# The multifaceted role of ischemia/reperfusion in sickle cell anemia

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**Sickle cell anemia is a unique disease dominated by hemolytic anemia and vaso-occlusive events. The latter trigger a version of ischemia/reperfusion (I/R) pathobiology that is singular in its origin, cyclicity, complexity, instability, perpetuity, and breadth of clinical consequences. Specific clinical features are probably attributable to local I/R injury (e.g., stroke syndromes) or remote organ injury (e.g., acute chest syndrome) or the systematization of inflammation (e.g., multifocal arteriopathy). Indeed, by fashioning an underlying template of endothelial dysfunction and vulnerability, the robust inflammatory systematization no doubt contributes to all sickle pathology. In this Review, we highlight I/R–targeting therapeutics shown to improve microvascular blood flow in sickle transgenic mice undergoing I/R, and we suggest how such insights might be translated into human therapeutic strategies.**

The sickle mutation in the *HBB* gene encoding β globin results in formation of sickle hemoglobin (HbS) rather than normal HbA. When homozygous, this causes sickle cell anemia (SCA), a unique disease characterized by hemolytic anemia, recurrent vascular occlusions, a systemic inflammatory state, substantial multiorgan disease, foreshortened lifespan, and much suffering. All this stems from the abnormal behaviors of HbS: deoxygenated HbS assembles reversibly into rigid polymers (1), and oxygenated HbS is modestly unstable (2). While the former dominates the disease, the latter does foster development of pathogenic red blood cell (RBC) membrane abnormalities, as reviewed elsewhere (3, 4).

Vaso-occlusions punctuate the clinical course with severe acute painful episodes that are unpredictable in occurrence and variable in frequency. Despite its prominence, sickle vaso-occlusion still presents abundant mysteries that have prompted counterpoised queries (5): "Why don't vaso-occlusions occur all the time?" versus "Why do vaso-occlusions occur at all?" The answer to both questions, we believe, is that SCA is a disease powered by ischemia/reperfusion (I/R) injury pathobiology, a conclusion grounded in data from sickle transgenic mice, SCA patients, and the general I/R literature. In particular, I/R pathophysiology underlies the unique inflammatory context of SCA, a state that is cyclic, systemic, intense, complex, unstable, and perpetual. Thus, we regard I/R as the modern formulation of the "vicious cycle" between erythrostasis and occlusion presciently posited by Ham and Castle in 1942 (Figure 1).

In this Review, we briefly describe the concept of I/R injury and then emphasize the I/R features of the sickle context that match classical I/R models and diseases. We then link these features to the vascular wall pathobiology of SCA and thence to specific clinical features. We conclude by commenting on therapeutic implications and opportunities.

## I/R in general

At its core, I/R is the pathobiology of *resolving* ischemia — tissue injury not only derives from ischemia but also is paradoxically exacerbated by reperfusion-enabled reentry of oxygen (6–8). This pathobiology is an integral part of human diseases such as stroke, myocardial infarction, organ transplantation, bowel ischemia, acute renal injury, trauma, limb ischemia, postpriapic erectile tissue damage, and SCA (8). Predictably, I/R is a risk of therapeutic revascularization procedures.

In some respects, SCA mirrors the I/R of other diseases, but in other respects it differs substantially. To elucidate sickle I/R pathobiology, we here parse it into a stepwise pathogenic vector: (a) a triggering vascular event, (b) development of local I/R injury, (c) systematization of inflammation, (d) development of vascular wall disease, and (e) clinical consequences. A caution, however: SCA and I/R each involve vastly complex, intricately interactive, and temporally/locationally variable systems biologies. Neither can be reviewed thoroughly in the present context. So, this Review emphasizes the overlapping aspects of the two pathobiologies.

## The occlusive trigger in SCA

In classical I/R diseases, a triggering vascular occlusion typically involves an artery, but in SCA, the occlusions are microvascular events. A long-held assumption was that this results from HbS polymerization instigating RBC rigidification and shape change ("sickling"). However, for most sickle RBCs to undergo this calamitous transformation, something must slow their microvascular transit, i.e., provide sufficient time for polymer to form (1, 9).

The basis for such slowing is understood to be the abnormal adhesion of blood cells to microvascular endothelium, with most data pertaining to sickle RBCs (10–13) and/or leukocytes (14, 15).

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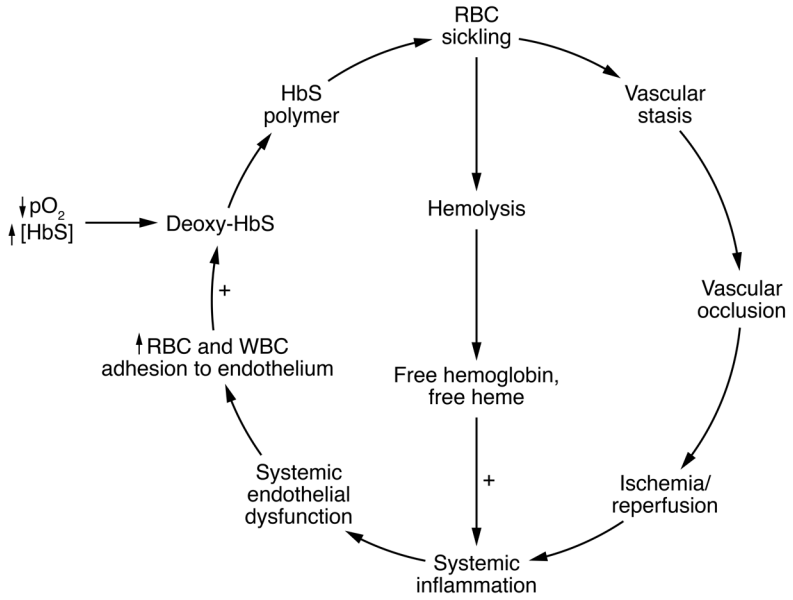


