Protein Description</th>
<th>Accession</th>
<th>Mw</th>
<th>Fold change</th>
<th>Mowse score</th>
<th>General</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apolipoprotein E</td>
<td>APOE_RAT</td>
<td>35.7</td>
<td>- 4.2</td>
<td>214</td>
<td>Mediates the binding, internalization, and catabolism of lipoprotein particles</td>
<td></td>
</tr>
<tr>
<td>Actin related Protein 2</td>
<td>ARP2_RAT</td>
<td>44.7</td>
<td>- 4.2</td>
<td>101</td>
<td>Regulation of actin polymerization</td>
<td></td>
</tr>
<tr>
<td>Calpain small subunit 1</td>
<td>CSS1_RAT</td>
<td>28.5</td>
<td>5.3</td>
<td>38</td>
<td>Regulation of calpain activity, cell migration</td>
<td></td>
</tr>
<tr>
<td>Calreticulin</td>
<td>CALR_RAT</td>
<td>48</td>
<td>- 6.0</td>
<td>347</td>
<td>Molecular calcium-binding chaperone, quality control in the ER, vascular regulatory antithrombotic role</td>
<td></td>
</tr>
<tr>
<td>Calumenin</td>
<td>CALU_RAT</td>
<td>37</td>
<td>- 6.2</td>
<td>60</td>
<td>Vascular regulatory, regulation of vit K-dependent carboxylation of multiple amino-terminal glutamate residues.</td>
<td></td>
</tr>
<tr>
<td>Complement C3 precursor</td>
<td>CO3_RAT</td>
<td>186</td>
<td>- 2.5</td>
<td>178</td>
<td>Activation of the complement system</td>
<td></td>
</tr>
<tr>
<td>Coronin1A</td>
<td>COR1A_RAT</td>
<td>51</td>
<td>- 2.9</td>
<td>74</td>
<td>A crucial component of the cytoskeleton of highly motile cells</td>
<td></td>
</tr>
<tr>
<td>Elongation factor 1 gamma</td>
<td>EF1G_RAT</td>
<td>50</td>
<td>- 2.2</td>
<td>92</td>
<td>Protein synthesis</td>
<td></td>
</tr>
<tr>
<td>Alpha Enolase</td>
<td>ENOA_RAT</td>
<td>47.1</td>
<td>3.0</td>
<td>68</td>
<td>Activator of the complement system and a mediator of the local inflammatory response glycolysis, plays a part in growth control, hypoxia tolerance and allergic responses</td>
<td></td>
</tr>
<tr>
<td>Fibrinogen alpha</td>
<td>FIBA_RAT</td>
<td>86.6</td>
<td>2.7</td>
<td>90</td>
<td>Yield monomers that polymerize into fibrin and acting as a cofactor in platelet aggregation.</td>
<td></td>
</tr>
<tr>
<td>Fibrinogen gamma</td>
<td>FIBG_RAT</td>
<td>50.6</td>
<td>3.3</td>
<td>357</td>
<td>Yield monomers that polymerize into fibrin and acting as a cofactor in platelet aggregation.</td>
<td></td>
</tr>
<tr>
<td>Guanine nucleotide binding protein 2</td>
<td>GBB2_RAT</td>
<td>37.3</td>
<td>-3.0</td>
<td>63</td>
<td>Glycoproteins bound to plasma memb., involved as a modulator or transducer in various transmembrane signaling systems</td>
<td></td>
</tr>
<tr>
<td>Tissue factor precursor</td>
<td>TF_RAT</td>
<td>33.4</td>
<td>2.1</td>
<td>20</td>
<td>Key component of extrinsic coagulation pathway.</td>
<td></td>
</tr>
<tr>
<td>Haptoglobin precursor</td>
<td>HPT_RAT</td>
<td>38.5</td>
<td>2.5</td>
<td>71</td>
