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Review Article

The stimulation of thrombosis by hypoxia

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ABSTRACT

Thrombus formation is increased under conditions of hypoxia in animal models of thrombosis and in human populations, but current therapies for thrombosis do not directly target hypoxia-responsive signaling pathways. The vascular response to hypoxia is controlled primarily by the hypoxia-inducible transcription factors (HIFs), whose target genes include several factors that regulate thrombus formation. In this article, we review the HIF-dependent and HIF-independent signaling pathways that regulate thrombus formation under hypoxic conditions. A better understanding of hypoxia-induced thrombus formation could lead to the development of novel prophylactic therapies for thrombosis.

1. Introduction

Venous thromboembolism (VTE), comprising of deep vein thrombosis (DVT) and pulmonary embolism (PE), remains a major health burden [1,2]. Risk factors for VTE include surgery, pregnancy, and systemic hypoxia (i.e. reduced oxygenation) [3,4]. In Europe, ~1.1 million VTE events occur per year, causing > 500,000 deaths annually [5]. In USA, VTE incidences of ~60% are found in patients undergoing orthopedic surgery [1] and total deaths from VTE are > 500,000 per year [6]. The global incidence of VTE has been estimated at ~2 per 1000 people per year, and thromboembolic conditions are estimated to account for ~1 in 4 deaths worldwide [7]. Despite progress in the development of effective treatments for thrombosis, current therapies still possess limitations, including increased risk of bleeding [8,9]. Direct oral anticoagulants are often contraindicated in elderly patients (> 80 years) and in patients with impaired renal function or advanced cancer [10,11]. The lack of safe and effective treatments for VTE raises an urgent need to better understand the mechanisms that regulate thrombus formation, which could lead to the identification of novel therapeutic targets, and eventually to the development of effective prophylactic therapies. In this review, we describe the regulation of thrombus formation by hypoxia and hypoxia-responsive signaling pathways.

1.1. Hypoxia-induced thrombosis

Thrombus formation occurs under conditions of increased coagulation, endothelial injury, and venous stasis (i.e. Virchow's Triad). Risk factors for thrombosis are directly or indirectly associated with one of these conditions and commonly known risk factors include immobilization and trauma [12]. Reduced oxygenation (i.e. hypoxia) is also a risk factor for thrombosis, since the incidence of thrombosis is increased under systemic or local hypoxia [13–16]. Hypoxia occurs when oxygen demand is greater than oxygen supply, for example when blood flow is reduced by immobility or disrupted by trauma. Reductions in oxygenation trigger a myriad of molecular and cellular signaling pathways that can contribute to the regulation of thrombus formation [17,18]. In other words, hypoxia is not only a consequence of vascular occlusion, but also stimulates thrombogenesis [19,20] (Fig. 1). Investigations of human populations and animal models of hypoxia and thrombosis suggest that hypoxia and its downstream signaling promote thrombus formation and propagation. These studies indicate that hypoxia-responsive signaling pathways could be therapeutically targeted to reduce VTE burden.

1.2. Hypoxia-inducible factors (HIFs)

The vascular response to hypoxia is controlled primarily by the

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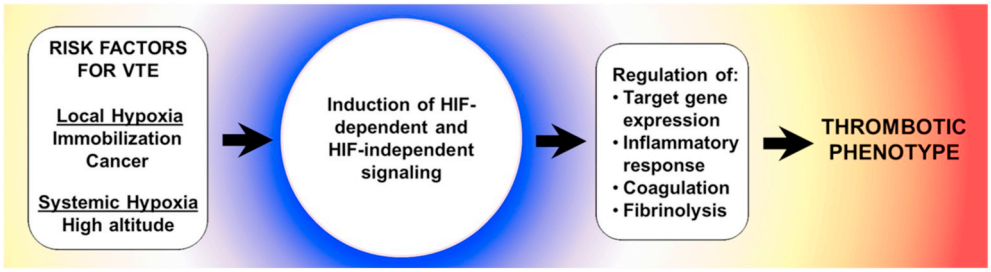

