LIST OF NOMINEES TWO MOST SIGNIFICANT SPECIFIC PUBLICATIONS/RESEARCH PAPERS RELEVANT TO THE RESEARCH WORK

S. No.	Authors	Title of the paper	Journal, (Year) Vol, Page No.	Status as Author	Impact Factor	Citation
1.	Gupta N, Sahu A, Prabhakar A, Chatterjee T, Tyagi T, Kumari B, Khan N, Nair V, Bajaj N, Sharma M, **Ashraf MZ*.**	Activation of NLRP3 inflammasome complex potentiates venous thrombosis in response to hypoxia.	**Proceedings of National Academy of Sciences – USA** (2017) 114(18):4763-4768.	Corresponding author	**12.7**	**141**
	The study elucidated the previously unknown cause of thrombus formation at high altitude. The publication highlighted the causal role of NLRP3-inflammasome and IL-1β in hypoxia-induced thrombosis. This work also established that thrombosis high altitude is centrally regulated by a complex network of coagulatory and inflammatory processes, critically linked through hypoxia inducible factors -1α (HIF-1α). The same work has also proposed the potential therapy for thrombosis at high altitude, (highlighted by Nature India, doi:10.1038/nindia.2017.143).					
2.	Tarun Tyagi, Shadab Ahmad, Neha Gupta, Anita Sahu, Yasmin Ahmad, Velu Nair, Tathagat Chatterjee, Nitin Bajaj, Shantanu Sengupta, Lilly Ganju, Shashi Bala Singh, **M. Zahid Ashraf***	Altered expression of platelet proteins and calpain activity mediate hypoxia-induced prothrombotic phenotype.	**Blood** (2014), 20;123(8):1250-60	Corresponding author	**22.6**	**117**
	The study published with editorial commentary has revealed the platelet specific novel biomarker for the detection of venous thrombogensis (formation of blood clots in legs, brain, lungs) at high altitude. The work also contributed exceptionally in proposing a novel biomarker 'Calpain' (Patent filed with IPO vide no. 733/DEL/2014) that will help in early diagnosis of thrombosis and provide timely treatment to Indian Army Jawans posted at extremely hostile terrain protecting our borders.					

Activation of NLRP3 inflammasome complex potentiates venous thrombosis in response to hypoxia

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Venous thromboembolism (VTE), caused by altered hemostasis, remains the third most common cause of mortality among all cardiovascular conditions. In addition to established genetic and acquired risk factors, low-oxygen environments also predispose otherwise healthy individuals to VTE. Although disease etiology appears to entail perturbation of hemostasis pathways, the key molecular determinants during immediate early response remain elusive. Using an established model of venous thrombosis, we here show that systemic hypoxia accelerates thromboembolic events, functionally stimulated by the activation of nucleotide binding domain, leucine-rich-containing family, pyrin domain containing 3 (NLRP3) inflammasome complex and increased IL-1β secretion. Interestingly, we also show that the expression of NLRP3 is mediated by hypoxia-inducible factor 1-alpha (HIF-1α) during these conditions. The pharmacological inhibition of caspase-1, in vivo knockdown of NLRP3, or HIF-1α other than IL-1β-neutralizing antibodies attenuated inflammasome activation and curtailed thrombosis under hypoxic conditions. We extend the significance of these preclinical findings by studying modulation of this pathway in patients with altitude-induced venous thrombosis. Our results demonstrate distinctive, increased expression of NLRP3, caspase-1, and IL-1β in individuals with clinically established venous thrombosis. We therefore propose that an early proinflammatory state in the venous milieu, orchestrated by the HIF-induced NLRP3 inflammasome complex, is a key determinant of acute thrombotic events during hypoxic conditions.

thrombosis | hypoxia | HIF-1α | NLRP3 inflammasome | IL-1β

Epidemiological studies during recent years have unprecedentedly highlighted venous thromboembolism (VTE) as a key comorbidity factor during several life-threatening medical conditions. In addition to clinical complications such as cancer (1), cardiovascular diseases (2), surgery (3), and trauma (4), hypoxia as experienced during ascent to high altitude has emerged as another predisposing factor for VTE (5–8). A significantly higher incidence of deep vein thrombosis and pulmonary embolism (8, 9), portal vein thrombosis (10), cerebral venous thrombosis (11), transient ischemic attacks, and stroke (12) has been observed at high to extreme altitude (13). Despite clinical relevance, a caveat in our basic understanding of early molecular events underlying hypoxia-induced venous thrombosis poses a major bottleneck for effective design of interventional approaches.

Recent studies have highlighted a strong link between hypoxia responses and inflammation, involving activation of multiple cell types including lymphocytes, platelets, and endothelium (14, 15). Plausibly, in addition to direct mechanism involving hypoxia-induced modulation of hemostasis and coagulation factors (5, 16), pleiotropic modalities such as sterile inflammation could be involved. The role of this axis and key mediators in hypoxia-induced hypercoagulation, however, remains to be experimentally validated. The activation of coagulation pathways per se has been shown to promote inflammation in the system (17, 18). This secondary inflammation can engage in a positive feed-forward loop to aggravate the prothrombotic phenotype. Thus, the cause or consequence relationship between inflammation and hypercoagulation during hypoxia remains far from being resolved.

We recently showed that hypobaric hypoxia promotes a prothrombotic propensity through the involvement of a crucial cysteine protease, calpain (19). In continuing work, we blended an unbiased systems-level approach with targeted pharmacological inhibition and in vivo siRNA-mediated knockdown strategies to show that the activation of nucleotide binding domain, leucine-rich-containing family, pyrin domain containing 3 (NLRP3) inflammasome complex augments thrombus formation in response to hypoxia. We reinforced significance of this key preclinical finding from the animal model by demonstrating the activation of the NLRP3 gene in patients, who developed thrombosis at high altitudes. We also present evidence that the activation of this complex is an early response to hypoxia and is critically regulated by hypoxia-inducible factor 1-alpha (HIF-1α).

Results

For all experiments described here, we have used uniform group nomenclature. After inferior vena cava (IVC) ligation (also refer to *SI Materials and Methods*), the group of animals kept in normal atmosphere conditions was designated as thrombotic (T), whereas the ligated ones kept under simulated hypobaric hypoxia were identified as hypoxia thrombotic (HT). Sham surgery controls kept under normal environmental conditions were designated as normoxic (N), and those kept in simulated hypoxia were identified as hypoxic (H).

Significance

Hypoxia predisposes otherwise healthy individuals to venous thrombosis, but the underlying mechanism has been unclear. Our study revealed a causal role for nucleotide binding domain, leucine-rich-containing family, pyrin domain containing 3 (NLRP3) inflammasome and IL-1β during hypoxia-induced venous thrombosis. We further show a direct association between NLRP3 and hypoxia-inducible factor 1-alpha (HIF-1α) during these conditions. Specific interventions within the hypoxia–HIF-1α–NLRP3–IL-1β axis in the venous milieu significantly reduced venous thrombosis in our animal model. Notably, we also observed modulation of similar pathways in patients diagnosed with altitude-induced venous thrombosis. Our study thus revealed thrombosis at high altitude to be centrally regulated by a complex network of coagulatory and inflammatory processes, critically linked through HIF-1α.

Author contributions: M.S. and M.Z.A. designed research; N.G., A.S., A.P., T.C., T.T., B.K., and N.B. performed research; N.K. and V.N. contributed new reagents/analytic tools; N.G., A.S., A.P., M.S., and M.Z.A. analyzed data; and N.G., M.S., and M.Z.A. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1620458114/-/DCSupplemental.

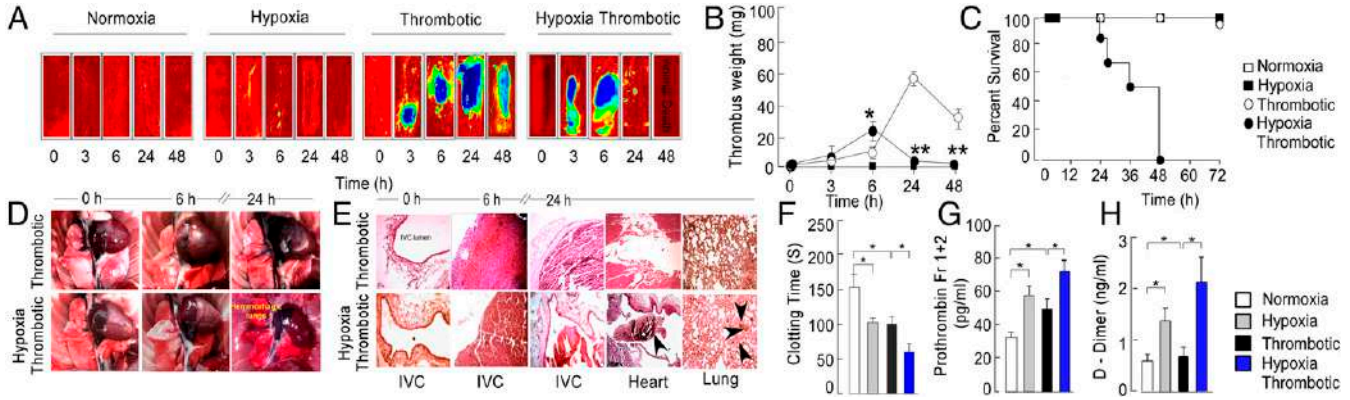

Fig. 1. Systemic hypoxia accelerates venous thromboembolism. (*A*) Representative heat maps representing IVC in situ. (*B*) Thrombus weight. (*C*) Percentage survival rate calculated using Log-rank (Mantel-Cox) test ($P < .001$). (*D*) Representative in situ images of gross lungs obtained from thrombotic ($n = 5$) and hypoxia thrombotic groups ($n = 7$). (*E*) Representative hematoxylin-eosin-stained cross-sectional images of IVC, lungs, and heart from indicated groups. Estimation of (*F*) clotting time, (*G*) secretory prothrombin fragment 1+2, and (*H*) D-dimer at the 6-h point. $*P < 0.05$ and $**P < 0.01$.

Hypoxic Exposure Aggravates Venous Thrombosis, Leading to Fatal Thromboembolic Consequences. We first established temporal kinetics of thromboembolic events in four groups of animals. In situ examination at various intervals (3, 6, 24, and 48 h) revealed significantly larger thrombus in the HT group (Fig. 1 *A* and *B*). Further, thrombus weight peaked earlier in the HT group (6 h). We observed a reduction in thrombus weight (Fig. 1*B*) with extended hypoxic exposure (≥24 h), along with a higher mortality rate in this group (Fig. 1 *C* and Fig. S1*A*). The thrombus from the HT group was denser compared with that for the T group at early times, suggesting exaggerated thrombogenesis with hypoxia exposure (Fig. S1 *A* and *B*). Further, the lumen of IVC in the HT group appeared to be completely obstructed after 6 h of hypoxic exposure with prominent recanalization compared with the T group (Fig. S1*A*), suggesting an early onset of thrombolytic process as well. Thrombotic animals in normoxia (T) showed progressive increase in thrombus formation (until 24 h), but negligible mortality (Fig. 1 *A*–*C* and Fig. S1*A* and *B*).

Necroptic examination in the HT group suggested mortality to be associated with hemorrhagic lungs (Fig. 1*D*), whereas no sign of bleeding or thrombus could be noticed in the lungs of animals from the NT group. Thromboembolus was found in the heart and lungs of animals in the HT group (24 h), with concomitant evidence for resolution from the IVC (Fig. 1 *D* and *E* and Fig. S1*A*), suggesting a shift of hemostatic equilibrium toward fibrinolytic pathway at this point. We therefore investigated both thrombotic and fibrinolytic axes in these animals.

We recorded parameters associated with prothrombotic conditions, including clotting time, prothrombin fragment 1+2 (corresponding to cleavage of prothrombin by factor Xa), and D-dimers in all groups. The clotting time was significantly shortened in the HT group (Fig. 1*F*). Further, a higher level of prothrombin fragment 1+2 (20) and D-dimer (Fig. 1 *G* and *H*) supported an accelerated thrombogenic activity in this group.

We also observed increased expression of free plasminogen activation inhibitor-1 (PAI-1) (Fig. S1*C*) in HT animals. The levels of fibrinolytic enzyme urokinase plasminogen activator (uPA) decreased, whereas tissue plasminogen activator (tPA) levels (Fig. S1 *D* and *E*) increased in thrombotic animals. Fibrinolytic molecules were also elevated in the H group, suggesting that hypoxia per se could modulate hemostasis pathways. These results suggested that systemic hypoxia exacerbated hypercoagulation, with compensatory hyperfibrinolysis that likely culminated in disseminated intravascular coagulation, leading to sudden animal death.

Evidence for Modulation of Hypoxia Response Pathways During Pathogenesis of Venous Thrombosis. Our observation that systemic hypoxia accelerated thrombotic cascade prompted us to investigate the functional involvement of hypoxia response pathways. As shown in Fig. 2 *A*–*C*, similar to nonligated animals exposed to hypoxia, the expression of HIF-1α and its target genes [vascular endothelial growth factor (*VEGF*) and erythropoietin (*EPO*)] was significantly increased in thrombotic groups (highest in HT group), supporting the possibility that the hypoxic milieu is an integral component involved during perturbation of hemostasis.