**Figure 1. The pathophysiology of sickle cell anemia is cyclic, with a robust inflammatory state that is driven by blood cell adhesion to endothelium, leading to I/R.**

In sickle transgenic mice, such adhesion in postcapillary venules causes sluggish flow, sometimes outright blockage, with retrograde RBC sickling observed as a secondary event (12, 13). The adhesion biology of this is addressed in a later section. Heterocellular interactions, platelets, and neutrophil extracellular traps can contribute to occlusion as well (15, 16).

## Ischemia and local I/R injury

Notwithstanding its unique initiation, the evolution of ischemia in SCA no doubt matches that in other diseases and experimental I/R models: a progression beginning with rundown of ATP and culminating in mitochondrial failure (8). The pathobiology of I/R injury lies in between the extremes of full tissue recovery (from early restoration of blood flow) and tissue death from reperfusion failure. During this phase, conversion of xanthine oxidoreductase to xanthine oxidase (XO) begins (17).

In large-vessel I/R diseases, the severity of local I/R injury depends on extent and duration of the ischemia, although susceptibility varies by organ and cell type (8). As a microvascular disease, SCA also must derive severity from occlusive multifocality, perhaps — but not necessarily — requiring stochastic locational convergence for actual I/R injury to occur. Occlusive events occur recurrently — and, we suspect, incessantly, propelling the perpetuity of the sickle inflammatory state.

I/R impact can also be influenced by genetics, epigenetics, differential organ susceptibilities, and the microbiome, as reviewed elsewhere (8). SCA additionally involves some fundamental vulnerabilities. Before discussing the I/R reperfusion phase and its sequelae, we describe the instructive experimental sickle models.

## Sickle transgenic mouse models

Much insight is provided by sickle transgenic mice in which human α- and $\beta^s$-globin genes have been added or swapped in for murine globin genes. Several such sickle mouse models have been available, with sickle-like phenotypes ranging from mild to severe.

At their unmanipulated baseline, such mice exhibit many aberrances, including: abnormal blood cell activation, oxidant generation, elaboration of inflammatory mediators, and coagulation activation; endothelial cell dysfunction; growth factor imbalances; tissue hypoxia; and organ pathologies. Although parallel data on SCA patients are vexingly unsystematic, they clearly confirm that these murine aberrances also are features of human SCA. This reflects the centrality of systemic inflammation.

Additional insight derives from sickle mice transiently provoked by exposure to modest hypoxia (7%–11% $O_2$ for 1–4 hours), followed by reoxygenation via return to room air, with endpoints monitored anywhere from 5 minutes to 24 hours thereafter (18). This provocation, termed H/R, is intended to instigate transient RBC sickling and occlusion(s), followed by resolution. In normal mice, such H/R provocation causes little change, but in sickle mice it induces unmistakable I/R pathobiology.

## Reperfusion and local injury

Reoxygenation of the ischemic area causes local I/R Injury, the defining feature of I/R pathobiology. The early footprints of this in non-sickle I/R injury models (7, 8) are also evident in H/R–provoked sickle mice, as follows:

Newly formed XO uses xanthine and oxygen to generate superoxide ($O_2^-$), and XO layers onto distant endothelium (17, 19). Generation of $O_2^-$ and $H_2O_2$ is boosted, including within endothelium, and hydroxyl radical is generated (17, 19, 20). NF-κB activation ensues in endothelium, blood cells, and tissues (17, 21–24). Complement is activated and deposited on cells and tissues (25). Blood quickly acquires inflammation promoters, e.g., TNF, IL-1β, HMGB1 (26–28). RBCs disgorge HbS, mimicking the hyperhemolysis of acute vaso-occlusive episodes in SCA (29). Leukocytes become locally activated (27, 30), releasing cytokines and chemokines, as do tissue mast cells (31).

In due course, the sickle mice develop additional aberrances. Endothelial cells become activated, as evidenced by increased expression of P-selectin, VCAM-1, and tissue factor (Figure 2), appearing at time scales of minutes, 4 to 8 hours, and 12 to 18 hours, respectively (14, 18, 21, 22, 27). Convincingly, H/R provocation of mild-phenotype sickle mice converts their nearly normal endothelium to an activated one resembling that of unprovoked severe-phenotype sickle mice (18). P-selectin–dependent leukoadhesion to microvascular endothelium greatly increases, and leukocyte emigration ensues (14). Occlusive events and microinfarcts develop in the brain (32, 33), and more obvious damage appears in the liver and kidney (23, 34). Even without H/R provocation, severe-phenotype sickle mice exhibit substantial pathology such as infarctions in liver, kidney, spleen, and brain (35).