Fig. 1. Hypoxia is a link between risk factors for thrombosis and thrombogenesis. Risk factors for thrombosis can result in hypoxia, leading to activation of hypoxia-regulated genes that mediate coagulation or fibrinolysis. Hypoxia-regulated genes may represent putative targets in the prevention of thrombosis. Abbreviations: HIF, hypoxia-inducible factor; VTE, venous thromboembolism.

hypoxia-inducible transcription factors (HIFs). HIFs are heterodimeric nuclear transcription factors consisting of α and β subunits, which together regulate transcription of genes that mediate the homeostatic responses to reduced oxygenation [21]. The α sub-units of HIF1 and HIF2 (i.e. HIF1α and HIF2α respectively) are hypoxia-dependent, but the HIFβ sub-unit is constitutively expressed in all nucleated cell types. Under normoxic conditions, oxygen-dependent hydroxylation of the HIFα sub-units occurs at distinct proline residues, driven by prolyl-hydroxylase domain (PHD) enzymes 1–3. HIFα hydroxylation facilitates binding with the von Hippel-Lindau protein, which interacts with elongin C and recruits the ubiquitin ligase complex causing ubiquitination and rapid proteosomal degradation [21,22]. Expression of the HIFα sub-units is suppressed under normoxia, but expression increases exponentially as oxygen concentration declines. Under hypoxic conditions, hydroxylation activity of the PHD enzymes is suppressed, allowing the HIFα sub-units to accumulate in the nucleus, dimerize with HIFβ and bind to the hypoxia-responsive element (HRE) in the promotor or enhancer region of its target genes to activate their transcription. HIF targets include factors that promote thrombosis, such as plasminogen activator inhibitor (PAI) 1, but not all hypoxia-induced factors are pro-thrombotic, and not all hypoxia-induced factors that enhance thrombus formation contain an HRE. In other words, hypoxia-induced changes in the expression of pro- or anti-thrombotic factors can be controlled directly via HIFs or HIF target genes or indirectly via HIF-independent mechanisms. For example, hypoxia also activates early growth response (EGR) 1, which is known to regulate thrombus formation [23,24]. Hypoxia-responsive signaling pathways can also regulate thrombogenesis indirectly through the induction of pro-inflammatory mediators such as tumor necrosis factor (TNF) α and interleukin (IL) 1 [19]. The identification of hypoxia-responsive transcription factors, target genes, or signaling responses that control thrombus formation could represent an important step towards the development of novel and safe prophylactic therapies that reduce thrombosis.

2. Observational studies of hypoxia-induced thrombosis

Circumstantial evidence that hypoxia triggers thrombogenesis was provided by Hamer et al., who measured venous hypoxia in 2 patients with varicose veins [25]; these authors showed that localized hypoxia exists in venous valve pockets under undisturbed streamlined flow [25]. In a mouse model of DVT induced by blood flow restriction and endothelial disturbance of the inferior vena cava, newly formed venous thrombus is 10-fold less oxygenated compared with venous blood, and HIF1α and HIF2α levels are increased within newly formed compared with resolving venous thrombus and stabilized in the surrounding vessel [26–28]. The population studies described below have also assessed thrombotic burden under conditions of hypoxia, including in humans that are immobilized, reside at high altitude, or have solid tumors.