We studied the expression of 84 well-documented hypoxia-responsive genes, using real-time PCR analysis under specific conditions (Fig. S2*A*). We observed nearly 77% of these genes to be differentially expressed after 6 h of exposure to hypoxia (Fig. S2*B*); 76% of these genes were differentially regulated by IVC ligation alone (T), and 86% by IVC ligation followed by exposure to hypoxia (HT) (Fig. S2*B*). Further, the majority of genes up-regulated by hypoxia were present in the T dataset (Fig. S2*C*). The expression value of most of these genes was uniformly highest in the HT group, further suggesting exacerbation of responses by systemic hypoxia (Fig. S2 *D* and *E*).

Genome-Wide Expression Analysis Suggests Hierarchical Activation of Intense Inflammatory Response During Hypoxia-Induced Thrombus Formation. We next used RNA sequencing to obtain an unbiased cross-sectional snapshot of changes in gene expression in all groups. Nearly 606 unique transcripts (Entrez Gene) were significantly modulated by at least ±twofold in the H group (Dataset S1). The T and HT groups showed a much higher number of differentially expressed genes (4,966 and 4,664, Entrez Gene, respectively; Dataset S1). As evident from Fig. 3*A*, 487 hypoxia-specific genes (H) were also differentially expressed in thrombotic groups (T and HT). A significant number of differentially expressed genes were unique to T and HT groups (which did not appear in the H group), which was a clear indication that specific additional pathways were perturbed during thrombotic (T and HT) conditions (Fig. 3*A* and Fig. S3*A*).

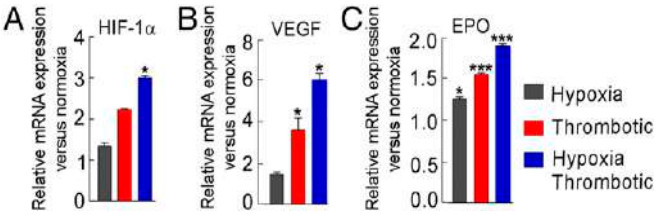

Fig. 2. Functional role of hypoxia response pathways during thrombosis. Relative expression of HIF-1α (*A*), VEGF (*B*), and EPO (*C*) at the 6-h point. 18S rRNA was used as internal control, and expression was normalized to that of the normoxic group (N). Mean ± SEM ($n = 6$) is shown. $*P < 0.05$; $***P < 0.001$.

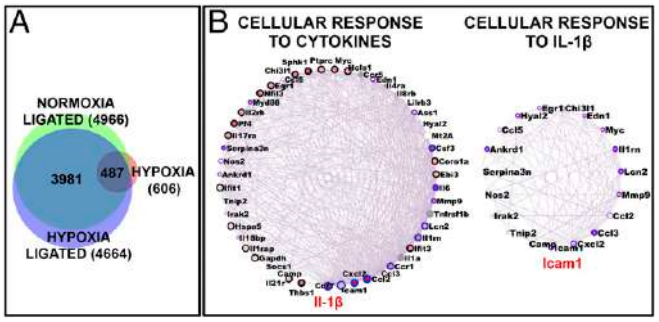

Fig. 3. Genome-wide expression and network analysis during thrombosis. Transcriptome sequencing was performed on RNA isolated from IVC of animals from all four groups, followed by data mining. (*A*) Intersection among differentially expressed genes, represented using Venn diagram. (*B*) Key networks (degree-sorted circle view) representative of inflammatory responses (significantly enriched from up-regulated genes in thrombotic group). Highest-degree nodes (IL-1β and ICAM 1) are indicated in red.

We extracted nonoverlapping biological processes, significantly modulated in the HT group, using GeneMania (21) (Fig. 3*B* and Fig. S3 *B* and *C*). The pathways enriched from the set of down-regulated genes principally indicated perturbation of metabolic processes characteristic of hypoxic stress (Fig. S3*C*). The processes enriched from up-regulated genes (Fig. S3*B*) suggested activation of angiogenesis, oxidative stress response, and calcium ion homeostasis in addition to other hypoxia response pathways. The dataset also revealed modulation of key processes regulating thrombotic propensity, including coagulation and platelet activation (Fig. S3*B*). We also observed processes representing strong and hierarchical activation of immune response in the HT group, as represented by networks related to early immune response such as cytokine signaling, activation of innate immune response, immunological synapse, and activation of adaptive immune system (involving T lymphocytes) (Fig. S3*B*). The subsequent analysis of these data yielded two lines of evidence suggesting that IL-1β could be the central regulator of such immune response in these animals. First, we observed a distinct network of genes regulating cellular responses to IL-1β (Fig. 3*B*). Second, multiple measures of centrality in immune response to specific subnetworks presented IL-1β as an important "bottleneck" gene, whose deletion led to collapse of the biological network. As can be readily observed from Fig. 3*B*, IL-1β is the most connected (highest degree) node or hub gene in the network related to cellular responses to cytokines.

Activation of NLRP3 Inflammasome in Response to Hypoxia-Induced Thrombosis. We next measured levels of IL-1β secreted in the localized IVC milieu after 6 h of hypoxia exposure and, in agreement with our transcriptome data, observed a significant increase in concentration of IL-1β in the T and HT groups (Fig. 4*A*). The concomitant activation of two signaling pathways is critical for the secretion of functionally active IL-1β: the first signal ensures transcriptional up-regulation of IL-1β, and the second leads to the assembly of a pro-IL-1β-processing inflammasome complex. Our transcriptome data (Fig. S4*A*) were distinctively evident for the activation of these arms in thrombotic groups. Real-time PCR analysis confirmed significant increase in the expression of IL-1β (Fig. 4*B*), caspase-1 (Fig. 4*C*), and NLRP3 (Fig. 4*D*) transcripts in the HT group. Further, caspase-1/ICE activity and NLRP3 expression were significantly elevated in the IVC milieu (tissue homogenate; Fig. 4 *E* and *F*) and plasma (Fig. S4 *B* and *C*) of animals under these conditions. Taken together, these data suggest an early activation of the NLRP3 inflammasome pathway in thrombotic animals. In addition to IL-1β, NLRP3 inflammasome also regulates IL-18 secretion. Consistent with this proposition, we also observed a significant increase in IL-18 levels (Fig. 4*G*) in IVC of animals in thrombotic groups (T and HT).

We observed increased recruitment of neutrophils (elastase-positive foci) and macrophages (mac-387–positive regions) in the IVC of animals from the HT group (Fig. S4*D*). The increase in myeloperoxidase activity and monocyte chemotactic protein-1 levels (Fig. S4 *E* and *F*) further supported recruitment of neutrophils and macrophages during thrombosis.

The secretion of IL-1β and activation of IL-1 receptor signaling initiates a cascade of events leading to modulation of expression of numerous other cytokines. We therefore investigated changes in the levels of cytokines, using cytokine-specific antibody arrays. We observed an increase in the secretion of proinflammatory cytokines in thrombotic animals (Fig. 4*H* and Fig. S4*G*). Taken together, we concluded that hypoxic signal culminates in the activation of NLRP3 inflammasome and active IL-1 signaling that eventually results in a prothrombotic state.

Hypoxia Response Pathways Functionally Regulate Expression of NLRP3 and IL-1β During Thrombosis. To investigate the relationship between hypoxia and inflammasome activation, we first used a pharmacological inhibitor of hypoxia response pathway, CAY10585, which inhibits accumulation and transcriptional activity of HIF-1α and thus, decreases the expression of HIF-1α target genes (22). As shown in Fig. S5*A*, CAY10585 treatment led to decreased expression of HIF-1α and its target genes (*VEGF* and *EPO*). We also observed conspicuous differences in the expression of 84 hypoxia pathway genes (modulated by hypoxia; Fig. S2 *A*–*E*) in CAY10585-treated groups (Fig. S5*B*).

We next tested whether CAY10585 modulated expression of NLRP3 pathway genes in our model system. As shown in Fig. 5 *A* and *B*, the expression of NLRP3 and IL-1β transcripts was significantly lowered in the animals pretreated with this inhibitor. These observations suggested a key role of hypoxia in regulating the expression of NLRP3 inflammasome components.

To establish the functional relevance of inhibition by CAY10585, we next recorded thrombus weight, prothrombin fragment 1+2, and D-dimer in the presence of this inhibitor. We observed a reduction in thrombus weight (Fig. 5*C*), level of prothrombin fragment 1+2 (Fig. 5*D*), and D-dimer (Fig. 5*E*) in the HT group, pretreated with this inhibitor and thus clearly

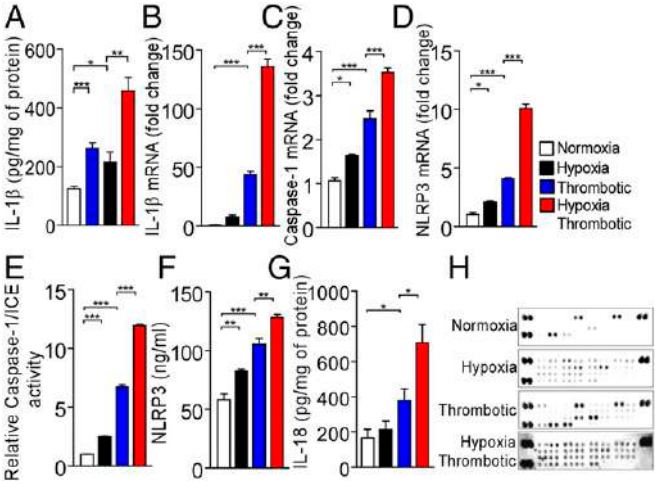

Fig. 4. Activation of NLRP3 inflammasome during hypoxia-induced thrombosis. (*A*) Estimation of IL-1β by ELISA in tissue homogenates after 6 h of exposure. (*B*–*D*) Relative levels of expression of IL-1β (*B*), Caspase-1 (*C*), and NLRP3 (*D*) transcripts (real-time PCR) at indicated points in the HT group. 18S rRNA was used as an internal control, and expression was normalized to that of animals in normoxic group. (*E*) Relative Caspase-1/ICE activity in indicated groups. Estimation of NLRP3 (*F*) and IL-18 (*G*) levels by ELISA after 6 h exposure. (*H*) Estimation of cytokines (using cytokine arrays) from tissue homogenates from all four groups. The results are representative of a minimum of three independent experiments. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

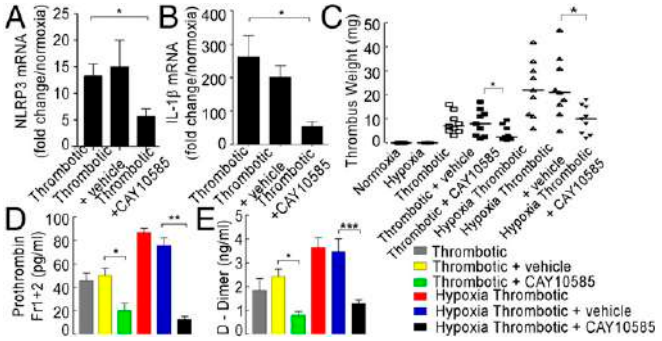

Fig. 5. Hypoxia-induced proinflammatory state is regulated by transcriptional activity of HIF-1α. HIF inhibitor, CAY10585 (100 μg/kg) was administered (intravenous) before IVC ligation and hypoxic challenge for 6 h. Relative expression of (*A*) NLRP3 and (*B*) 1L-1β transcripts (real-time PCR). (*C*) Dot plot showing medians of thrombus weight, (*D*) Levels of prothrombin fragment 1+2, (*E*) D-dimer (ELISA) estimation in plasma samples. Mean ± SEM is shown ($n \geq 6$). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

supporting a functional role of hypoxia response pathways in the activation of the NLRP3 inflammasome and thrombogenesis.

Evidence for a Functional Role of HIF-1α During Hypoxia-Induced NLRP3 Expression. We next tested whether HIF-1α, the central regulator of hypoxia responses (23), was involved in hypoxia-induced expression of NLRP3 and thrombogenesis. We used an in vivo siRNA approach. As shown in Fig. 6*A*, animals in the HT group (6 h), treated with HIF-1α siRNA, showed a significant reduction in the accumulation of the HIF transcript. The expression of NLRP3 (Fig. 6*B*), IL-1β (Fig. 6*C*), and caspase-1 (Fig. S6*A*) transcripts was significantly reduced in these groups. Further, caspase-1/ICE activity (Fig. S6*B*) and NLRP3 protein levels (Fig. S6*C*) were also diminished in the plasma of these groups. The animals in siRNA-treated groups showed significant reduction in thrombus weight (Fig. 6*D*), with an increase in clotting (Fig. 6*E*) and prothrombin time (PT; Fig. S6*D*). The knock-down of HIF-2α in our experiments led to a significant increase in HIF-1α expression, a likely compensatory response (Fig. 6*A*). Concurrent with an increase in expression of HIF-1α, the expression of NLRP3, IL-1β, and caspase-1 was also elevated, along with increase in thrombus weight, clotting time, and PT (Fig. 6 *A*–*E* and Fig. S6*D*).