<td>Combines with free plasma hemoglobin, preventing loss of iron through the kidneys, secreted in response to hypoxia as well as acute inflammation</td>
<td></td>
</tr>
<tr>
<td>Heat shock cognate 71</td>
<td>HSP7C_RAT</td>
<td>70.8</td>
<td>2.3</td>
<td>150</td>
<td>Chaperon, regulation of transcription, response to stress</td>
<td></td>
</tr>
<tr>
<td>Protein Name</td>
<td>Species</td>
<td>Gene</td>
<td>Protein</td>
<td>Other Information</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-----------------------------------</td>
<td>----------</td>
<td>-------</td>
<td>----------</td>
<td>------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T kininogen 1</td>
<td>KNT1_RAT</td>
<td>47.7</td>
<td>3.6</td>
<td>117</td>
<td>Acute phase protein</td>
<td></td>
</tr>
<tr>
<td>Myosin light chain PP 6</td>
<td>MYL6_RAT</td>
<td>16.9</td>
<td>5.9</td>
<td>87</td>
<td>Motor protein, also involved in collagen induced platelet activation</td>
<td></td>
</tr>
<tr>
<td>Tropomyosin alpha 1</td>
<td>TPM1_RAT</td>
<td>32.6</td>
<td>4.2</td>
<td>50</td>
<td>Binds to actin filaments, cytoskeletal reorganization</td>
<td></td>
</tr>
<tr>
<td>Tropomyosin beta</td>
<td>TPM2_RAT</td>
<td>32.8</td>
<td>4.6</td>
<td>96</td>
<td>Binds to actin filaments, cytoskeletal reorganization</td>
<td></td>
</tr>
<tr>
<td>Tropomyosin alpha 4</td>
<td>TPM4_RAT</td>
<td>28.5</td>
<td>2.0</td>
<td>64</td>
<td>Binds to actin filaments, cytoskeletal reorganization</td>
<td></td>
</tr>
<tr>
<td>Serrotransferrin precursor</td>
<td>TRFE_RAT</td>
<td>76.3</td>
<td>2.4</td>
<td>108</td>
<td>Precursor to macromolecular activators of phagocytosis which enhance leukocyte phagocytosis via FCYRII receptor</td>
<td></td>
</tr>
<tr>
<td>Janus Kinase 3</td>
<td>JAK3_RAT</td>
<td>122</td>
<td>3.6</td>
<td>26</td>
<td>Cytokine mediated signaling and regulation of cytosolic calcium.</td>
<td></td>
</tr>
<tr>
<td>Alpha-1-antitrypsin</td>
<td>A1AT_RAT</td>
<td>46.1</td>
<td>4.2</td>
<td>50</td>
<td>Inhibitor of serine proteases.</td>
<td></td>
</tr>
<tr>
<td>Rho Guanine nucleotide exchange factor 7</td>
<td>ARHG7_RAT</td>
<td>73</td>
<td>4.2</td>
<td>19</td>
<td>Involved in RAC1 dependent signaling, cell migration, attachment and cell spreading.</td>
<td></td>
</tr>
<tr>
<td>Vitamin D binding Protein</td>
<td>VTDB_RAT</td>
<td>53.5</td>
<td>2.0</td>
<td>99</td>
<td>Carries Vitamin D in plasma, has T lymphocyte surface Association</td>
<td></td>
</tr>
<tr>
<td>Alpha-2-macroglobulin receptor-associated protein precursor</td>
<td>AMRP_RAT</td>
<td>42</td>
<td>5.7</td>
<td>33</td>
<td>Binds to members of LDL Receptor family and inhibits binding of their ligands</td>
<td></td>
</tr>
<tr>
<td>Non-muscle caldesmon (L-caldesmon)</td>
<td>CALD1_RAT</td>
<td>60.5</td>
<td>3.1</td>
<td>49</td>
<td>Actin and myosin binding protein implicated in regulation of actomyosin interactions</td>
<td></td>
</tr>
</tbody>
</table>
Figure Legends