2.1. Immobility

Delayed blood renewal due to immobilization, such as in limb paralysis, hospitalized individuals [29], or long-haul flights [30], results in localized hypoxia (e.g. in valve pockets of the deep veins). Observational studies in humans have shown that immobility and blood flow restriction is positively correlated with an increased risk of thrombosis; for example, a systematic review and meta-analysis of 43 epidemiological studies including 24,181 VTE patients evaluated immobilization as a risk factor for VTE and concluded that immobilization confers an approximately 2-fold increase in the risk of VTE, possibly due to muscular and diaphragm dysfunction that decrease venous blood flow in the legs and lead to hypoxic activation of coagulation [31]. Five individual cases of VTE associated with physical restraint have also been described, with the authors concluding that VTE in association with physical restraint can occur in the absence of pre-existing risk factors [32]. In an audit of 208 patients with tendo Achilis injury, the incidence of symptomatic VTE was approximately 6% during cast immobilization of the lower limb for at least 1 week, and more proximal DVT and PE were observed with increased duration of immobility, suggesting that risk of DVT increases with duration of immobilization [33]. Another study has shown that approximately 1 in 6 patients experience a VTE following cast immobilization of the lower limb when thromboprophylaxis is not administered [34]. It is important to be aware, however, that cast immobilizations often occur following major trauma, which itself is a risk factor for thrombosis. In a study of different postures in humans, it was shown that venous flow-rate is approximately halved when subjects are standing or sitting compared with lying supine [35]. The potential role of prolonged stasis in the stimulation of thrombosis was highlighted as far back as 1954 in warnings that venous thrombosis can be induced by flights, automobile trips, and even theater visits [30].

2.2. High altitude

When oxygen levels in ambient air are reduced (e.g. at high altitude), the incidence of thrombosis increases [36] (Table 1). For example, a prospective analysis of 20,257 hospitalized patients showed that people living at high altitude (> 3000 m above sea level) and at extreme altitude (> 5000 m above sea level) for approximately 11 months have a 30-fold increased risk of developing VTE compared

Table 1
Altitude and the risk of thrombosis.

Altitude (m above sea level)	Oxygenation (% O_2 in air)	Duration of exposure (months)	Risk of thrombosis (fold increase)	Reference
3000–5000	~17.3–18.6	12	30	[37]
3048–6096	~16.6–18.6	13	25	[38]
2210	~19.4	60	≥2	[39]

Table 2
Thrombo-inflammatory HIF targets. Abbreviations: CXCL, chemokine-X-chemokine ligand; ICAM, intra-cellular adhesion molecule; IL, interleukin; NFκB, nuclear factor κB; NLRP3, NLR family pyrin domain containing 3; TACE, tumor necrosis factor-α converting enzyme/ADAM17; TF, tissue factor; TFPI, tissue factor pathway inhibitor; TLR, toll-like receptor; TNFα, tumor necrosis factor α; VEGF, vascular endothelial growth factor.

Factor	Upstream regulator	Expression change	Reference(s)
CCL5	HIF1	Increase	[57]
CCR5			[57]
CXCL12			[58]
CXCR4			[59,60]
ICAM			[60]
NFκB			[61,62]
NLRP3			[19]
TACE			[63]
TF			[64]
TLR4			[65]
TNFα			[63,66]
Protein S		Decrease	[67]
IL6	HIF1/HIF2	Increase	[66,68]
PAI1			[24,55]
IL1β			[19,60,68]
CXCL8			[60]
IL12			[68,69]
VEGF			[60,70]
CXCL2	HIF2	Increase	[68]
TFPI		Decrease	[54,71]