We next analyzed the NLRP3 promoter for putative HIF-responsive elements/sites. Our in silico analysis returned three sequences closely matching HIF-responsive element consensus (Fig. S6*E*). We therefore performed chromatin immunoprecipitation experiments, using two different HIF-1α antibody clones and probes spanning NLRP3 promoter. As shown in Fig. 6*F*, we consistently observed recruitment of HIF-1α at one of these sites [−975 w.r.t transcription start site (TSS)], implicating functional involvement of HIF-1α in regulating NLRP3 expression during HT conditions.

NLRP3 Inflammasome Axis Inhibition Curtails Hypoxia-Induced Thrombosis. To establish whether the activation of NLRP3 inflammasome played a causal role in the initiation and propagation of thrombosis in our model system, we used three different inhibition strategies and, subsequently, performed in situ thrombus examination, recorded thrombus weight and length, and checked in vivo levels of prothrombin fragment 1+2 and D-dimer, in addition to monitoring the aggregation of platelets isolated from these animals.

We first knocked down NLRP3 transcript in the animals, using in vivo grade siRNA (10 mg/kg body weight), aiming to check the assembly of the NLRP3 inflammasome complex. We observed a significant reduction in caspase-1/ICE activity (Fig. S7*A*), in addition to reduced thrombus (Fig. 7 *A*–*C*), in IVC of the HT group treated with NLRP3 siRNA. We also observed a significant reduction in the levels of prothrombin fragment 1+2 and the D-dimer (Fig. 7 *D* and *E*, respectively). These results suggested a causal role for NLRP3 during thrombosis. The platelets isolated from animals pretreated with NLRP3 siRNA, before IVC ligation, also showed reduced aggregation in response to ADP (used as physiological agonist) (Fig. 7*F*). This observation suggested an upstream role of NLRP3 inflammasome to platelet activation, which is a vital step in the thrombogenic cascade.

In the next set of experiments, we used SML0499 to inhibit the catalytic activity of caspase-1, required for the production of active IL-1β from its proform. The in situ thrombus examination, thrombus weight and length, prothrombin fragment 1+2, D-dimer, and ex vivo platelet aggregation assay using ADP are shown in Fig. S7 *B*–*G*. The cumulative results from all these assays showed that inhibition of caspase-1 activity reduced thrombogenesis under hypoxic conditions.

Finally, we injected specific antibodies against active IL-1β and thus limited its bioavailability, essential for signaling via cognate IL-1 receptors. As presented in Fig. S7 *H*–*M*, these animals also showed a reduction in thrombus formation, as evident from a similar set of parameters described earlier. Taken together, these results (Fig. 7 *A*–*F* and Fig. S7 *B*–*M*) demonstrated an indispensable role for NLRP3 inflammasome-mediated active IL-1β generation in hypoxia-induced thrombus formation. The expression analysis in specific cell types [peripheral blood mononuclear cells (PBMNs), platelets, and vessel wall] suggested that both NLRP3 and IL-1β increased significantly in the PBMNs, apart from thrombus isolated from a localized (ligated) venous site (Fig. S8).

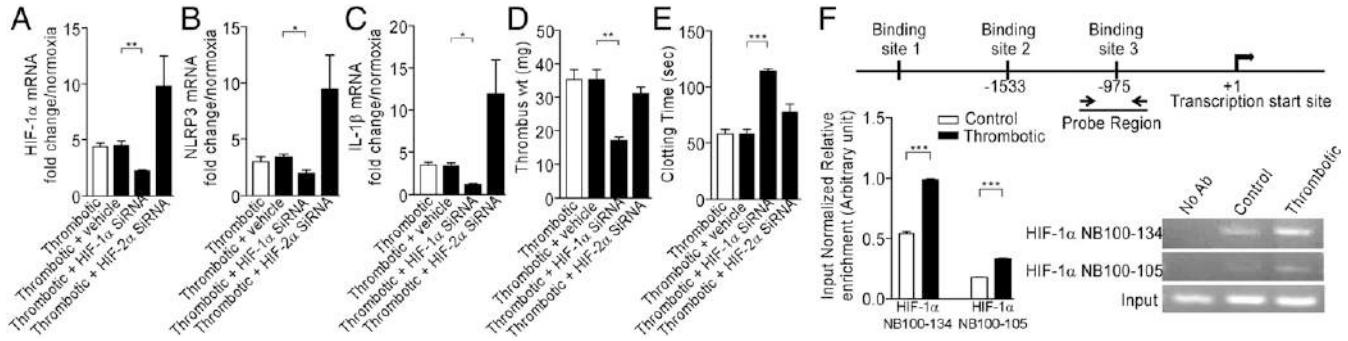

Fig. 6. HIF-1α regulates NLRP3 expression and thrombogenesis. Animals were treated with in vivo grade HIF-1α and HIF-2α siRNA. After RNA isolation from indicated groups, real-time PCR was performed for (*A*) HIF-1α, (*B*) NLRP3, (*C*) IL-1β. Thrombus weight (*D*) and clotting time (*E*) were also recorded. (*F*) Chromatin immunoprecipitation with two different HIF-1α antibody (indicated) and primer pairs spanning putative sites (indicated). The enrichment of NLRP3 promoter region in chromatin immunoprecipitation experiments was quantitated and plotted to obtain the bar graph (mean ± SEM) shown in the figure. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

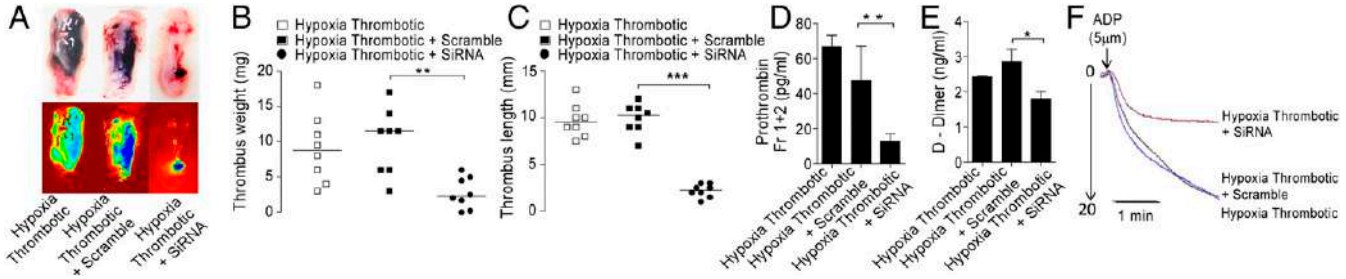

Fig. 7. Knock-down of NLRP3 curtails hypoxia-Induced thrombosis. NLRP3 was knock-down using in vivo grade siRNA complexes and specific parameter studied. (*A*) Photomicrographs of thrombosed IVC; (*B*) thrombus weight; (*C*) thrombus length (median indicated); (*D*) Prothrombin fragments 1+2; (*E*) D-dimer, and (*F*) platelet aggregation assay in groups (indicated in figure), 6 h postinduction. All datasets are representative of a minimum of three independent experiments. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

Evidence for the Involvement of the NLRP3 Inflammasome in Human Patients with Altitude-Induced Thrombosis. We next sought to investigate the potential involvement of the NLRP3 inflammasome in clinically confirmed cases of VTE ($n = 18$) occurring in response to the hypoxic environment. The demographic, clinical, and specific genetic parameters of patients with VTE are presented in Fig. S9. We observed a relatively higher number of patients lacking thrombophilic traits [including deficiency of protein C, protein S, and ATIII, in addition to activated protein C (APC) resistance], major SNPs [factor V Leiden, prothrombin, tissue factor pathway inhibitor (TFPI), fibrinogen-β, methylene tetrahydrofolate reductase (MTHFR), and PAI-1], and other additional risk factors (including lipid profile, homocysteine, and blood glucose levels) known to be associated with a predisposition to VTE (Fig. S9). These observations likely suggested that VTE episodes in these individuals were potentially triggered by environmental conditions (hypoxia) prevailing at altitudes.

To test the likely involvement of the NLRP3 inflammasome pathway, we next studied relative expression of key genes of this pathway in these patients. As shown in Fig. 8 *A–D*, we observed an increase in NLRP3, caspase-1, IL-1β, and IL-18 mRNA expression in patients compared with healthy age-matched controls. Furthermore, caspase-1/ICE activity was significantly elevated along with increased levels of NLRP3 (protein) and IL-1β in the patient samples (plasma, Fig. 8 *E–G*). This dataset supported involvement of the NLRP3 inflammasome pathway in the pathogenesis of VTE in individuals exposed to the hypoxic challenge.

Fig. 8*H* schematically depicts the scheme of events causally underlying activation of thrombosis in hypoxic environments.

Discussion

The present study revealed a causal role of strong inflammatory response involving NLRP3 and IL-1β in activating hypoxia-induced thrombogenic cascade in the venous milieu. Of critical note is the fact that HIF-1α, known to regulate a plethora of human diseases (24), emerged as the key node connecting hypoxia responses to proinflammatory state via its ability to regulate the expression of NLRP3 (transcript) under these conditions. Conceivably, the evidence for a direct connection between HIF-1α and NLRP3 is likely to have general implications, especially as a target for intervention in other pathological conditions emanating from hypoxia and the proinflammatory state.

The biological activation of IL-1β requires parallel activation of pathways, culminating in transcriptional up-regulation of IL-1β, increased expression of NLRP3, and enzymatic activation of caspase-1 (25). Some recent reports suggested platelets as a likely source of IL-1β (26–29), produced by virtue of a stored repertoire of molecules (mRNA, inflammasome components) and cue-dependent processing/secretion during thrombogenesis. Thus, an important question pertaining to the possibility of platelet-origin IL-1β in sustaining an intense phenotype, such as that observed in our study, remains paradigmatic. Our present dataset provides some additional information in this regard. We observed up-regulation of IL-1β transcript in addition to other inflammasome components in mononuclear cells (PBMNs), apart from thrombus isolated from localized (ligated) venous site, and this up-regulation could be prevented using HIF inhibitor, CAY10585, or HIF-1α-specific siRNA. It thus is reasonable to assume that the immune cells are likely to play an important role in regulating the intensity of venous thrombosis, likely through de novo cue-dependent transcriptional up-regulation of IL-1β and inflammasome pathway genes (NLRP3, caspase-1). Finally, the fact that we also observed a concomitant increase in the relative expression of NLRP3, caspase-1, IL-1β, and IL-18 transcripts in peripheral blood cells of volunteers who developed VTE at high altitudes lends strong support to this proposition.

The hypoxic milieu in vivo has been proposed as a critical regulator of sterile inflammation and consequent pathological effects. The mechanistic basis appears to include modulation of intrinsic mitochondrial redox homeostasis (30), in addition to activation of toll-like-receptors and various danger signals such as ATP release from necrotic cells (31). The issue of hypoxia-induced

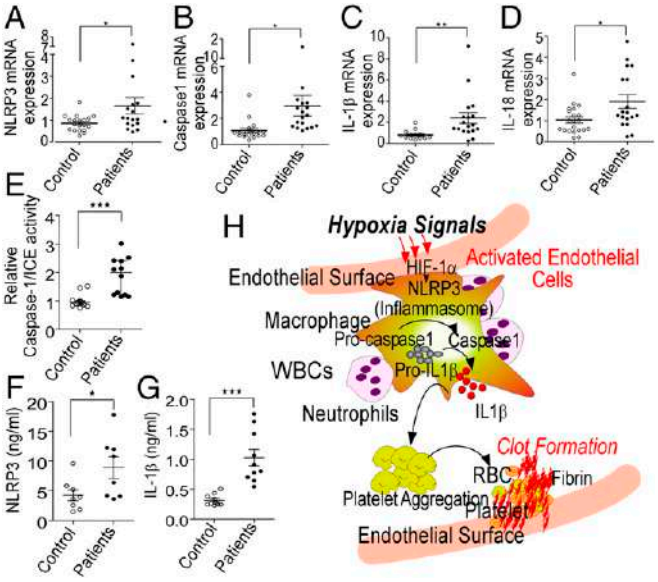

Fig. 8. Evidence for involvement of NLRP3 inflammasome components in patients with altitude-induced venous thrombosis. (*A–D*) RNA was isolated from PBMNs from the blood samples of patients ($n = 18$), and real-time PCR for indicated genes was performed. β-actin was used as an internal control. The relative expression of NLRP3 (*A*), caspase-1 (*B*), 1L-1β (*C*), and IL-18 (*D*) transcripts. (*E*) Caspase-1/ICE activity in plasma samples from patients and controls ($n = 12$). Estimation of NLRP3 (*F*) and IL-1β (*G*) levels in plasma samples from patients and controls ($n \geq 8$) Median values for individual groups are also shown. $*P < 0.05$; $**P < 0.01$. (*H*) Diagrammatic representation of an inferred scheme of events during hypoxia-induced thrombosis.

inflammasome activation and the proinflammatory state breeding pathophysiological outcomes at high altitude encompasses conflicting studies and opinions (15, 32). Our transcriptome data revealed dense gene networks related to strong proinflammatory responses, involving both innate and adaptive immune cells (Fig. 3). Further, as described here, hypoxia-induced thrombosis could be circumvented by inflammasome inhibition, suggesting an early role of inflammation in this process. We also showed that inhibition of hypoxia response pathways (using pharmacological inhibitor or siRNA) prevented transcriptional up-regulation of NLRP3 and IL-1β with significant antithrombotic effects. Taken together, these results posit that hypoxia regulates significant pathological effects via its ability to promote the proinflammatory state.