### Therapeutic corroboration

That these footprints of I/R injury are actually involved in sickle pathogenesis is confirmed by corresponding therapeutic inter-

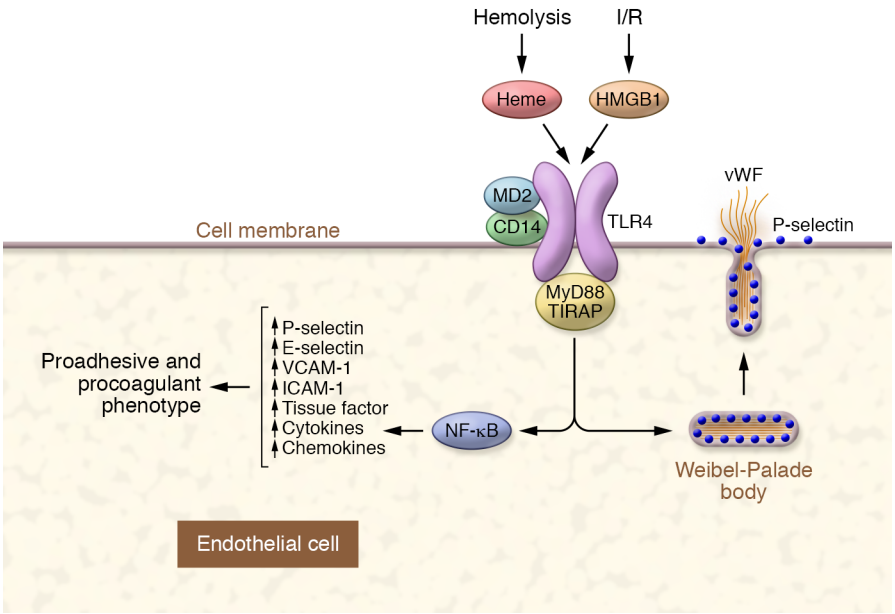


**Figure 2. Biological convergence of heme and HMGB1 as inflammatory instigators.** Activation of TLR4 leads to the secretion of Weibel-Palade bodies as well as proadhesive and procoagulant phenotypes in vascular endothelial cells.

ventions. Many strategies and agents are shown to exert vascular endothelial benefit in H/R–provoked sickle mice (36, 37). Among these strategies, we emphasize those shown to actually improve microvascular blood flow: (a) oxidant suppression using allopurinol to inhibit XO (17), superoxide dismutase (20), catalase (20), polynitroxyl albumin (38), or functional deficiency of NADPH oxidase (39); (b) NF-κB inhibition using sulfasalazine (20), $p50^{-/-}$ (24, 27), dexamethasone (22), and the hydroxamic acid derivatives didox and trimidox (40); (c) inflammatory blunting using etanercept to block TNF (27), TAK-242 to inhibit TLR4 signaling (26), and antibodies against C5 or C5aR to impede complement (25); (d) application of endothelial-sparing agents such as lovastatin (18), the histone deacetylase inhibitor trichostatin A (41), haptoglobin and hemopexin (42), and added heme oxygenase-1 (43) or CO (44, 45); and (e) antiadhesive strategies such as heparinoids (46), 2-fluorfucose (47), blockade of P-selectin in cremaster (14), cerebral (48), and dermal vessels (26), and blockade of VCAM-1 or ICAM-1 in dermis (26). This spectrum of effective strategies and agents in H/R–provoked sickle mice is consistent with the understanding of I/R injury generally (6, 7, 8, 49).

#### Perfusion paradox

Postischemic reperfusion can spread locally activated leukocytes such that they plug nearby capillaries, creating a "no reflow" area that expands the local I/R injury (8). This can instigate a "perfusion paradox" whereby macrovascular hyperperfusion and microvascular hypoperfusion exist concurrently, e.g., as documented in sickle mouse brain (50) and kidney, (34) as well as in the brain in SCA patients (51, 52).

## Systematization of inflammation

In I/R diseases, reperfusion can disperse inflammatory influences beyond the initial injury area, thus bathing endothelium throughout the vascular tree. In severe cases, such as SCA, the resulting systemic inflammation intensifies and arborizes as additional maladaptive changes are recruited and the amount of affected endothelium extends. Homeostasis becomes increasingly compromised as the milieu gains complexity. This state is thoroughly evident in unmanipulated sickle mice at their baseline and in SCA patients. Some of its prominent features are as follows.

#### Cyclicity and perpetuity

In the specific sickle context, this systematization step fosters development of an inflammatory and adhesive endothelium that captures blood cells, leading to microvascular occlusion (12–14). Thus, occlusion causes adhesion causes occlusion, potentially ad infinitum. For this reason, we believe that the unique I/R–driven cyclicity is the governing instigator of the sickle inflammatory state (Figure 1).

In SCA, I/R events are recurrent — and probably incessant. Evidence for the latter is provided by SCA individuals with a very mild disease history, who are wholly asymptomatic and far remote from acute events, but still exhibit chaotic fluctuations of inflammatory biomarkers, as illustrated elsewhere (53). Absent symptoms, this identifies an underlying unsteadiness and/or intermittency. Yet, during such periods, patient hemolysis appears steady. Our interpretation, therefore, is that the so-called SCA "steady state" between acute events is actually a period when occlusions continue to occur, but are of insufficient magnitude to trigger clinical awareness.