with people living at non-high altitude (0-800 m) [37]. Furthermore, the incidence of VTE is increased by 25-fold in lowland dwellers after they have been exposed to a high-altitude environment (10,000–20,000 ft) for approximately 13 months compared with those staying in lowland [38]. Even moderate altitude serves as risk factor for VTE; for instance, a study of humans living at an altitude of 7250 ft. for 5 years showed a > 2-fold increase in the incidence of PE compared with those living at sea level [39]. Jha et al. conducted a genome-wide expression analysis of a population of venous thrombosis patients living at high altitude (> 3648 m) for 1–5 months and identified that under environmental hypoxia at high altitude, genes involved in hypoxia signaling pathways represent a determining factor for development of thrombosis; hypoxia-responsive genes that were differentially expressed at high altitude included angiogenin, RNase A family 5, early growth response 1, lamin A, matrix metallopeptidase 14, neurofibromin 1; PDZ and LIM domain 1, procollagen-lysine 1, 2-oxoglutarate 5-dioxygenase 1, solute carrier family 6 member 4; solute carrier family 9 member 1, and endothelial TEK tyrosine kinase [40]. In a separate study, effects of high altitude on platelet function and fibrinogen levels were investigated in 40 healthy men who started at sea level and were re-assessed following 3 and 13 months at approximately 4500 m altitude [41]. In this study, platelet count decreased by 12% after 3 months of high altitude and by 31% after 13 months in comparison with basal levels, but mean platelet volumes increased by 40 and 50% after 3 and 13 months of high altitude respectively versus basal levels [42]. Authors of this study also showed that levels of fibrinogen in the plasma were elevated by 53% after 3 months of high altitude [42]. These findings imply that platelet burden and fibrinogen level can be altered by high altitude and may contribute to the development of a prothrombotic phenotype, while the authors of this study suggested that increased substrate availability for coagulation but not increased platelet activity leads to increased thrombosis at high altitude [42]. In a study of a population of Chuvash polycythemia patients, the 598C > T mutation in the von Hippel Lindau (VHL) gene was associated with higher mortality due to peripheral thrombotic events and cerebral vascular events [43]. The effect of altitude on thrombotic prevalence in patients with polycythemia vera was also investigated in a retrospective population study of 71 patients living at an altitude of at least 5000 ft. compared with 166 patients residing at sea level [16]. In this study, an approximate 4-fold increase in odds ratio of thrombosis was observed in the patients residing at altitude compared to sea level patients [16].

2.3. Congenital disorders of hypoxia-sensing pathways

The role of the HIF-PHD-VHL system in controlling erythropoiesis has been shown by genetic studies of individuals with congenital polycythemia; in these patients, excess red blood cell production and increased hematocrit levels are associated with an increased risk for thrombosis [44,45]. Germline mutations in genes such as VHL, EGLN1 (encoding PHD2), EPAS1 (encoding HIF2α), and the EPO receptor can also result in altered erythropoiesis [44–46]. As mentioned above, in patients with Chuvash polycythemia, the 598C > T mutation in the VHL gene is associated with higher mortality due to peripheral thrombotic and cerebral vascular events [43]. Missense mutation of $VHL^{598\ C>T}$ (VHL^{R200W}) is a loss-of-function mutation causing reduced affinity of VHL for HIF1α, which reduces HIFα proteasomal degradation and increases transcriptional upregulation of HIF target genes including HIF2-dependent erythropoietin expression [47,48]. Mutations in $EGLN1^{950\ C>G}$ ($PHD2^{P317R}$) and $EGLN1^{1112\ G>A}$ ($PHD2^{P371H}$) are other types of loss-of-function mutations in which individuals develop sagittal sinus thrombosis [45,46]. In addition, germline mutations for EPAS1 (HIF2α) including $EPAS1^{1609G>T}$, $EPAS1^{1604T>C}$, and $EPAS1^{1620C>G}$ are gain-of-function polycythemic mutations that involve genetic alteration close to the HIF2α hydroxylation site [49,50], thereby preventing its hydroxylation and subsequent proteosomal degradation.

2.4. Malignancy

Hypoxia and HIF induction are established characteristics of many solid tumor types. The positive association between cancer and thrombosis was found in humans approximately 150 years ago and this condition is now referred to as Trousseau's syndrome [51]. Patients with different types of cancer demonstrate varying degrees of increased risk of thrombosis [52]. Overall, a cancer patient is approximately 4-fold more likely to suffer from VTE compared with a non-cancer patient, and VTE is the second most common cause of death in cancer patients after cancer progression [51,52]. However, the increased risk of VTE in cancer patients cannot be attributed to the onset of hypoxia alone, given that many pro-coagulant and inflammatory factors are increased following malignancy (or trauma), such as increased tissue factor (TF) expression, along with increased platelet turnover and activity. As well as hypoxia and HIF activation in solid tumors, the formation of pulmonary microthrombi is associated with upregulations in the pulmonary levels of HIF1α and HIF2α [53]. Notably, HIF1 and HIF2 targets include thrombo-inflammatory factors (Table 2) as well as factors that directly regulate coagulation and fibrinolysis, such as prothrombotic TF and PAI1 and anti-thrombotic TF pathway inhibitor (TFPI) (Fig. 2) [24,51,54–56].