Figure 1. Hypoxia exposure results in hypercoagulation. Rats were exposed to simulated hypoxic conditions as described in methods; tail vein bleeding assay, PT and aPTT assays were performed. (A) Rat tail was transected 4 mm from the tip and immersed in warm normal saline (37°C) and the time taken for complete cessation of blood flow was noted (which was significantly lower in hypoxia exposed rats than in controls). (B) In the filter paper method, the tail tip was blotted gently onto Whatman paper at one min intervals. The diameter of blood spots and the duration of bleeding were significantly lower in exposed rats compared to control rats. (C and D) PT and aPTT were measured in citrated plasma and reflected a similar trend as bleeding time. Data are presented as mean ± SEM, (n=10). *P < .05 vs control.

Figure 2. Agonist dependent increase in platelet adhesion, aggregation and ATP release in exposed animals. (A-F) Platelets were isolated from both control and hypoxia exposed rats, fluorescently labelled and allowed to adhere to collagen or fibrinogen precoated plates for upto 60 minutes at 37°C. (A,D) Representative images of fluorescence microscopy based platelet adhesion on type I collagen (10µM) and fibrinogen (20µM) coated plates using fluorogenic dye calcein. Images were captured using FITC filter on Motic Inverted Microscope AE31(x200 original magnification). (B,C,E and F) Quantitation of platelet adhesion data were expressed as average area or size of adhered platelet clumps and per cent area covered by adhered platelets on collagen coated plate (top panel) and fibrinogen coated plate (bottom panel) after 60 minute incubation. Quantitation was performed by Motic ImagePlus 2.0 software. (G) For platelet aggregation assay, platelet-rich plasma from rats of indicated groups was incubated at 37°C for atleast 3 min was induced by ADP with stirring at 1200 rpm and optically monitored. The rate
and extent of ADP induced platelet aggregation was higher in hypoxia exposed animals compared to controls. Representative aggregation curves are shown in response to ADP (2.5 and 5µM). (H) Bar graph shows aggregation results expressed as maximal amplitude of aggregation. (I) Representative ATP release curve in response to ADP analysed using luciferase assay. (J) Quantitation of aggregation and ATP release were expressed as maximum amplitude. Data are presented (mean ± SEM) as average results of at least three independent experiments, (n= 6). *P < .05, **P < .01 vs control. See Supplementary Methods for details.

Figure 3. Exposure to hypoxia induces higher surface expression of platelet activation markers and increased clot retraction. (A-D) Washed platelets from hypoxia exposed and control animals were either stimulated with ADP (activated) or left unstimulated (resting). The αIIbβ3 and CD41 surface expressions were determined by flowcytometric analysis using FITC-conjugated antibodies. Flowcytometry histograms from representative experiments (A, B) and quantitation of fluorescence expressed as mean fluorescence intensities (C,D) are shown. Data are presented as mean ± SEM, a typical result of at least three independent experiments (n≥6, *P< .05 vs activated control). (E, F) Clot retraction assay was performed in PRP isolated from control and exposed animals as described in Methods. The clot retraction in exposed animals was found to be significantly greater than in control animals. Shown are representative images of the clot retraction assay for different incubation periods and the clot size was quantified using ImageJ software and expressed as per cent retraction of clot (mean ± SEM) (n=6). *P < .05, **P < .01. See Supplementary Methods for details.
Figure 4. Platelet proteome analysis from control and exposed rats. Platelets were isolated from both control and hypoxia exposed animals, total platelet protein was prepared for platelet proteome analysis. Each protein lysate was subjected first to isoelectric focussing, followed by SDS-PAGE in second dimension as described in Methods. The differentially expressed proteins were identified using MALDI TOF MS/MS. (A) Representative 2DE gel image of platelet proteome (4-7 pI range, 13 cm). (B) The pie chart of platelet protein features from 2DE gel after differential analysis of gel images by Progenesis SameSpots software, shows that 27% of platelet protein features in range of pI 4-7 were altered in hypoxia exposed platelets. (C) A typical heat map of selected portion from 2DE gels showing representative differential spots in 3D, prepared by using ImageJ software. (D-E) Bioinformatic analysis of identified proteins was performed with GeneCodis web tool (http://genecodis.cnb.csic.es), (D) GO cellular compartment analysis and (E) GO molecular function analysis. See Supplementary Methods for details.