Virtually all mechanistic understanding, elucidated to date, pertaining to specific forms of thrombosis keep complying with Virchow's Triad, although in somewhat kaleidoscopic molecular patterns regulating individual hypercoagulable states (33). In keeping with the essence of this fact, venous and arterial thrombosis also appears to entail principally similar events, but conspicuously divergent origins. Although early endothelial injury is an established modus operandi of arterial thrombosis, it appears to be dispensable during early stages of venous forms that precipitate under diverse conditions and stimuli. A recent study showed that the NLRP3 inflammasome inhibitor, Arglabin, curtailed the atherogenic effect of high-fat diet in ApoE2.K_i mice (34). In view of such information, it is tempting to speculate that activation of NLRP3 inflammasome complex could constitute a unifying molecular cornerstone between diverse pathological states and various forms of thrombosis.

Hypercoagulable state is also known to predispose an individual to elevated risk for pulmonary embolism, which is a bigger clinical challenge and often more fatal, arising from increased thrombus dissemination. We too observed a somewhat similar phenomenon in our animal model with disseminated intravascular coagulation or hypercoagulation concomitant with elevated fibrinolytic activity, under hypoxic condition (Fig. 1). Conversely, a thrombotic state could also manifest as a result of skewing of homeostasis toward hypercoagulation due to a less-effective fibrinolytic system. Taken together, such arguments define an apparent paradigm for clinical significance and an area of future investigation. In view of our results, we posit that the strength of biological cues propagating individual pathways (coagulatory-fibrinolytic) critically regulate resultant effects with phenotypic manifestation such as localized thrombosis, consumptive coagulopathy, or pulmonary embolism.

In summary, our study revealed an important target, NLRP3 inflammasome, for hypoxia-induced venous thrombosis in addition to reinforcing an intriguing complexity involving intricately interacting coagulatory, thrombolytic, and inflammatory hubs at its core.

Materials and Methods

Detailed materials and methods are included in *SI Materials and Methods*.

Animal Experiments. All experiments were conducted in compliance with guidelines of the Committee for Purpose of Control and Supervision of Experiments on Animals, Government of India. Male Sprague–Dawley rats, weighing 250–300 g, were used and exposed to hypobaric hypoxia, using environmental chamber simulating 429 torr. The IVC ligation model for in vivo thrombosis was used as previously described by us (19).

Human Studies. Human studies were conducted in strict compliance with the ethical standards of Indian Council of Medical Research. Informed consent was obtained from the subjects as per Declaration of Helsinki. Young male patients with VTE (n = 18) evacuated from high-altitude regions to tertiary care facilities (Command Hospital Chandimandir, Chandigarh or Army Hospital, New Delhi) were enrolled. Equal numbers of healthy, age-matched male subjects with no prior history for VTE were included as controls.

Statistics. Data are presented as mean ± SEM. The statistical significance of differences was evaluated using unpaired t test or Mann-Whitney test. Bonferroni post hoc test was done for multiple group comparison, using Prism 5 (GraphPad) software. The statistical significance of differences were represented as $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

ACKNOWLEDGMENTS. We acknowledge Dr. Shashi B Singh, Directorate General Armed Forces Medical Services, Dr. Srishti Gupta, Dr. Iti Garg, and Dr. R. J. Tirpude for their support. This study was funded by Defence Research and Development Organization Project SL-10/DIP-255.

1. Zwicker JI, et al. (2009) Tumor-derived tissue factor-bearing microparticles are associated with venous thromboembolic events in malignancy. *Clin Cancer Res* 15:6830–6840.
2. Zhou X, et al. (2010) Incidence and risk factors of venous thromboembolic events in lymphoma. *Am J Med* 123:935–941.
3. Chan MY, Andreotti F, Becker RC (2008) Hypercoagulable states in cardiovascular disease. *Circulation* 118:2286–2297.
4. Demers C, et al. (1998) Incidence of venographically proved deep vein thrombosis after knee arthroscopy. *Arch Intern Med* 158:47–50.
5. Bendz B, Rostrup M, Sevre K, Andersen TO, Sandset PM (2000) Association between acute hypobaric hypoxia and activation of coagulation in human beings. *Lancet* 356:1657–1658.
6. Lapostolle F, et al. (2001) Severe pulmonary embolism associated with air travel. *N Engl J Med* 345:779–783.
7. Smallman DP, McBratney CM, Olsen CH, Slogic KM, Henderson CJ (2011) Quantification of the 5-year incidence of thromboembolic events in U.S. Air Force Academy cadets in comparison to the U.S. Naval and Military Academies. *Mil Med* 176:209–213.
8. Ward M (1975) *Mountain medicine: A clinical study of cold and high altitude* (Crosby Lockwood Staples, London).
9. Zangari M, et al. (2013) Could hypoxia increase the prevalence of thrombotic complications in polycythemia vera? *Blood Coagul Fibrinolysis* 24:311–316.
10. Anand AC, Saha A, Kumar R, Sharma V, Jha SK (2000) Portal system thrombosis: A new dimension of high altitude illnesses. *Trop Gastroenterol* 21:172–173.
11. Cheng S, Chng SM, Singh R (2009) Cerebral venous infarction during a high altitude expedition. *Singapore Med J* 50:e306–e308.
12. Jha SK, Anand AC, Sharma V, Kumar N, Adya CM (2002) Stroke at high altitude: Indian experience. *High Alt Med Biol* 3:21–27.
13. Gupta N, Ashraf MZ (2012) Exposure to high altitude: A risk factor for venous thromboembolism? *Semin Thromb Hemost* 38:156–163.
14. Mannucci PM, Gringeri A, Peyvandi F, Di Paolantonio T, Mariani G (2002) Short-term exposure to high altitude causes coagulation activation and inhibits fibrinolysis. *Thromb Haemost* 87:342–343.
15. Eltzschig HK, Carmeliet P (2011) Hypoxia and inflammation. *N Engl J Med* 364:656–665.
16. Rider P, et al. (2012) The transcription of the alarmin cytokine interleukin-1 alpha is controlled by hypoxia inducible factors 1 and 2 alpha in hypoxic cells. *Front Immunol* 3:290.
17. Mileno MD, et al. (1995) Coagulation of whole blood stimulates interleukin-1 beta gene expression. *J Infect Dis* 172:308–311.
18. Reitsma PH, Rosendaal FR (2004) Activation of innate immunity in patients with venous thrombosis: The Leiden Thrombophilia Study. *J Thromb Haemost* 2:619–622.
19. Tyagi T, et al. (2014) Altered expression of platelet proteins and calpain activity mediate hypoxia-induced prothrombotic phenotype. *Blood* 123:1250–1260.
20. Páramo JA (2010) Prothrombin fragments in cardiovascular disease. *Adv Clin Chem* 51:1–23.
21. Warde-Farley D, et al. (2010) The GeneMANIA prediction server: Biological network integration for gene prioritization and predicting gene function. *Nucleic Acids Res* 38:W214–220.
22. Lee K, et al. (2007) (Aryloxyacetylamino)benzoic acid analogues: A new class of hypoxia-inducible factor-1 inhibitors. *J Med Chem* 50:1675–1684.
23. Semenza GL (2007) Life with oxygen. *Science* 318:62–64.
24. Semenza GL (2000) HIF-1 and human disease: One highly involved factor. *Genes Dev* 14:1983–1991.
25. Martinon F, Mayor A, Tschopp J (2009) The inflammasomes: Guardians of the body. *Annu Rev Immunol* 27:229–265.
26. Lindemann S, et al. (2001) Activated platelets mediate inflammatory signaling by regulated interleukin 1beta synthesis. *J Cell Biol* 154:485–490.
27. Denis MM, et al. (2005) Escaping the nuclear confines: Signal-dependent pre-mRNA splicing in anucleate platelets. *Cell* 122:379–391.
28. Hottz ED, et al. (2013) Platelets mediate increased endothelium permeability in dengue through NLRP3-inflammasome activation. *Blood* 122:3405–3414.
29. Brown GT, Narayanan P, Li W, Silverstein RL, McIntyre TM (2013) Lipopolysaccharide stimulates platelets through an IL-1β autocrine loop. *J Immunol* 191:5196–5203.
30. Usui F, et al. (2015) Inflammasome activation by mitochondrial oxidative stress in macrophages leads to the development of angiotensin II-induced aortic aneurysm. *Arterioscler Thromb Vasc Biol* 35:127–136.
31. Weinberg SE, Sena LA, Chandel NS (2015) Mitochondria in the regulation of innate and adaptive immunity. *Immunity* 42:406–417.
32. Wanderer AA (2011) Hypoxia and inflammation. *N Engl J Med* 364(20):1976.
33. López JA, Chen J (2009) Pathophysiology of venous thrombosis. *Thromb Res* 123:S30–S34.
34. Abderrazak A, et al. (2015) Anti-inflammatory and antiatherogenic effects of the NLRP3 inflammasome inhibitor arglabin in ApoE2.Ki mice fed a high-fat diet. *Circulation* 131:1061–1070.
35. Trapnell C, et al. (2012) Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. *Nat Protoc* 7:562–578.

Regular Article

PLATELETS AND THROMBOPOIESIS

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

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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.

Oxygen-compromised environments, such as high altitude, air travel, and sports, and pathological conditions, such as 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, 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 calpain small subunit 1 expression. The inhibition of calpain activity demonstrated reversal of 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 at extreme altitude had elevated plasma calpain activities and increased soluble P-selectin level. In summary, this study suggests that augmented calpain activity is associated with increased incidence of thrombosis under hypoxic environments. (*Blood*. 2014;123(8):1250-1260)

Introduction

Hypoxia, experienced either during physical activities such as ascent to mountains, air travel, or sports activities or with pathological conditions such as solid tumors has been suggested to be associated with thrombotic episodes.[1-5] 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.[6-9] 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, focusing on hematological factors and on proteins involved in thrombin generation and fibrinolysis, to understand altitude-induced thrombotic events.[10-13] 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; only a few reports focused on platelet reactivity at high altitude.[14-17] In fact, in chronic obstructive pulmonary disease and sleep apnea, hypoxia has been associated with increased platelet reactivity.[18,19] 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 acts as an important secondary messenger to regulate the fundamental platelet reactivity via the key downstream signaling cascades.[20]

The proteome analysis, which is used for identifying novel proteins and pathways, has become an ideal tool to study anucleated cells such as platelets. Previously, platelet proteomic analysis revealed differential regulation of proteins in response to agonists and in diseases such as acute coronary syndrome.[21,22] 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

Submitted May 10, 2013; accepted November 21, 2013. Prepublished online as *Blood* First Edition paper, December 2, 2013; DOI 10.1182/blood-2013-05-501924.

Part of this study was presented as a poster at the 22nd International Congress on Thrombosis, Nice, France, October 6-9, 2012.

The online version of this article contains a data supplement.

There is an Inside *Blood* commentary on this article in this issue.