3. Experimental studies of hypoxia-induced thrombosis

Experimental evidence that thrombogenesis is triggered by hypoxia was provided in the seminal study by Hamer et al., who measured venous blood oxygenation in luminal and valvular pockets of 8 dogs during streamlined blood flow and under conditions of intermittent pulsatile blood flow [25]. It was found that undisturbed blood within the valve pockets became hypoxic, but that blood oxygenation levels in these same pockets rose to that of luminal blood when vessels were pulsated to empty and re-fill the valve pockets. Early thrombus formation was also triggered in the valve cusps during non-pulsatile flow only, supporting the possibility that localized hypoxia following blood stasis stimulates thrombogenesis in venous valve pockets. A more recent experimental study in mice has shown that systemic exposure to 6% oxygen for 24 h followed by 1–3 h of reoxygenation in 21% oxygen,

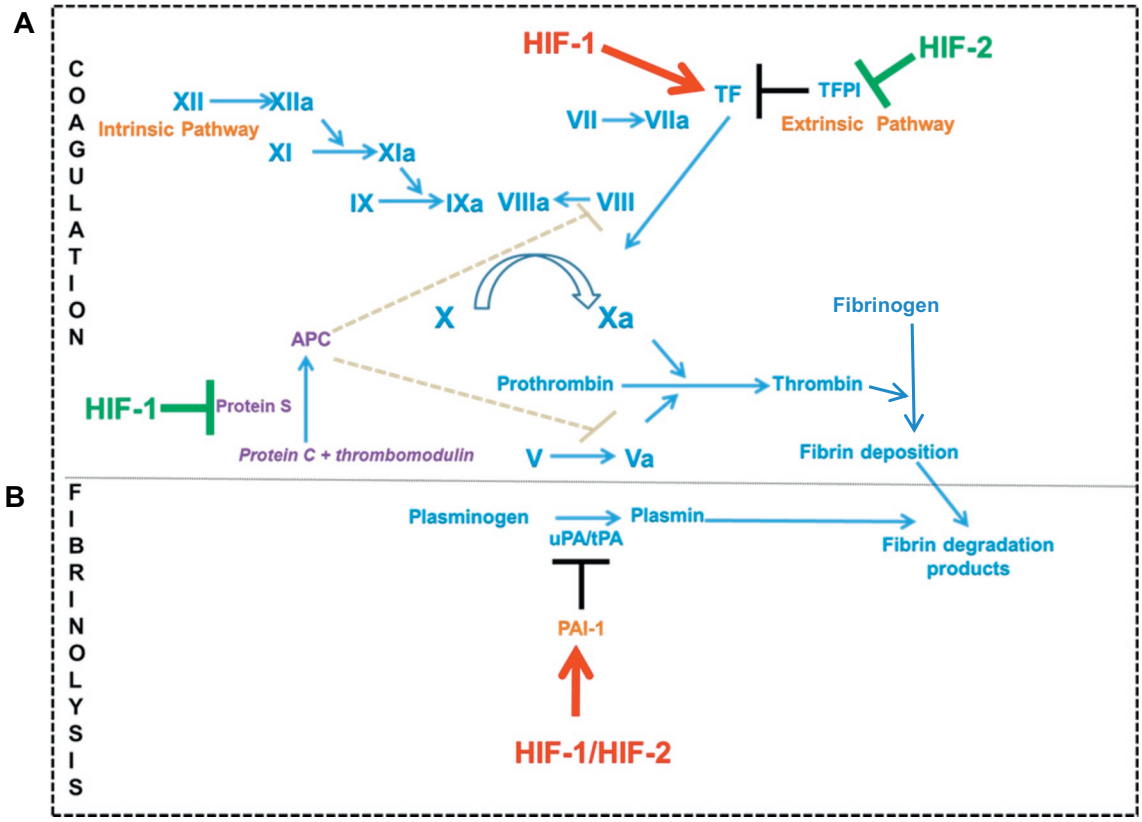

Fig. 2. Influence of HIFs on coagulation and fibrinolysis. (A) The coagulation cascade and (B) fibrinolysis pathways can be altered via HIF-mediated activation (red) or inhibition (green) of thrombotic or anti-thrombotic factors respectively. Abbreviations: HIF, hypoxia-inducible factor; TF, tissue factor; TFPI, TF pathway inhibitor; tPA, tissue type plasminogen activator; PAI1, plasminogen activator inhibitor 1; uPA, urokinase type plasminogen activator. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

increases the incidence and burden of stenosis-induced thrombosis in the inferior vena cava from 13% to 73% compared with mice kept at 21% oxygen throughout; furthermore, the hypoxia-induced increases in thrombosis were dependent upon von Willebrand factor binding to the GPIbα receptor [72]. In rats, an oxygenation level of 11% for 6 h potentiated venous thrombosis in the inferior vena cava by approximately 2-fold versus normoxic controls [19]. Experimental studies have gone on to investigate the molecular and cellular regulation of thrombus formation following hypoxia. Mechanisms by which hypoxia can stimulate a pro-thrombotic response include platelet activation and HIF-mediated or HIF-independent increases in pro-thrombotic factors or decreases in anti-thrombotic factors.

3.1. Platelet activation