Consequently, thought has generally focused on recurrent incitement as a defining feature of sickle inflammation. Recently, however, data from sickle mice have suggested that pathophysiology may additionally involve a failure of normal inflammation resolution mechanisms (54). If so, SCA shares this with other chronic inflammatory diseases, e.g., rheumatoid arthritis.

#### Inducers of sterile inflammation

It has become evident that damage-associated molecular patterns (DAMPs) (55) that are sterile inflammation-inducing, TLR4-signaling ligands may well be dominant instigators in SCA. For exam-

**Table 1. Clinical disease features likely involving I/R pathobiology in sickle cell anemia**

| |
|---|
| **Local I/R injury** |
| Stroke (silent, clinical, hemorrhagic transformation) |
| Cognitive deficits |
| Kidney disease |
| Bone pain |
| Abdominal pain |
| Postpriapic erectile dysfunction |
| Nociceptive hypersensitivity |
| Retinopathy |
| Liver disease |
| Perfusion paradox |
| **Remote organ injury** |
| Acute chest syndrome |
| **Systematization of inflammation** |
| All of the above |
| Endothelial dysfunction |
| Arterial vasculopathy |

ple, H/R–provoked sickle mice develop increased plasma HMGB1 (28) and hemoglobin/heme (26). In SCA patients, both are elevated at baseline and both increase further in association with acute vaso-occlusive events (28, 29). Interestingly, there can be TLR4 signaling synergy between HMGB1 and either hemoglobin or heme (28). We hypothesize that heparan sulfate (56), released by inflammatory glycocalyx degradation, plays a similar role. Inhibition of DAMP signaling via TLR4 knockout improves microvascular flow in H/R–provoked sickle mice (26).

## Unusual vulnerabilities in SCA

### Endothelial dysfunction

Sickle mice (57) and SCA patients (58, 59) exhibit persistent endothelial dysfunction, itself a hallmark feature of I/R. This must bestow exquisite vulnerability on the vascular wall with regard to impact of any new insult. A suggestive example from a non-sickle study revealed that when endothelial nitric oxide synthase (eNOS) is deficient, the pulmonary vasoconstrictive effect of hypoxia is greatly magnified (60); this is of concern in sickle sleep apnea (61).

### DAMPs

By activating the NLRP3 inflammasome, DAMPs can create a primed state in endothelial cells, monocytes, and platelets — in essence, this represents a "hair trigger" for subsequent release of IL-1β and HMGB1 (62–64). Opportunity for pathogenic synergy is presented by the superimposed, high expression of TLR4 in sickle monocytes (65). Such leukocyte activation can result from their activating interaction with sickle RBCs (66, 67), and we suspect the same is true of endothelial cells because of the injury response they manifest in response to sickle RBCs (68, 69).

### Genetics

Polymorphisms in non-globin genes can modulate I/R impact (8). Suggesting this, the Duffy-null state highly prevalent in sub-Saharan Africa bestows increased severity in cases of acute lung injury (70), a classical complication of SCA. Another example is a TLR4 polymorphic haplotype, prevalent only in sub-Saharan Africa, that augments inflammatory signaling by TLR4 ligands (71).

### Conditioning and concurrency

Remarkably, a facet of I/R injury generally is that mild ischemia, insufficient to induce injury, can actually exert a "conditioning" effect that can ameliorate a subsequent I/R injury, even at a distant location (8). So, it seems highly likely that at any given time, the milieu of an SCA patient is an unpredictable mixture of effects from new I/R injuries plus resolving I/R injuries plus conditioning effects from mild events. We suggest that this ever-changing milieu contributes to the characteristic instability of the sickle inflammatory state. Whether conditioning can be exploited for benefit in SCA deserves clinical investigation. A hint of support lies in a demonstration that exercise training of sickle mice reduces their systemic inflammation (72).

### Hemolysis and its products

Kato et al. have described SCA patients as being either hemolytic or vaso-occlusive in phenotype (73). However, the I/R and hemolytic components of SCA are inextricably linked. Both derive from HbS polymerization and RBC sickling, and the DAMPs produced by the two processes (HMGB1 and heme) biologically converge at TLR4 signaling (Figure 2). Thus, our view is that the two processes are codominant, each contributing to every clinical feature.

Not surprisingly, therefore, it has thus far been impossible to meaningfully parse out the proportionate contributions of hemolysis and I/R. However, two new sickle pharmaceuticals perhaps could be experimentally exploited to do so. Crizanlizumab blocks P-selectin–mediated adhesion biology; it blunts vaso-occlusive severity but has no effect on hemolysis (74). Conversely, voxelotor locks HbS molecules into the oxyHb configuration; it reduces hemolysis but has no effect on vaso-occlusive severity (75).

Intravascular hemolysis exposes multiple harmful perturbants (4). Cell-free oxyHbS consumes NO in extracellular space (76). Released RBC microparticles (77) can activate monocytes, damage endothelium (78, 79), and enhance thrombin generation (80). Released arginase can depress plasma L-arginine. Cell-free oxyHbS quickly auto-oxidizes to release free heme (2) that avidly enters and oxidizes plasma low-density lipoproteins (81); heme similarly enters endothelial membranes, exerting injurious inflammatory effects (82). That this is relevant is suggested by the ameliorative effect of an apolipoprotein A mimetic in sickle mice (83). Indeed, our data suggest that one-half of heme's inflammatory impact on endothelium is TLR4-independent.