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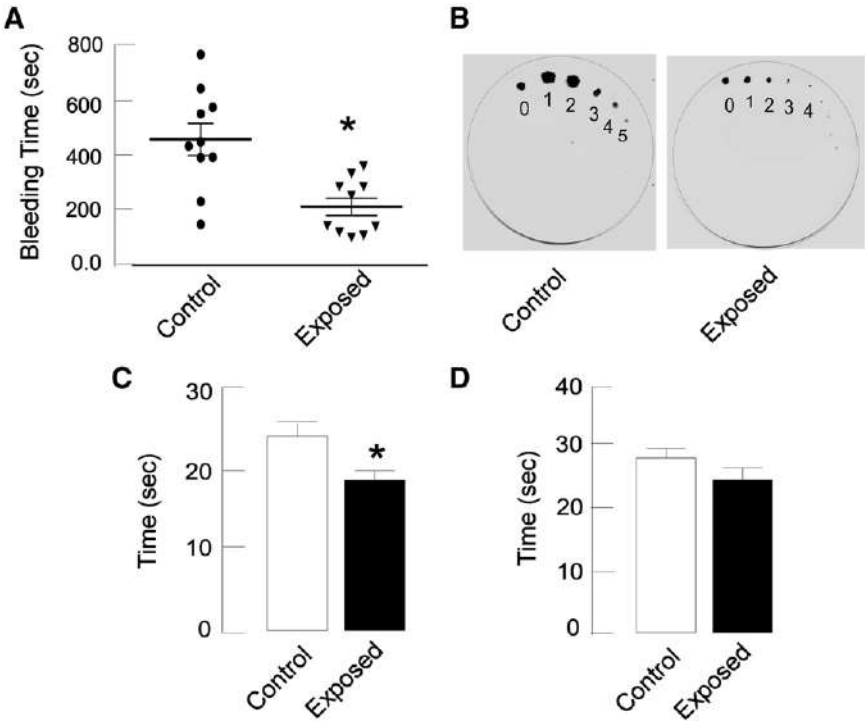

Figure 1. Hypoxia exposure results in hypercoagulation. Rats were exposed to simulated hypoxic conditions as described in Materials and methods; tail vein bleeding assay, PT, and activated partial thromboplastin time (aPTT) assays were performed. (A) Rat tail was transected 4 mm from the tip and immersed in warm normal saline (37°C), and the time taken for complete cessation of blood flow was noted (it 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 1-minute intervals. The diameter of blood spots and the duration of bleeding were significantly lower in exposed rats compared with control rats. (C-D) PT (C) and aPTT (D) 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.

proteins, which are involved in coagulation, calcium homeostasis, signal transduction, acute phase response, and cytoskeletal reorganization. The disturbed calcium homeostasis was further explored by quantifying cytosolic Ca^{2+} levels and calpain activity in platelets of hypoxic animals. Calpain small subunit 1 (CAPNS1), which functions like a chaperone for calpain proteases[23] and controls cell spreading and migration,[24] 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,[25,26] the prothrombotic role for calpain under hypoxia was further confirmed by in vivo model of hypoxia-induced thrombosis. To investigate the potential of these preclinical investigations for clinical relevance, the calpain activity and soluble P-selectin (sP-selectin) level were analyzed in plasma samples of patients who developed deep vein thrombosis (DVT) at high altitudes (>3648 meters). The results from these studies for the first time suggest that a hypoxic environment alters the platelet proteome and induces platelet hyperreactivity, leading to a prothrombotic phenotype that is mainly mediated by activation of calpain.

Materials and methods

Materials

α-thrombin, adenosine diphosphate (ADP), and chronolume luciferin-luciferase reagents were purchased from Chrono-log. Anti-CD41 and anti-$\alpha IIb\beta_3$ antibodies were purchased from Abcam. Immobilized pH gradient gel strips, ampholytes, and mineral oil for 2-dimensional electrophoresis were products of GE Healthcare, and trypsin was from Promega. Rat-specific enzyme-linked immunosorbent assay (ELISA) kits were purchased from Bmassay and USCN Life Sciences Inc. PD150606 was from Tocris Biosciences. The reverse transcription-polymerase chain reaction (RT-PCR) kit for platelet RNA analysis and all other reagents were from Sigma-Aldrich.

Animal exposure to simulated high-altitude conditions

All experiments were conducted in accordance with the Committee for the Purpose of Control and Supervision of Experiments on Animals, Government of India. Male Sprague-Dawley rats weighing 150 to 250 g were kept under standard laboratory conditions. The animals were exposed to simulated high-altitude (hypobaric hypoxia) conditions in a specially designed animal decompression chamber, which was maintained at pressure of 282 torr (equivalent to an altitude of 7620 meters with 8% oxygen) and at 10°C for various durations. Animals were exposed to simulated hypobaric hypoxia with cold surrounding, as at high-altitude regions, the associated low temperatures may also affect the biological systems. After optimization of the temperature range, 10°C was selected as a suitable temperature and was used for further hypoxic exposure studies. On the basis of initial hematological and coagulation screening data and previous studies, platelet studies were conducted after 6 hours of 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[27] with some modifications (see the supplemental Methods on the *Blood* Web site for details).

Human studies

Young (<40 years) male patients with lower limb DVT evacuated to either Western Command Hospital Chandimandir, Chandigarh, or Army Research and Referral Hospital, 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 meters). Patients with preexisting systemic diseases, malignancy, any prior surgery, or vasculitis were excluded. All patients had their diagnosis confirmed by objective imaging methods. A complete thrombophilia screening assessment, composed of protein C and S deficiency, antithrombin III deficiency, factor V Leiden, and prothrombin 20210G/A polymorphism screening, was also performed; laboratory investigations details are provided in the supplemental Methods. Equal numbers of healthy, age-matched male participants 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 of the mean (SEM). The statistical significance between 2 groups was tested by nonparametric unpaired 2-tailed Student *t* test, using Prism 5 software, and for multiple-group

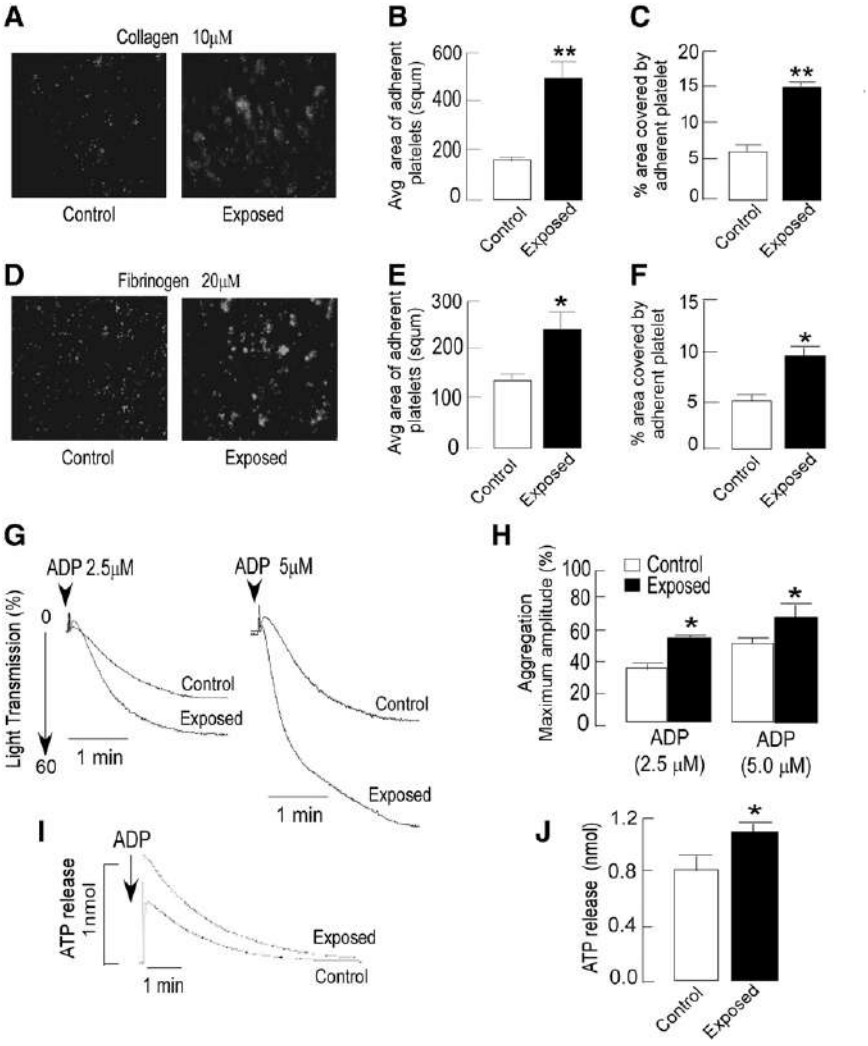

Figure 2. Agonist-dependent increase in platelet adhesion, aggregation, and ATP release in exposed animals. (A-F) Platelets were isolated from both control and hypoxia-exposed rats, fluorescently labeled, and allowed to adhere to collagen or fibrinogen precoated plates for up to 60 minutes at 37°C. (A,D) Representative images of fluorescence microscopy-based platelet adhesion on type 1 collagen-coated (10 μM) and fibrinogen-coated (20 μM) plates, using fluorogenic dye calcein. Images were captured using a fluorescein isothiocyanate filter on a Motic Inverted Microscope AE31(×200 original magnification). (B-C,E-F) Quantitation of platelet adhesion data was expressed as average area or size of adhered platelet clumps and percentage area covered by adhered platelets on the collagen-coated plate (top) and the fibrinogen-coated plate (bottom) after a 60-minute incubation. Quantitation was performed by Motic ImagePlus 2.0 software. (G) For the platelet aggregation assay, platelet-rich plasma from rats of indicated groups, incubated at 37°C for at least 3 minutes, 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 with control animals. Representative aggregation curves are shown in response to ADP (2.5 and 5 μM). (H) A bar graph shows aggregation results expressed as maximal amplitude of aggregation. (I) Representative ATP release curve in response to ADP analyzed using a 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 3 independent experiments (n = 6). $^*P < .05$, $^{**}P < .01$ vs control. See the supplemental Methods for details.

comparison, 1-way analysis of variance was applied, followed by Dunnett's test. A P value less than .05 was considered significant.

Results

Hypoxic exposure results in hypercoagulative state

To investigate whether hypoxic exposure results in a prothrombotic phenotype in rats, bleeding time (by tail immersion and filter paper methods) and coagulation assays (prothrombin time, aPTT) were performed after hypoxic exposure. A significant shortening of bleeding times (202 ± 29 seconds) was observed in exposed animals compared with controls (440 ± 54 seconds; $P < .05$; 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 level, hematocrit levels, or platelet count in hypoxia-exposed animals, with the exception of a decrease in white blood cell count (supplemental Table 1).

As hypoxia exposure to rats also involved cold surroundings (chamber temperature of 10°C), a bleeding time assay was also performed in rats exposed to hypoxia under normothermic surrounding (chamber temperature of 28°C) to determine the possible effect (if any) of moderate cold surroundings on hypercoagulative tendency. Exposure under normothermic conditions resulted in a significant reduction in bleeding time, as under hypothermic conditions (supplemental Figure 1A). 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 percentage area covered by the same were significantly higher in the exposed group compared with the controls (Figure 2A-F); the increase was greater on the collagen surface. Next, we tested whether platelet aggregation and dense granule release were affected by 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 the exposed group compared with the response to ADP (2.5 and 5 μM; $P < .05$), whereas only a 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 ($P < .05$)

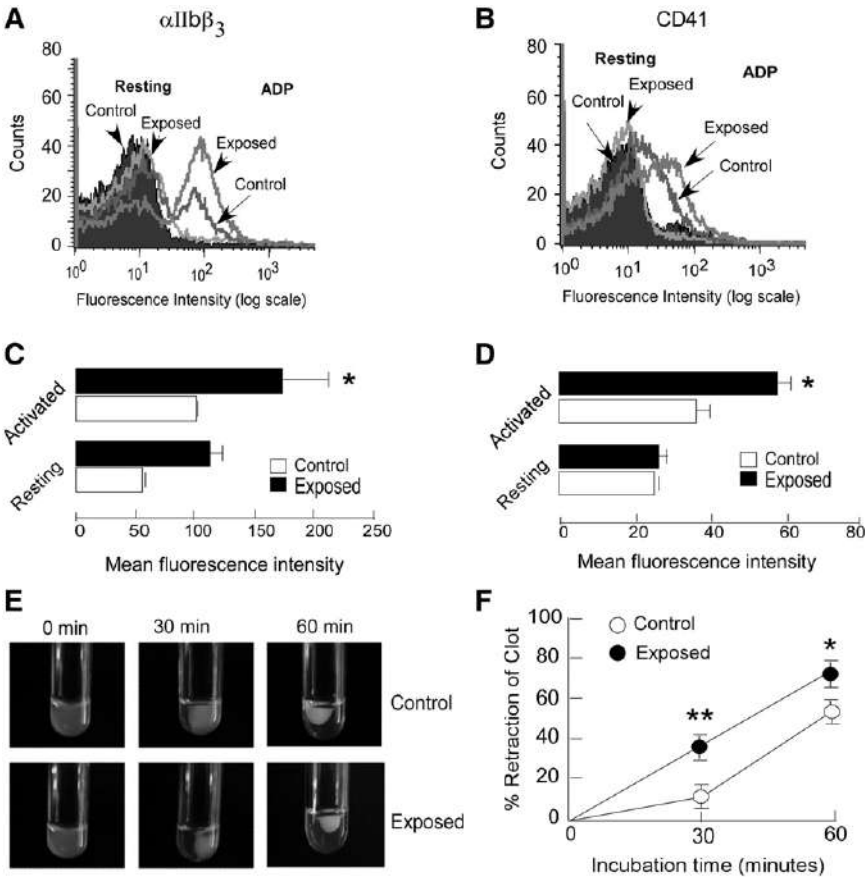

Figure 3. Exposure to hypoxia induces higher surface expression of platelet activation markers and increased clot retraction. (A-D) Washed platelets from hypoxia-exposed and control animals were either stimulated with ADP (activated) or left unstimulated (resting). The $\alpha IIb\beta_3$ and CD41 surface expressions were determined by flow cytometric analysis, using fluorescein isothiocyanate-conjugated antibodies. Flow cytometry 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 3 independent experiments ($n \geq 6$; $^*P < .05$ vs activated control). (E-F) Clot retraction assay was performed in platelet rich plasma (PRP) isolated from control and exposed animals, as described in the supplemental 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; the clot size was quantified using ImageJ software and expressed as percent retraction of clot (mean ± SEM; $n = 6$). $^*P < .05$, $^{**}P < .01$ vs control. See the supplemental Methods for details.

in platelets from exposed animals compared with those from controls (Figure 2I-J). Activated platelets bear increased numbers of various transmembrane proteins, which serve as platelet activation markers. A flow cytometry-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 the $\alpha IIb\beta_3$ receptor complex[28] demonstrated an enhanced surface expression of this complex that reflects greater platelet activation on induction with ADP in exposed animals than that seen in controls (Figure 3A,C). CD41, another platelet activation marker, also exhibited a similar response (Figure 3B,D). Because 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 in vitro clot retraction assay. As shown in Figure 3E-F, hypoxic exposure resulted in higher reduction in clot size or increased the clot retraction. The clot retraction in exposed groups was 30% and 20% more than that of control groups at 30 and 60 minutes, respectively. These observations reflected higher platelet reactivity and greater platelet–fibrinogen interactions in exposed animals, which corroborates with the other platelet assays such as aggregation, activation, and adhesion.