In platelets isolated from a cohort of patients with metabolic and peripheral artery disease, exposure to 5% oxygen for 2 h resulted in an increase in the expression of the platelet activation protein, P-selectin, and an increase in the expression of the fibrinogen binding protein, GpIIb/IIIa, compared with platelets exposed to 21% oxygen [73]. Under a level of hypoxia of 5% oxygenation for 2 h, human platelet function and protein expression are altered partly due to activation of the redox sensor, ERK5 [73]. Inhibition of ERK5 also reduced thrombotic burden in an experimental study of murine limb ischemia, while platelet specific ERK5 knockdown led to an inhibition of platelet activity compared with levels found in non-ischemic sham controls [73]. Tyagi et al. studied the role of the hypoxia-induced platelet regulator, calpain, in platelet hyper-reactivity and function and showed that hypoxia-induced calpain in platelets contributes to the development of a pro-thrombotic platelet phenotype [20]. When Tyagi et al. studied inferior vena cava thrombosis in rats kept at 8% oxygenation for 6 h, the authors found that hypoxia enhanced calpain activity by approximately 3-fold via CAPSN1 and promoted thrombus formation [20].

3.2. Coagulation cascade and fibrinolytic pathway

Under hypoxic conditions, TFPI expression is transcriptionally repressed by HIF2α in breast cancer cells, indicating that suppression of anti-coagulant factors (as well as induction of pro-coagulant factors) can occur downstream of hypoxia to enhance thrombosis [54]. In mice, exposure to 6% oxygen for 8 h gave rise to an increase in thrombosis in the pulmonary vasculature, which was associated with an increase in recruitment of mononuclear phagocytes and a 20-fold increase in TF transcript expression in lung tissue samples [74]. It has also been shown in lung cancer cells that enhanced TF signaling is responsible for hypoxia- and HIF1α-dependent increases in plasma coagulation [56]. TF binding to FVII/FVIIa initiates blood coagulation by activating clotting factors FX and FIX [75]. Given that TF triggers the extrinsic pathway of the coagulation cascade, it is likely that HIF1α-dependent increases in TF expression trigger thrombus formation via increases in FX and thrombin. Meanwhile, endothelial exposure to hypoxia activates a pro-thrombotic phenotype by suppressing endothelial expression of the anti-coagulant molecule, thrombomodulin, while anoxia results in a reduction in the fibrinolytic potential of endothelial cells [76–78]. The anti-coagulant plasma glycoprotein, protein S, is also downregulated by hypoxia through HIF1α induction in hepatocarcinoma cells and downregulated in mouse plasma by overexpression of hepatic HIF1α; these HIF1-dependent decreases were found to be associated with increases in thrombin expression [14,67].