## Vascular wall: the interface between biology and pathology

The vascular wall bears the brunt of I/R–driven inflammation, with endothelium being its pivotal pathobiologic target. This is critical because normal vascular health requires successful endothelial integration of myriad inputs to organize adaptive homeostatic responses. In doing so, it adaptively links different biophysiologic functions, e.g., inflammation, coagulation, vasoregulation,

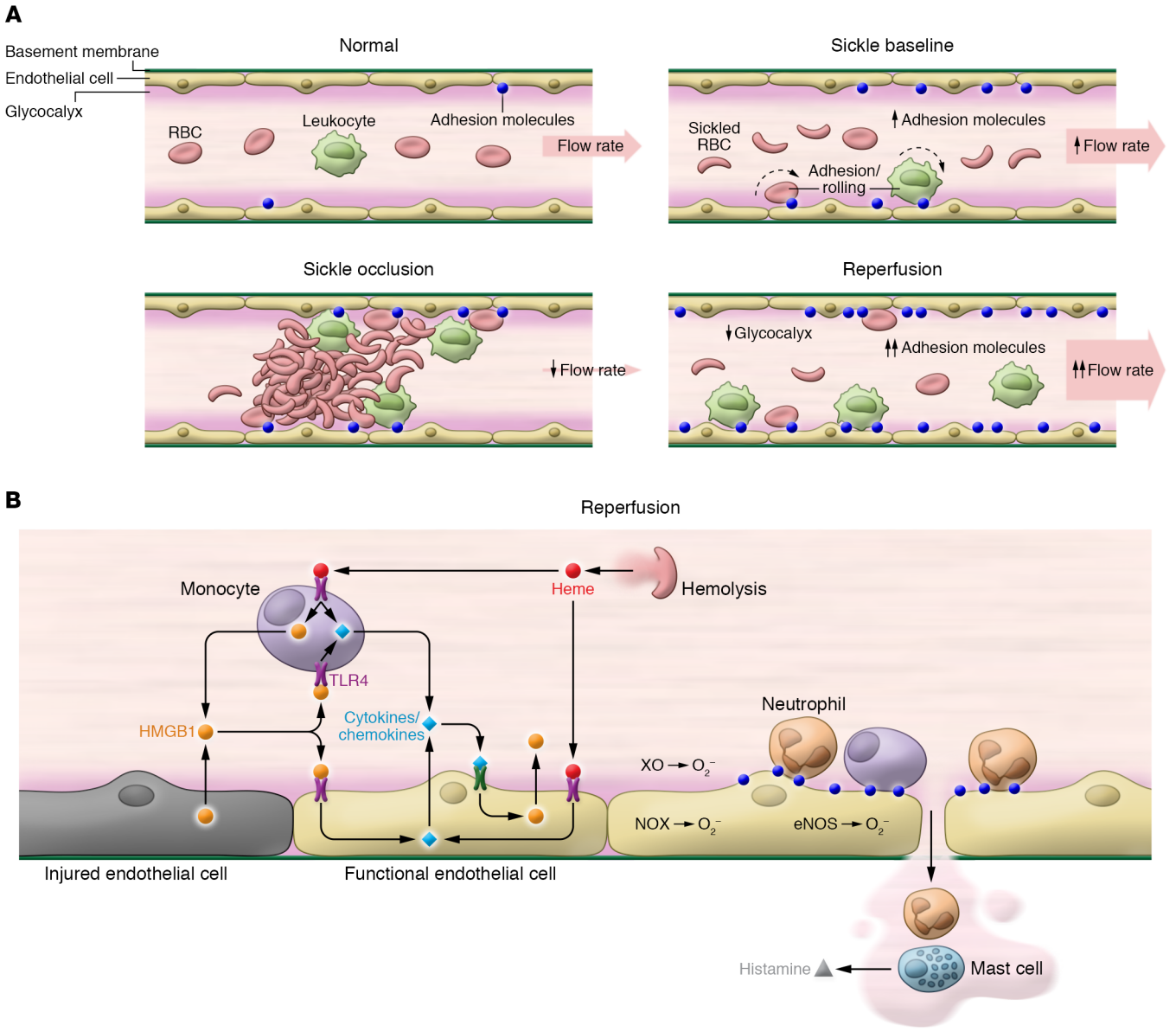


**Figure 3. Key early features of I/R in the sickle context.** (**A**) Compared with normal, baseline sickle vascular biology includes somewhat increased adhesion molecule expression, some baseline rolling of red cells and leukocytes on endothelium, and increased flow rate. Sickle vaso-occlusion is triggered by red cell and/or leukocyte adhesion in postcapillary venules, resulting in retrograde sickling and obstruction. Upon reperfusion, adhesion molecule expression and blood cell rolling and adhesion are far more prominent, the glycocalyx has thinned, and flow is notably hyperemic. (**B**) An expanded view of the reperfusion in **A**, showing prominent early pathobiology of local I/R injury in SCA.

etc. In SCA, normality is overtaken by I/R, with prominent maladaptive consequences for the vascular wall (Figure 3).