Platelet function was also tested in rats exposed to hypoxia under normothermic surroundings (chamber temperature of 28°C) to determine whether moderate hypothermic surroundings (chamber temperature of 10°C) are contributing to platelet hyperreactivity under hypoxic environment. The hypoxia exposure under normothermic conditions resulted in a significant enhancement of rate and extent of platelet aggregation, as under hypothermic conditions (supplemental Figure 1B-D). 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 2-dimensional gel electrophoresis, followed by identification of differentially expressed proteins by matrix-assisted laser desorption-ionization time-of-flight tandem mass spectrometry (MS/MS) (Figure 4A-C). Initial optimizations suggested that a pH range of 4 to 7 was ideal for 2-dimensional-polyacrylamide gel electrophoresis on platelet samples, as this range provided the detection of the maximum number of protein features and improved resolution compared with other narrow pH ranges.

Software analysis of gel images resulted in the detection of more than 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 a MascotMowse score equal to or greater than 50 (Table 1), and 19 were upregulated, whereas 8 proteins were suppressed.

Bioinformatic analysis of identified differentially expressed proteins revealed that all these proteins belong to different cellular locations, such as the cell membrane, cytoskeletal, mitochondrial, endoplasmic reticulum, vesicular, and so on (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, and so on (Table 1). Furthermore, these proteins correspond to diverse molecular functions, including nucleotide binding, hormone binding, calcium channel

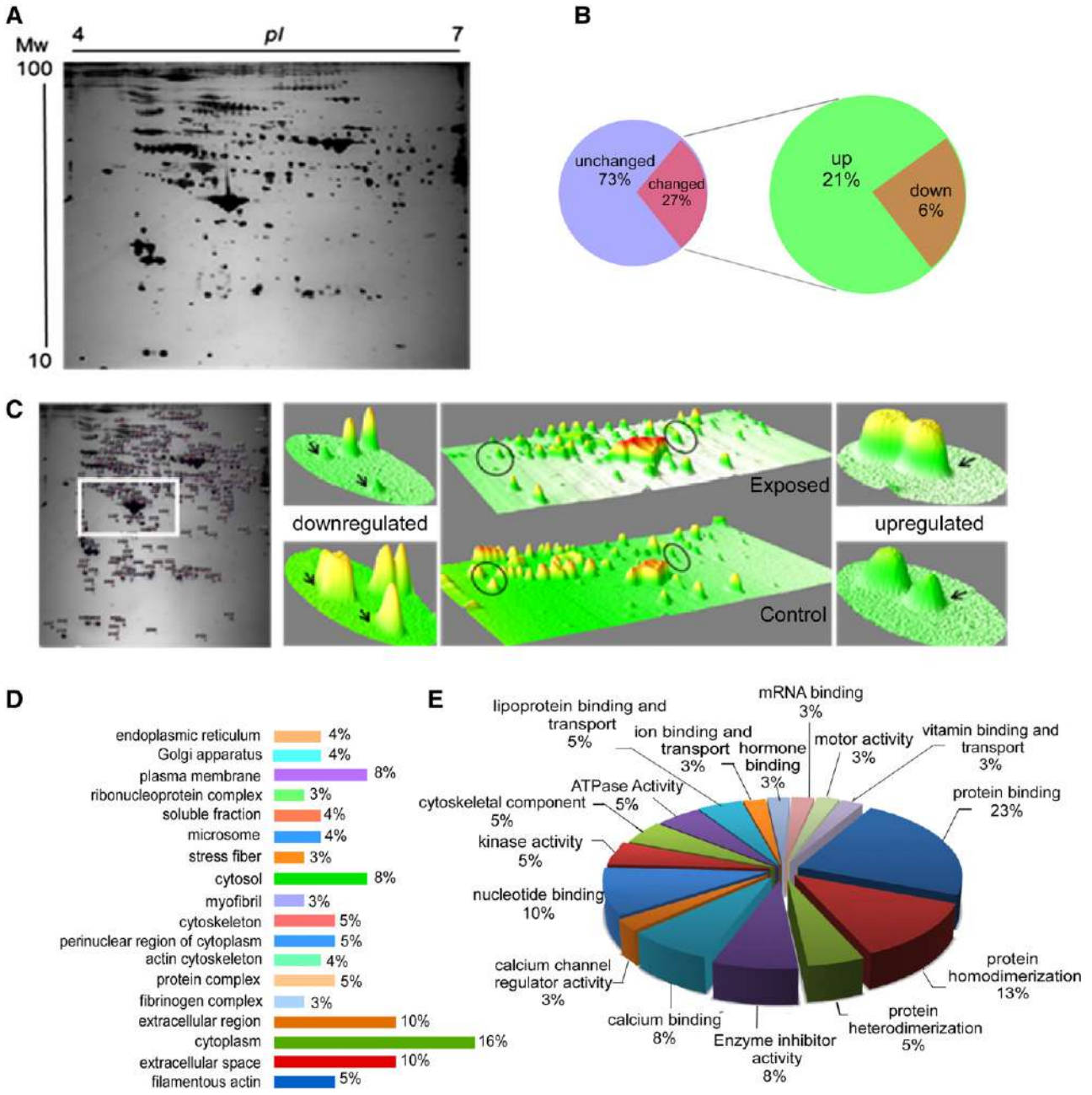

Figure 4. Platelet proteome analysis from control and exposed rats. Platelets were isolated from both control and hypoxia-exposed animals, and total platelet protein was prepared for platelet proteome analysis. Each protein lysate was subjected first to isoelectric focusing, followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in the second dimension, as described in the supplemental Methods. The differentially expressed proteins were identified using matrix-assisted laser desorption-ionization time-of-flight mass spectrometry. (A) Representative 2-dimensional gel electrophoresis (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 pH 4 to 7 were altered in hypoxia-exposed platelets. (C) A typical heat map of a selected portion from 2DE gels showing representative differential spots in 3 dimensions, prepared using ImageJ software. (D-E) Bioinformatic analysis of identified proteins was performed with the GeneCodis Web tool (http://genecodis.cnb.csic.es). (D) Gene Ontology cellular compartment analysis and (E) Gene Ontology molecular function analysis. See the supplemental Methods for details.

regulation, ATPase activity, calcium binding, enzyme inhibition, and response to ions such as Fe^{3+}, Mg^{2+}, Ca^{2+}, and so on (Figure 4E).

On the basis of the 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 γ, calreticulin, calumenin, CAPNS1, nonneural α enolase, Janus kinase 3, and ρ guanine exchange factor 7, 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). ELISA results for 3 of the proteins including fibrinogen β, fibrinogen α, and coronin 1A were not in accordance with the proteomic findings, and thus were not followed-up further (data not shown).

Proteins with prothrombotic nature, which includes important coagulation factors, tissue factor (TF), and fibrinogen, were

Table 1. Platelet proteins differentially expressed in hypoxia-exposed animals

Protein description	Accession	Mol wt	Fold change	Mowse score	General function
Apolipoprotein E	APOE_RAT	35.7	−4.2	214	Mediates the binding, internalization, and catabolism of lipoprotein particles
Actin related Protein 2	ARP2_RAT	44.7	−4.2	101	Regulation of actin polymerization
Calpain small subunit 1	CSS1_RAT	28.5	5.3	38	Regulation of calpain activity, cell migration
Calreticulin	CALR_RAT	48	−6.0	347	Molecular calcium-binding chaperone, quality control in the endoplasmic reticulum, vascular regulatory antithrombotic role
Calumenin	CALU_RAT	37	−6.2	60	Vascular regulatory, regulation of vitamin K-dependent carboxylation of multiple amino-terminal glutamate residues
Complement C3 precursor	CO3_RAT	186	−2.5	178	Activation of the complement system
Coronin1A	COR1A_RAT	51	−2.9	74	A crucial component of the cytoskeleton of highly motile cells
Elongation factor 1 γ	EF1G_RAT	50	−2.2	92	Protein synthesis
α Enolase	ENOA_RAT	47.1	3.0	68	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
Fibrinogen α	FIBA_RAT	86.6	2.7	90	Yield monomers that polymerize into fibrin and acts as a cofactor in platelet aggregation
Fibrinogen γ	FIBG_RAT	50.6	3.3	357	Yield monomers that polymerize into fibrin and acts as a cofactor in platelet aggregation
Guanine nucleotide binding protein 2	GBB2_RAT	37.3	−3.0	63	Glycoproteins bound to plasma membrane, involved as a modulator or transducer in various transmembrane signaling systems
TF precursor	TF_RAT	33.4	2.1	20	Key component of extrinsic coagulation pathway
Haptoglobin precursor	HPT_RAT	38.5	2.5	71	Combines with free plasma hemoglobin, preventing loss of iron through the kidneys, secreted in response to hypoxia as well as acute inflammation
Heat shock cognate 71	HSP7C_RAT	70.8	2.3	150	Chaperon, regulation of transcription, response to stress
T kininogen 1	KNT1_RAT	47.7	3.6	117	Acute-phase protein
Myosin light chain PP 6	MYL6_RAT	16.9	5.9	87	Motor protein, also involved in collagen-induced platelet activation
Tropomyosin α 1	TPM1_RAT	32.6	4.2	50	Binds to actin filaments, cytoskeletal reorganization
Tropomyosin β	TPM2_RAT	32.8	4.6	96	Binds to actin filaments, cytoskeletal reorganization
Tropomyosin α 4	TPM4_RAT	28.5	2.0	64	Binds to actin filaments, cytoskeletal reorganization
Serrotransferrin precursor	TRFE_RAT	76.3	2.4	108	Precursor to macromolecular activators of phagocytosis which enhance leukocyte phagocytosis
Janus Kinase 3	JAK3_RAT	122	3.6	26	Cytokine-mediated signaling and regulation of cytosolic calcium.
α-1-antitrypsin	A1AT_RAT	46.1	4.2	50	Inhibitor of serine proteases; platelet isoform secreted in α granules
ρ Guanine nucleotide exchange factor 7	ARHG7_RAT	73	4.2	19	Involved in ras-related C3 botulinum toxin substrate 1-dependent signaling, cell migration, attachment, and cell spreading.
Vitamin D binding Protein	VTDB_RAT	53.5	2.0	99	Carries vitamin D in plasma, has T lymphocyte surface association
α-2-macroglobulin receptor-associated protein precursor	AMRP_RAT	42	5.7	33	Binds to members of low-density lipoprotein receptor family and inhibits binding of their ligands
Nonmuscle caldesmon (L- caldesmon)	CALD1_RAT	60.5	3.1	49	Actin and myosin binding protein implicated in regulation of actomyosin interactions

upregulated, whereas those having antithrombotic activity, including calcium-binding proteins calreticulin and calumenin,[30,31] were suppressed under hypoxic conditions. The identified proteins also included some upregulated proteins playing major roles in the platelet activation process. A recent study observed TF expression in human platelets, but not in platelets from mice.[32] Also, there is conflicting information regarding TF expression in platelets; thus, we have also examined the TF mRNA to verify its expression in platelets from control and hypoxia-exposed animals, using RT-PCR (supplemental Figure 2). 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 the proteomic and ELISA data and suggest that differential TF expression regulation in rat platelets may be a result of signal-dependent splicing, as demonstrated in human platelets earlier.[33]

Collectively, the hypoxia-induced changes in the expression pattern of platelet proteins indicate that hypoxic exposure shifts the platelet proteome toward the prothrombotic state.