PAI1 belongs to the serpin superfamily and stimulates thrombus propagation by inhibiting tissue- and urokinase-type plasminogen activator-mediated fibrinolysis. Previous reports showed that 4 h of

hypoxia induces PAI1 in a time-dependent manner by up to 6-fold in murine macrophages [79]. It was later demonstrated that PAI1 contains 2 HRE sequences and that its expression is regulated by HIF1 and HIF2 in mouse hepatoma cells [55]. Gupta et al. then showed that exposure of thrombosed rats to 11% oxygen for 6 h increased the expression of PAI1 by approximately 2-fold in the hypoxia-induced thrombus compared with the thrombus of normoxia-exposed controls using a rat model of inferior vena cava thrombosis induced by blood flow restriction [19]. In vitro, PAI1 expression in murine macrophages is regulated by the activation of EGR1, HIF1α, and CCAAT/C/EBPα [24]. Perhaps co-incidentally, thrombosis is also a common complication in pressure ulcer patients in whom HIF1α is induced during ulcer development, and experimental inhibition of HIF1α dramatically reduces PAI1 and attenuates thrombus formation in compressed mouse skin [80]. These findings together suggest that prophylactic targeting of the HIF1-PAI1 signaling axis represents a putative strategy of reducing thrombus formation.

3.3. Inflammation and the inflammasome

In addition to the role of stasis-induced hypoxia in venous thrombus formation, the role of vascular wall hypoxia and inflammation has also been demonstrated in arterial thrombogenesis. In a rabbit model of arterial thrombosis, in which thrombosis was induced in the femoral artery of cholesterol-fed animals by repeated balloon injury, vascular wall hypoxia was detected in the vessel neointima (using pimonidazole hydrochloride as a hypoxia marker) and this was positively associated with the thrombogenic potential of the atherosclerotic plaque and with intra-plaque thrombus formation [81]. This study also found expression of HIF1α and nuclear factor kappa B (NF-kB) p65 in human coronary thrombotic plaque samples [81]. Furthermore, in macrophage-rich neointimal areas within rabbit femoral arteries, the magnitude of the hypoxic areas were correlated with the number of HIF1α-, TF-, and NF-kβ p65-positive myeloid cells [81]. To elucidate the mechanism by which high altitude induces thromboembolism, Gupta et al. used an unbiased whole transcriptome analysis to evaluate the genes that were differentially expressed at the site of hypoxia-induced versus normoxic thrombosis using a rat model of inferior vena cava blood flow restriction [19]. The authors of this study showed a direct association between HIF1α and NLRP3-Caspase 1-IL1β signaling [19] and showed that interventions against HIF1α or the NLRP3-Caspase 1-IL1β signaling axis reduced hypoxia-induced thrombus formation in rats [19]. Importantly, the translational implications of these experimental findings were supported by their observation of similar signaling pathway modulation in venous thrombosis patients at high altitude [19]. It is therefore possible that the NLRP3-Caspase 1-IL1β signaling pathway is a novel putative target for prophylactic therapies against thrombosis at high altitude. It is important to note here, however, that inhibitors of HIF1 signaling also impair thrombus resolution in a mouse model of inferior vena cava DVT induced by blood flow restriction and endothelial disturbance [82,83]. Given that therapies that act on hypoxia responsive pathways will likely impact upon a wide range of physiological and pathological processes, the timing and delivery of such treatments as well as their possible unwanted side-effects must be carefully considered.

4. Conclusions

Observational and experimental studies show that conditions of hypoxia are associated with increased risk of thrombosis. Given that hypoxia and HIF target genes modulate coagulation, fibrinolysis, and thrombus resolution, hypoxia-responsive signaling mechanisms that regulate thrombosis may represent putative therapeutic targets.

Declaration of competing interest

None.

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Author contributions

N.G. drafted the manuscript and figures. Y.Y.Z. edited the manuscript. C.E.E. drafted and revised the manuscript.

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