### Oxidant generation

In I/R generally, multiple sources of oxidant generation are extant and vary over time and among organs; most prominent are XO, NADPH oxidase, cytochrome P450, and uncoupled eNOS (6–8, 49).

In sickle mice at baseline, $O_2^-$, $H_2O_2$, and hydroxyl radical are generated in excess (17), and this worsens after H/R provocation. Also at baseline, they exhibit abnormal XO on the endothelial surface (19) and eNOS uncoupling (57). Biochemical footprints of oxidative/nitrosative stress are apparent: tissue nitrotyrosine (19) and lipid peroxidation products (17) are present, and activities of heme oxygenase-1 (84) and Nrf2 (85) are increased. NADPH oxidase is found activated in phagocytic cells (67, 86), RBCs (87), and endothelial cells (20), just as it is in arteriopathies generally (88). Indeed, in sickle mice, NADPH oxidase underlies cerebral vessel P-selectin exposure and occlusion (39).

Notably, the sickle milieu includes numerous NADPH oxidase activators, one of which is exposure of leukocytes and endothelial cells to sickle RBCs (67, 68). Also suspect is the anemic hyperemia, because abnormal shear stress can activate endothelial NADPH oxidase (89). In H/R-provoked sickle mice, the arterial endothelial oxidant level rises dramatically (20), and neurochemical perturbants

increase further (90). Peculiarly, an increased inducible NO synthase (iNOS)/eNOS activity ratio is evident in sickle mouse kidney (34) and liver (23). In this regard, SCA mimics sepsis, in which increased iNOS activity coexists with a deficiency of eNOS activity (91).

#### Glycocalyx thinning

The endothelial thick glycocalyx separates endothelial surface and free flowing blood in all blood vessels (92, 93). In inflammatory contexts it is degraded (94), so we suspect this is present in SCA. This would be highly pathogenically relevant because glycocalyx integrity is required for multiple endothelial homeostatic functions, including proper mechanosensing of shear stress, repelling of adhesive blood cells, and several others.

#### Endothelial hyperpermeability

Proper regulation of endothelial barrier permeability controls accessibility of subendothelial space to water, solutes, and inflammatory cells. Barrier hyperpermeability is a hallmark feature of I/R pathophysiology (8, 95), and it is evident in sickle mice and patients (35). This is specifically relevant to brain and lung disease in SCA. H/R provocation of sickle mice activates mast cells (31) that can permeabilize monolayers of lung (96) and brain (97) endothelial cells. A variety of permeabilizing agents are abundant in SCA. High doses of heme, enough to cause cell and host death, can permeabilize pulmonary endothelial layers (96). Elevated TNF in sickle mice (27) and patients is relevant, as it not only permeabilizes endothelial and epithelial barriers but also impedes adaptive fluid reuptake.

#### Endothelial adhesion biology

In inflammatory syndromes, endothelial activation can derive from many perturbants, a plethora of which are documented in blood of sickle mice and patients (98). An example in H/R–provoked sickle mice is that activated monocytes produce TNF that activates venular endothelium to upregulate tissue factor (TF) and VCAM-1 (27). That SCA patients do have activated endothelium is evidenced by elevated levels of: endothelial-derived microparticles, some expressing TF and VCAM-1 (77); soluble adhesion molecules (98); and circulating endothelial cells (CECs) that are abnormally positive for TF, VCAM-1, ICAM-1, and E-selectin (P-selectin was not tested) (99–101). Sickle mice at baseline have correspondingly activated CECs and endothelium in situ (101).

*RBC adhesion*. In vitro experiments have identified about 20 different mechanisms that can mediate sickle RBC adhesion to cultured endothelial cells in vitro (11), some shown in flow chambers (e.g., TNF-provoked adhesion via RBC $\alpha_4\beta_1$ and endothelial VCAM-1; ref. 102). However, only three mechanisms have been validated in sickle mice vivo under flow: P-selectin (103), $\alpha_v\beta_3$ (104), and ICAM-4/LW (105).

*WBC adhesion*. Two studies in the sickle mouse at baseline identified abnormal leukoadhesion with slowing of blood flow in cremaster and cerebral venules (14, 48). It was inhibited by P-selectin blockade but not by E-selectin blockade.

In another cremaster venular model, E-selectin was implicated in sickle mice challenged with TNF (15, 106). In that model, adherent leukocytes can, if they engage E-selectin, activate $\alpha_M\beta_2$ integrin that then actually captures sickle RBCs, a heterocellular adhesion event that is mitigated by blockade of E-selectin. The devil being in the details, this E-selectin model (15, 106) employs a severe-phenotype sickle mouse (that at baseline has plasma TNF approximately 15 pg/mL and already exhibits substantial TNF-dependent pathobiology; ref. 27) and injects it with an additional 500,000 pg TNF, a dose that causes lethality within hours (106).

*I/R in general*. In non-sickle I/R models, leukoadhesion roles for various adhesion molecules have been identified (6, 8, 107). In these models, P-selectin is virtually always implicated in leukocyte rolling, the first step in eventual capture, while E-selectin is implicated in some I/R models but not in others.