Figure 5. ELISA based validation and protein-protein interaction analysis of identified differential proteins. Selective differential proteins identified by MS/MS were used for heat map generation and 3D view analysis. The differentially expressed proteins were quantified in platelet samples from control and exposed rats by ELISA and the platelet specific protein-protein interaction analysis was performed with an open web source ‘PlateletWeb’ (http://plateletweb.bioapps.biozentrum.uni-wuerzburg.de). (A) Representative protein spots of identified proteins on 2DE gel with heat map in 3D view. (B) ELISA based validation of protein levels in platelet samples expressed as mean ± SEM (n=6, *P < .05, **P < .01). (C) Interaction maps of differentially expressed proteins showing their interacting partners using PlateletWeb database. The proteins directly involved in coagulation cascade are highlighted.
Figure 6. Increased calpain activity after hypoxia exposure in rats and human DVT patients and antithrombotic effect of calpain inhibition in vivo and ex vivo. Platelets were isolated from indicated groups and processed for either florescence based calpain activity or intracellular calcium assays as described in supplemental methods. (A) Quantitation of calpain activity in platelets demonstrating higher proteolytic activity of calpain in exposed animals. (B) Intraplatelet free calcium levels measured by Fura-2 based fluorogenic assay showed increased iCa$^{2+}$ in exposed animals. (C-D) Representative aggregation curves showing reversal of hypoxia induced platelet aggregation by, pre-incubation with calpain inhibitor PD150606 (50µM) ex vivo (C) and by preinfusion of PD150606 (1mg/kg body weight) in vivo (D). (E-I) The antithrombotic and platelet inhibitory effect of pre-infusion of PD150606 via tail vein in rat model of stasis induced thrombosis as described in ‘Methods’. (E) Representative images of extracted portions of inferior vena cava with thrombus (top panel) from thrombotic animals with their heat maps (bottom panel) showing smaller thrombus in the case of PD150606 pre-infusion. (F) Quantitations of size of the thrombus isolated from IVC portions of thrombotic animals (n=8). (G) Decreased calpain activity in platelets isolated from thrombotic animals pre-infused with PD150606. (H) Representative platelet aggregation curves showing strong negative effect of PD150606 pre-infusion in thrombotic rats as compared to vehicle control or no infusion. Data are presented as mean ± SEM (n=8) and analysed by unpaired t test. *$P<.05$, **$P<.01$ vs thrombosis + vehicle (I) Hematoxylin-eosin stained sections of thrombus with vessel wall showing morphological differences (x200 original magnification). (J-K) The plasma samples from human DVT patients from HA regions and from control individuals were analysed for soluble P selectin levels and calpain activity as described (J) Higher soluble P-selectin levels in
plasma samples (data presented as box and whiskers plot) indicated hyperactive platelets in HA induced DVT patients. (K) Calpain activity in human plasma samples of HA induced DVT patients compared to age and sex matched healthy controls shown as a scatter plot (n=10). The activity in patients was significantly ($P = .0014$) higher in comparison to controls. Data are presented as mean ± SEM, n=10 in each group, and analyzed by t-test as compared to respective controls.
Figure 1
Tyagi et al, 2013

A

B

C

D

From www.bloodjournal.org by guest on November 1, 2017. For personal use only.
Figure 2
Tyagi et al, 2013

A
Collagen 10μM
Control Exposed

B
Area of adherent platelets

C
% area covered by adherent platelets

D
Fibrinogen 20μM
Control Exposed

E
Area of adherent platelets

F
% area covered by adherent platelets

G
ADP 2.5μM
Light Transmission (%)
Control Exposed
1 min

H
 aggregation

I
ATP release (nmol)
ADP
Control Exposed
1 min

J
ATP release (nmol)
Control Exposed

32
Figure 3
Tyagi et al, 2013

A

\[ \alpha I I \beta \]

Counts

10^0 10^1 10^2 10^3

Fluorescence Intensity (log scale)

B

CD41

Counts

10^0 10^1 10^2 10^3

Fluorescence Intensity (log scale)

C

Activated

Resting

Control
Exposed

Mean fluorescence intensity

D

Activated

Resting

Control
Exposed

Mean fluorescence intensity

E

0 min 30 min 60 min

Control
Exposed

F

% Retraction of Clot

Control
Exposed

Incubation time (minutes)

**

*
Figure 4
Tyagi et al, 2013

A

Mw

100

10

4

pl

7

B

unchanged

73%

changed

27%

up

21%

down

6%

C
downregulated

Exposed

Control

upregulated

D

endoplasmic reticulum

4%

Golgi apparatus

4%

plasma membrane

8%

ribonucleoprotein complex

3%

soluble fraction

4%

microsome

4%

stress fiber

3%

cytosol

8%

myofilbr

3%

cytoskeleton

6%

perinuclear region of cytoplasm

6%

actin cytoskeleton

4%

protein complex

5%

fibrinogen complex

3%

extracellular region

10%

extracellular space

10%

filamentous actin

5%

E

lipoprotein binding and transport

5%

ion binding and transport

3%

calcium channel regulator activity

10%

calcium binding

8%

Enzyme inhibitor activity

8%

mRNA binding

3%

ATPase Activity

3%

nucleotide binding

10%

protein homodimerization

13%

protein binding

23%

motor activity

3%

vitamin binding and transport

3%

kinase activity

5%

cytoskeletal component

5%

protein binding
Figure 6
Tyagi et al, 2013

A

B

C

D

E

F

G

H

I

J

K

From www.bloodjournal.org by guest on November 1, 2017. For personal use only.
Altered expression of platelet proteins and calpain activity mediate hypoxia induced prothrombotic phenotype

Tarun Tyagi, Shadab Ahmad, Neha Gupta, Anita Sahu, Yasmin Ahmad, Velu Nair, Tathagat Chatterjee, Nitin Bajaj, Shantanu Sengupta, Lilly Ganju, Shashi Bala Singh and Mohammad Z. Ashraf