Calpains play a vital role in hypoxia-induced prothrombotic phenotype

The analysis of hypoxia-induced differentially expressed proteins directed our focus toward calcium-triggered events. Calcium-based regulation of intracellular events is known to be central in platelet activation. Calpain, a thiol protease, has been found to be regulated by calcium influx and oxidative stress.[34,35] Upregulation of CAPNS1 in platelets from exposed animals was evident from the proteomic and ELISA results (Figures 4-5). To confirm the activation of platelet calpain, platelet intracellular calcium and calpain activity were

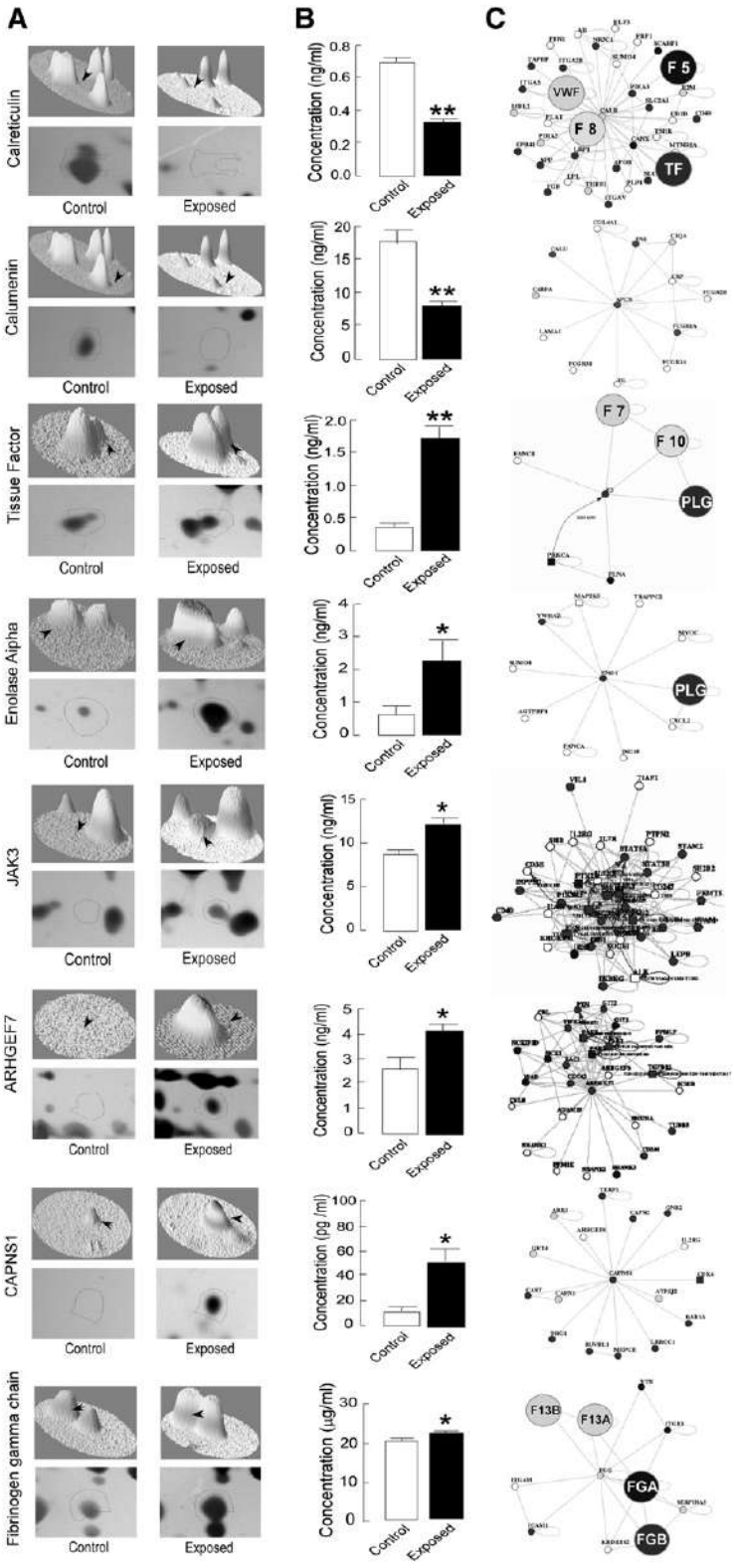

Figure 5. ELISA-based validation and protein–protein interaction analysis of identified differential proteins. Selective differential proteins identified by MS/MS were used for heat map generation and 3-dimensional view analysis. The differentially expressed proteins were quantified in platelet samples 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 3-dimensional 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 the PlateletWeb database. The proteins directly involved in coagulation cascade are highlighted.

measured in PRP and were found to be significantly higher in exposed animals compared with in controls (Figure 6A-B). Thus, differential regulation of calcium-binding proteins, elevated platelet intracellular Ca^{2+}, and higher calpain activity in hypoxic animals suggested that a disturbed Ca^{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),

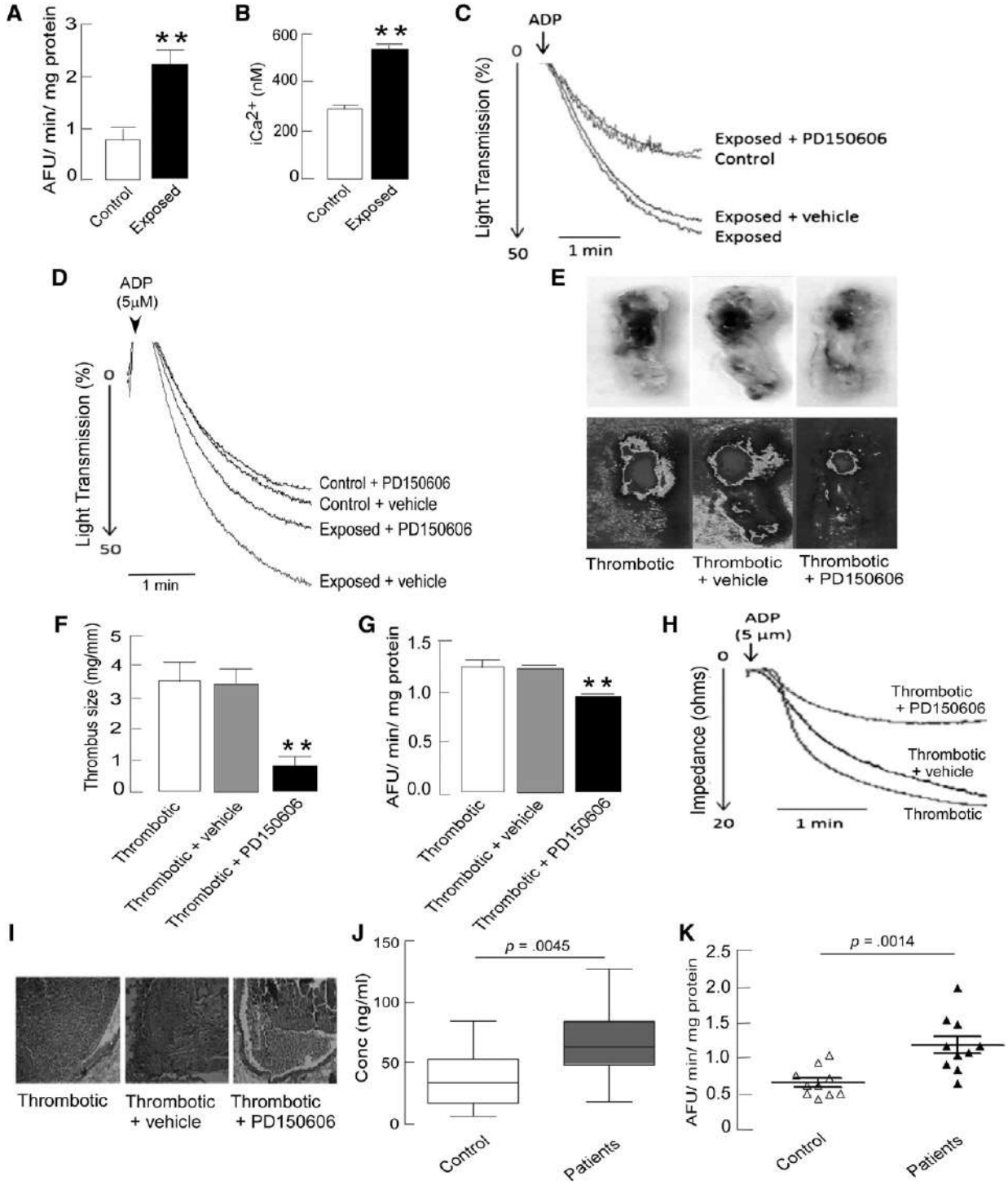

Figure 6. Increased calpain activity after hypoxia exposure in rats and human DVT patients, and the antithrombotic effect of calpain inhibition in vivo and ex vivo. Platelets were isolated from indicated groups and processed for either florescence-based calpain activity or intracellular calcium assays, as described in the supplemental Methods. (A) Quantitation of calpain activity in platelets demonstrating higher proteolytic activity of calpain in exposed animals. (B) Intraplatelet calcium levels measured by Fura-2 based fluorogenic assay showed increased platelet iCa^{2+} in exposed animals. (C-D) Representative aggregation curves showing reversal of hypoxia-induced platelet aggregation by preincubation of PRP with calpain inhibitor PD150606 (50 μM) ex vivo (C) and by preinfusion of PD150606 (1 mg/kg body weight) in vivo (D). (E-I) The antithrombotic and platelet inhibitory effects of preinfusion of PD150606 via tail vein in rat model of stasis-induced thrombosis, as described in the supplemental Methods. (E) Representative images of extracted portions of IVC with thrombus (top) from thrombotic animals with their heat maps (bottom), showing smaller thrombus in the case of PD150606 preinfusion. (F) Quantitations of the size of the thrombus isolated from the IVC portions of thrombotic animals (n = 8). (G) Decreased calpain activity in platelets isolated from thrombotic animals preinfused with PD150606. (H) Representative platelet aggregation curves showing a strong negative effect of PD150606 preinfusion in thrombotic rats compared with vehicle control or no infusion. Data are presented as mean ± SEM (n = 8) and analyzed by unpaired *t* test. $^*P < .05$, $^{**}P < .01$ vs thrombotic + vehicle. (I) Hematoxylin-eosin-stained sections of thrombus with vessel wall, showing morphological differences (×200 original magnification). (J-K) The plasma samples from human DVT patients from high-altitude regions and from control individuals were analyzed for soluble P selectin levels and calpain activity, (J) Higher sP-selectin levels in plasma samples (data presented as box and whiskers plot) indicated hyperactive platelets in high-altitude-induced DVT patients. (K) Calpain activity in human plasma samples of high-altitude-induced DVT patients compared with age- and sex-matched healthy controls, shown as a scatter plot (n = 10). The activity in patients was significantly ($P = .0014$) higher in comparison with controls. Data are presented as mean ± SEM (n = 10 in each group) and analyzed by *t*-test compared with respective controls.

which binds to CAPNS1,[36] was used for further animal studies. Preincubation of PRP with PD150606 (50 μM) for 10 minutes at 37°C resulted in the reversal of hypoxia-induced platelet hyperreactivity (Figure 6C). Further, the preinfusion of animals with PD150606 (1 mg/kg body weight) via tail vein before 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 seconds with PD150606 vs 212 ± 22 seconds 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 resulted in the generation of thrombus as a result of 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.[27,37] Animals preinfused with calpain inhibitor, PD150606 (1 mg/kg body weight), demonstrated significantly reduced thrombus size (0.80 ± 0.33 mg/mm) compared with those preinfused with vehicle (3.41 ± 0.51 mg/mm; $P < .01$) (Figure 6E-F). Similarly, calpain activity and platelet aggregation were found to be significantly lower in animals preinfused with PD150606 compared with vehicle controls (Figure 6G-H). 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 a decompression chamber). Thrombus formation was found to be accelerated after 6 hours of hypoxic exposure, as reflected by a significantly enhanced thrombus size in these animals compared with in unexposed ligated animals (supplemental Figure 3). Furthermore, the effect of preinfusion with PD150606 was more pronounced, as indicated by thrombus size in exposed ligated animals compared with in their unexposed counterparts (supplemental Figure 3). To exclude the off-target effect (if any) of a single calpain inhibitor (PD150606), 2 additional known 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 (supplemental Figure 4). However, PD150606 proved to be the most potent among these 3 inhibitors.

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, calpain activity was found to be significantly higher in human patients' plasma samples compared with in the controls (1.213 ± 0.121 arbitrary fluorescent units/min/mg vs 0.70 ± 0.062 arbitrary fluorescent units/min/mg; $P = .0014$; n = 10; Figure 6K). All 10 patients selected for this study were negative for 2 common thrombophilia traits: factor V Leiden (1691G/A, rs6025) and prothrombin (20210G/A, rs1799963) mutations. Moreover, we did not find any association between thrombophilia factors and enhanced calpain activity in these patients (see supplemental Table 2 for details). There was no significant difference in the mean age of patients (35 years) and controls (32 years), and all were healthy with a normal body mass index range (<29.9 kg/m^2). We also analyzed the levels of sP-selectin, a soluble marker of platelet activation in the plasma samples of patients and controls. Plasma sP-selectin was significantly higher in patients compared with controls, which is suggestive of increased platelet reactivity in the patients (Figure 6J). These results emphasize the translational implication of preclinical 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 prothrombotic,[10-12] certain studies have challenged these observations.[38,39] Hence, in the current study, we tried to investigate and understand the effect of acute hypoxic exposure on coagulation and platelets.