*H/R sickle mouse*. The far greater leukoadhesion/emigration in cremaster venules of H/R–provoked sickle mice was mitigated by blockade of P-selectin but not of E-selectin (14). Similar data were obtained using cerebral venules (48). In dermal microvessels, leukoadhesion was limited by blockade of P-selectin, ICAM-1, and VCAM-1 (22). We note corresponding clinical trial data from SCA patients: P-selectin blockade using crizanlizumab exerted significant interventional and prophylactic efficacy, but E-selectin blockade with rivipansel failed to meet endpoints regarding vaso-occlusive severity (74, 75). Thus, in our opinion the available data most vividly highlight a role for P-selectin in adhesion biology of SCA and in sickle I/R. However, the vast complexity of adhesion biology argues that far more study, in physiologically relevant models, is required to truly understand functional adhesion biology in the sickle I/R context.

#### Endothelial activation: coagulation

Predictably, the endothelial-based, bidirectional activating linkage between inflammation and coagulation is evident in SCA as a hypercoagulable state (80) with abnormal activation of coagulation, fibrinolysis, and platelets. An example of its complexity is that the abnormal activation of platelets in SCA elevates plasma thrombospondin (108), which inhibits ADAMTS13 (109), resulting in abnormal high–molecular weight forms of von Willebrand factor (110) that mediate sickle RBC adhesion to endothelium (110).

#### Endothelial vasoregulatory dysfunction

It has been argued that in SCA the cause of eNOS dysfunction is consumption of NO by plasma oxyHbS (73). Our view is that this simply *mimics* some aspects of eNOS dysfunction but does not actually cause it (4). Actual eNOS uncoupling, on the other hand, is a hallmark feature of I/R contexts (6–8) and is present in sickle mice (57, 83, 111) and patients (58, 59). The sickle milieu likely includes several abnormalities that are known causes of eNOS uncoupling: oxidation of tetrahydrobiopterin due to increased intracellular $O_2^-$ (112), inhibition from elevated asymmetric dimethyl arginine (113), and depletion of intracellular L-arginine by TNF-induced endothelial arginases (114, 115) and possibly by hypoxia-induced impairment of the L-arginine importer (116).

## Clinical consequences

I/R undoubtedly contributes to many features of SCA; we suspect it is sufficient for some and necessary for many (Table 1).

#### Local I/R injury to the brain

The brain is the organ most vulnerable to I/R injury (8). In SCA, strokes of at least three types occur: small "silent" strokes that

begin in childhood and occur lifelong, eventually in perhaps 50% of patients; hemorrhagic strokes in young adults; and cerebral artery clinical ischemic stroke affecting approximately 8% of children (117).

The concurrency of macrovascular hyperemia plus microvascular hypoperfusion in the SCA patient brain constitutes the perfusion paradox, revealing considerable shunting and oxygen demand/supply mismatching (52, 118–120). There is low vascular reserve, as the combination of endothelial dysfunction and anemic hyperemia heavily taxes adaptive capacity (51, 52). The regions that develop the silent strokes exhibit low perfusion, possibly from occlusion of penetrating venules (121), consistent with the postcapillary venular location of pathogenic blood cell adhesion in SCA. Thus, in sickle mice (50) and SCA patients (51, 52, 118, 119), parts of the brain are chronically hypoxic or on the borderline thereof.

Many aspects of I/R pathophysiology likely participate in these events. Cerebrovascular P-selectin expression triggered by NADPH oxidase impedes cerebral microvascular flow (39, 48), and H/R provocation triggers microinfarcts (32, 33). The brains of sickle mice and patients reveal abnormal blood-brain barrier hyperpermeability (35), a known feature of cerebral I/R models. Indeed, such hyperpermeability is implicated in lacunar strokes, stroke size, hemorrhagic transformation, and considerable cognitive disturbance (122, 123), thus paralleling SCA brain disease. It has been argued that, in general, normality of brain function requires normality of cerebrovascular endothelial function (124), highlighting the fundamental pathobiologic role of I/R–driven endothelial dysfunction in SCA.

#### Local I/R injury to other organs

The kidney's high osmolality, low pH, and low $pO_2$ are strong promoters of HbS polymerization, so it is not surprising that the organ exhibits high susceptibility to I/R injury (34). Thus, renal disease accounts for approximately 17% of mortality in SCA. Features of liver in SCA mimic those observed in experimental I/R models. While gut I/R has not been studied in the sickle context, we suspect its involvement because patients can manifest considerable abdominal pain during crises (and there can be celiac arteriopathy). I/R can contribute to nociceptive hypersensitivity. The bone marrow, frequently involved in the occlusive crises of SCA, would seem to be a setup for occurrence of I/R, given its low $pO_2$ and sluggish flow (125); however, this has not been studied.

#### Systematization of inflammation and arteriopathy

The I/R–driven systematization of inflammation is the most prominent facet of I/R in SCA, as it engenders the template of endothelial vulnerability that profoundly contributes to all clinical pathology. We here focus on the arterial vasculopathy of SCA that occurs in brain, lung, bowel, penis, kidney, and spleen (126).

Although the amount of reviewable histopathology is suboptimal, a major feature is intimal hyperplasia, the arterial wall's universal response to endothelial inflammation (126). In human medicine, arterial disease emerges from the chronicity of such injury, so we believe that the I/R–driven systematization of inflammation subserves arteriopathic evolution in SCA. In particular, in parallel with their inciting roles in the paradigmatic arteriopathy, atherosclerosis, the abnormal activation of blood monocytes (127), and DAMP-driven TLR4 signaling (26, 28, 128) likely play prominent roles in promoting SCA arteriopathy. Lesion locations, on the other hand, probably reflect added impact from various endothelial microenvironments (129).