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 multiple parameters including platelet adhesion, aggregation, and activation. The hyperreactivity of platelets was further supported by the enhanced surface expression of CD41 and $\alpha IIb\beta_3$, as well as by increased clot retraction in exposed animals. Earlier studies on platelet reactivity under a hypoxic environment produced conflicting results.[16,39] Rats were selected as the ideal animal model to perform this study because of 2 factors: first, rats are preferred over mice for conducting pathophysiological studies, and second, there have been significant similarities in rat and human platelet proteins, which encourages the use of rat models to study platelets and related prothrombotic events.[40] The current animal model involves hypobaric hypoxic exposure at a 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 surroundings on 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; that is, the exposed animals experienced significantly decreased bleeding time and increased platelet aggregation compared with controls (supplemental Figure 1). These preliminary observations emphasize that hypoxia is the key factor affecting platelets at high altitude.

Until the 1990s, it was believed that platelets carried proteins synthesized from their parent cell megakaryocytes. However, with the evidence of de novo protein synthesis and the discovery of protein synthesis machinery in platelets, these were found to be active protein-synthesizing anucleated cells. The subsequent discovery of platelet spliceosome and alternative splicing revealed a posttranscriptional mode of regulation of protein expression in platelets, making the platelet biology more complex. Therefore, we analyzed 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 comprised important coagulation cascade proteins such as fibrinogen and TF, some key signaling 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, which therefore was suggestive of the whole-platelet proteome alteration in the hypoxic environment. The increased levels of platelet TF in hypoxia supports our coagulation

assay data, where PT was found to be decreased after hypoxia. Hypoxia not only resulted in upregulation 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,[30,31] and maintaining calcium homeostasis.[20]

The enhanced CAPNS1-regulated calpain activity, along with elevated Ca^{2+}, may play an important role in hypoxia-induced thrombogenesis, as demonstrated by in vivo animal results. The in vivo hypoxic setting for thrombus formation was created by an IVC ligation approach that resulted in a hypoxic microenvironment due to stasis.[27,37] The upregulation of hypoxia-inducible factor 1α in both exposed (but nonligated) and thrombotic animals strongly supported our approach (data not shown). The pretreatment with 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 2 additional calpain inhibitors, MDL28170 and calpeptin, to rule out possible off-target effects.

On the basis of the current observations presented in this article, a few points about probable modes of action of calpain can be drawn. First, 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. Second, platelet hyperreactivity induced by hypoxia depends largely on calpain activity, as preinfusion of calpain inhibitor limits the hypoxia-induced platelet aggregation (Figure 6D). Third, 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) on agonist-induced platelet activation.[41] Therefore, from the current observations, it appears that hypoxic exposure accelerates the rate of transformation of calpain from its resting state to a fully activated state and positively regulates hypoxia-induced platelet reactivity. However, it remains to be explored whether hypoxia induces the known calpain reactions in platelets or triggers an altogether different set of biomolecular reactions. These nascent findings about the platelet–calpain relationship under hypoxia may provide future directions for deciphering the exact mechanism by which calpain protease systems mediate hypoxia-induced prothrombotic effects via regulating platelet function, as well as drive the hunt for novel substrates of calpain in platelets under a hypoxic environment. Another interesting approach may be investigating the role of calpain in modulating platelet proteome under hypoxia, in view of recently reported stabilization of the platelet proteome of diabetic patients by calpain inhibition.[42]

In addition to platelet activation, the prothrombotic nature of calpain can also be attributed to the diverse nature of its substrates, which comprise cytoskeletal proteins, membrane proteins, kinases, phosphatases, and ATPases.[43] More important, 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 because of 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, suggesting a potential link between oxygen-compromised status and thrombogenic index.

Acknowledgments

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

This work was funded by Defense Research and Development Organization, project SL-10 /DIP-255. T.T. is a senior research fellow of the 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 mass spectrometry 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 the human study; S.B.S. and L.G. edited the manuscript; and M.Z.A. designed the study, interpreted data, and drafted the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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References

1. Ward M. *Mountain Medicine: A Clinical Study of Cold and High Altitude.* London: Crosby Lockwood Staples; 1975.
2. Cucinell SA, Pitts CM. Thrombosis at mountain altitudes. *Aviat Space Environ Med.* 1987;58(11): 1109-1111.
3. Gupta N, Ashraf MZ. Exposure to high altitude: a risk factor for venous thromboembolism? *Semin Thromb Hemost.* 2012;38(2): 156-163.
4. Denko NC, Giaccia AJ. Tumor hypoxia, the physiological link between Trousseau's syndrome (carcinoma-induced coagulopathy) and metastasis. *Cancer Res.* 2001;61(3):795-798.
5. Zell L, Kindermann W, Marschall F, Scheffler P, Gross J, Buchter A. Paget-Schroetter syndrome in sports activities—case study and literature review. *Angiology.* 2001;52(5):337-342.
6. Singh I, Chohan IS. Blood coagulation changes at high altitude predisposing to pulmonary hypertension. *Br Heart J.* 1972;34(6):611-617.
7. Boulos P, Kouroukis C, Blake G. Superior sagittal sinus thrombosis occurring at high altitude associated with protein C deficiency. *Acta Haematol.* 1999;102(2):104-106.
8. Jha SK, Anand AC, Sharma V, Kumar N, Adya CM. Stroke at high altitude: Indian experience. *High Alt Med Biol.* 2002;3(1):21-27.
9. Anand AC, Saha A, Seth AK, Chopra GS, Nair V, Sharma V. Symptomatic portal system thrombosis in soldiers due to extended stay at extreme altitude. *J Gastroenterol Hepatol.* 2005;20(5):777-783.
10. Bendz B, Rostrup M, Sevre K, Andersen TO, Sandset PM. Association between acute hypobaric hypoxia and activation of coagulation in human beings. *Lancet.* 2000;356(9242): 1657-1658.
11. Mannucci PM, Gringeri A, Peyvandi F, Di Paolantonio T, Mariani G. Short-term exposure to high altitude causes coagulation activation and inhibits fibrinolysis. *Thromb Haemost.* 2002;87(2): 342-343.

12. Schreijer AJ, Cannegieter SC, Meijers JC, Middeldorp S, Büller HR, Rosendaal FR. Activation of coagulation system during air travel: a crossover study. *Lancet.* 2006;367(9513): 832-838.

13. Kotwal J, Apte CV, Kotwal A, Mukherjee B, Jayaram J. High altitude: a hypercoagulable state: results of a prospective cohort study. *Thromb Res.* 2007;120(3):391-397.

14. Gray GW, Bryan AC, Freedman MH, Houston CS, Lewis WF, McFadden DM, Newell G. Effect of altitude exposure on platelets. *J Appl Physiol.* 1975;39(4):648-652.

15. Hudson JG, Bowen AL, Navia P, Rios-Dalenz J, Pollard AJ, Williams D, Heath D. The effect of high altitude on platelet counts, thrombopoietin and erythropoietin levels in young Bolivian airmen visiting the Andes. *Int J Biometeorol.* 1999;43(2): 85-90.

16. Lehmann T, Mairbäurl H, Pleisch B, Maggiorini M, Bärtsch P, Reinhart WH. Platelet count and function at high altitude and in high-altitude pulmonary edema. *J Appl Physiol (1985).* 2006; 100(2):690-694.

17. Murayama M. Ex vivo human platelet aggregation induced by decompression during reduced barometric pressure, hydrostatic, and hydrodynamic (Bernoulli) effect. *Thromb Res.* 1984;33(5):477-485.

18. Wedzicha JA, Syndercombe-Court D, Tan KC. Increased platelet aggregate formation in patients with chronic airflow obstruction and hypoxaemia. *Thorax.* 1991;46(7):504-507.

19. Oga T, Chin K, Tabuchi A, et al. Effects of obstructive sleep apnea with intermittent hypoxia on platelet aggregability. *J Atheroscler Thromb.* 2009;16(6):862-869.

20. Rink TJ, Sage SO. Calcium signaling in human platelets. *Annu Rev Physiol.* 1990;52:431-449.

21. Parguiña AF, Grigorian-Shamajian L, Agra RM, et al. Proteins involved in platelet signaling are differentially regulated in acute coronary syndrome: a proteomic study. *PloS One.* 2010; 5(10):e13404.

22. Rex S, Beaulieu LM, Perlman DH, et al. Immune versus thrombotic stimulation of platelets differentially regulates signalling pathways, intracellular protein-protein interactions, and α-granule release. *Thromb Haemost.* 2009; 102(1):97-110.

23. Yoshizawa T, Sorimachi H, Tomioka S, Ishiura S, Suzuki K. Calpain dissociates into subunits in the presence of calcium ions. *Biochem Biophys Res Commun.* 1995;208(1):376-383.

24. Undyala VV, Dembo M, Cembrola K, et al. The calpain small subunit regulates cell-substrate mechanical interactions during fibroblast migration. *J Cell Sci.* 2008;121(Pt 21):3581-3588.

25. Moldoveanu T, Hosfield CM, Lim D, Elce JS, Jia Z, Davies PLA. A Ca(2+) switch aligns the active site of calpain. *Cell.* 2002;108(5):649-660.

26. Croce K, Flaumenhaft R, Rivers M, Furie B, Furie BC, Herman IM, Potter DA. Inhibition of calpain blocks platelet secretion, aggregation, and spreading. *J Biol Chem.* 1999;274(51): 36321-36327.

27. Zhou J, May L, Liao P, Gross PL, Weitz JI. Inferior vena cava ligation rapidly induces tissue factor expression and venous thrombosis in rats. *Arterioscler Thromb Vasc Biol.* 2009;29(6): 863-869.

28. Shattil SJ, Hoxie JA, Cunningham M, Brass LF. Changes in the platelet membrane glycoprotein IIb.IIIa complex during platelet activation. *J Biol Chem.* 1985;260(20):11107-11114.

29. Ginsberg MH, Frelinger AL, Lam SC, et al. Analysis of platelet aggregation disorders based on flow cytometric analysis of membrane glycoprotein IIb-IIIa with conformation-specific monoclonal antibodies. *Blood.* 1990;76(10): 2017-2023.

30. Kuwabara K, Pinsky DJ, Schmidt AM, et al. Calreticulin, an antithrombotic agent which binds to vitamin K-dependent coagulation factors, stimulates endothelial nitric oxide production, and limits thrombosis in canine coronary arteries. *J Biol Chem.* 1995;270(14):8179-8187.

31. Wajih N, Sane DC, Hutson SM, Wallin R. The inhibitory effect of calumenin on the vitamin K-dependent gamma-carboxylation system. Characterization of the system in normal and warfarin-resistant rats. *J Biol Chem.* 2004; 279(24):25276-25283.

32. Pawlinski R, Wang JG, Owens AP III, et al. Hematopoietic and nonhematopoietic cell tissue factor activates the coagulation cascade in endotoxemic mice. *Blood.* 2010;116(5):806-814.

33. Schwertz H, Tolley ND, Foulks JM, et al. Signal-dependent splicing of tissue factor pre-mRNA modulates the thrombogenicity of human platelets. *J Exp Med.* 2006;203(11):2433-2440.

34. Suzuki K, Sorimachi H, Yoshizawa T, Kinbara K, Ishiura S. Calpain: novel family members, activation, and physiologic function. *Biol Chem Hoppe Seyler.* 1995;376(9):523-529.

35. Ray SK, Fidan M, Nowak MW, Wilford GG, Hogan EL, Banik NL. Oxidative stress and Ca^{2+} influx upregulate calpain and induce apoptosis in PC12 cells. *Brain Res.* 2000;852(2):326-334.

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.

37. López JA, Chen J. Pathophysiology of venous thrombosis. *Thromb Res.* 2009;123(4 Suppl 4): S30-S34.

38. Bärtsch P, Straub PW, Haeberli A. Hypobaric hypoxia. *Lancet.* 2001;357(9260):955-956.

39. Toff WD, Jones CI, Ford I, et al. Effect of hypobaric hypoxia, simulating conditions during long-haul air travel, on coagulation, fibrinolysis, platelet function, and endothelial activation. *JAMA.* 2006;295(19):2251-2261.

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 evolution. *Proteomics.* 2010;10(13):2444-2457.

41. Saido TC, Suzuki H, Yamazaki H, Tanoue K, Suzuki K. In situ capture of mu-calpain activation in platelets. *J Biol Chem.* 1993;268(10): 7422-7426.

42. Randriamboavonjy V, Isaak J, Elgheznawy A, et al. Calpain inhibition stabilizes the platelet proteome and reactivity in diabetes. *Blood.* 2012; 120(2):415-423.

43. Randriamboavonjy V, Fleming I. All cut up! The consequences of calpain activation on platelet function. *Vascul Pharmacol.* 2012;56(5-6): 210-215.