*Circle of Willis*. Arteriopathy at the circle of Willis is strongly associated with childhood ischemic clinical stroke in SCA (117). Rather than one causing the other, however, it is suspected that each results from the anemic hyperemia of SCA. In support, experimental cerebral hyperperfusion was found to induce arteriopathy of the middle cerebral artery (130). The locational specificity could simply derive from abnormal shear stress patterns created by anemic hyperemia at cerebral artery divergences (131). The adverse effects of abnormal shear stress are many, but it can, e.g., induce endothelial NADPH oxidase activity (89).

*Pulmonary arterial hypertension*. Pulmonary arterial hypertension (PAH) surely is multifactorial in origin in SCA, just as it is in general medicine (132, 133). Adequate discussion of this is beyond the scope of this Review. Yet, in SCA, I/R–driven systematization of inflammation comprises a "perfect storm" that includes a great many relevant perturbants implicated in the general PAH medicine literature. Some of these are anemic hyperemia (134), DAMP signaling (135), growth factors, vasoregulators, coagulation/platelet activation, recurrent hypoxia from sleep apnea, and arterial glycocalyx degradation PAH (136). And, unique to the pulmonary artery, we hypothesize a meaningful role for endothelial injury that results from chronic endothelial molestation by the sickled RBCs in venous blood, as illustrated by in vitro experiments (68, 69).

#### Remote organ injury

Local I/R injury can be complicated by disease developing in a distant organ, an inflammatory acute lung syndrome being the most common example (8). Lung susceptibility derives from its unique features: constitutive endothelial P-selectin expression in pulmonary capillaries, enormous baseline marginated leukocyte pool, capillaries in series (137). In SCA, features of the acute chest syndrome perfectly match examples of remote organ injury in general medicine (36).

## Implications for therapeutics

The complex systems biology of the sickle I/R state involves a multitude of theoretical targets and, therefore, possible therapeutic approaches. The section above on "Therapeutic corroboration" describes nearly two dozen concrete examples of drugs that improve microvascular blood flow in H/R–provoked sickle mice. Although this implies that there may be many viable therapeutic approaches to sickle I/R, in reality each discrete step in Figure 1 comprises its own extraordinarily complex network.

Thus, it is probably naive to believe that sickle I/R can be effectively targeted with a single drug. And, of course, data from the sickle mouse may not be predictive of the SCA human. For example, the cardiac output of the mouse is 6-fold (by weight) greater than that of the human. Nonetheless, we suggest that this vastly intricate systems biology has two points of I/R vulnerability (we do not discuss targeting HbS polymerization herein).

#### Targetable vulnerabilities

One point of vulnerability may be the actual initiation step of I/R pathobiology, i.e., before it has arborized and reached mature

complexity. Targeting early events at this specific step with multiple drugs may be possible. We previously suggested an example combining three drugs already deployed in general medicine: allopurinol to inhibit XO (17), sulfasalazine to inhibit NF-κB (20), and etanercept to block TNF (27). Each individually improves microvascular flow in H/R–provoked sickle mice. We have elsewhere emphasized that proof of biological efficacy can be obtained on such drugs using very few subjects if tested longitudinally — we advise use of this strategy for efficacy screening. We also do believe that statins are logical for SCA. Their pleiotropic, endothelial-sparing effects are evident in non-sickle I/R models (138), in H/R–provoked sickle mice (18, 139), and perhaps in sickle patients (140). In fact, they are reported to exert tissue conditioning effects protective against subsequent I/R (141).

The second point of vulnerability lies in the central role that I/R–driven blood cell adhesion to endothelium plays in the perpetual cycling between occlusion and adhesion. So, targeting this adhesion is an indirect way to limit HbS polymerization. As articulated herein, our view is that fairly robust data argue for blocking P-selectin using crizanlizumab (74) or a heparinoid (142, 143).

## Conclusion

The pathophysiology of sickle cell anemia involves a unique form of I/R pathobiology that drives the robust inflammatory state, notable for its cyclicity, intensity, instability, complexity, and perpetuity. Certain specific clinical complications of SCA can be attributed, in large part, to local I/R injury (e.g., cerebrovascular disease) or I/R remote organ injury (e.g., acute chest syndrome) or I/R systematization of inflammation (e.g., arteriopathy). In the broad view, it is likely that the inflammatory systematization, via the template of endothelial dysfunction and vulnerability it establishes, contributes to all clinical disease in SCA.

Encouragingly, experimental studies have identified a number of strategies and multiple specific drugs that improve microvascular blood flow in sickle mice provoked via exposure to hypoxia/reoxygenation, a maneuver that induces an acute, bona fide I/R state in such mice. Unfortunately, a persistent stenosis in the bench-to-bedside translational pipeline has prevented promising approaches targeting I/R from reaching clinical investigation — despite our demonstration long ago that solid data confirmatory of in vivo human biological efficacy can be obtained by longitudinal study of as few as three SCA patients (53). We hope this Review will help persuade the sickle research community that such I/R–targeting approaches are worth testing in the clinic.

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