CRITICAL REVIEW

Multiple inducers of endothelial NOS (eNOS) dysfunction in sickle cell disease

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Abstract

A characteristic aspect of the robust, systemic inflammatory state in sickle cell disease is dysfunction of endothelial nitric oxide synthase (eNOS). We identify 10 aberrant endothelial cell inputs, present in the specific sickle context, that are known to have the ability to cause eNOS dysfunction. These are: endothelial arginase depletion, asymmetric dimethylarginine, complement activation, endothelial glycocalyx degradation, free fatty acids, inflammatory mediators, microparticles, oxidized low density lipoproteins, reactive oxygen species, and Toll-like receptor 4 signaling ligands. The effect of true eNOS dysfunction on clinical testing using flow-mediated dilation can be simulated by two known examples of endothelial dysfunction mimicry (hemoglobin consumption of NO; and oxidation of smooth muscle cell soluble guanylate cyclase). This lends ambiguity to interpretation of such clinical testing. The presence of these multiple perturbing factors argues that a therapeutic approach targeting only a single injurious endothelial input (or either example of mimicry) would not be sufficiently efficacious. This would seem to argue for identifying therapeutics that directly protect eNOS function or application of multiple therapeutic approaches.

1 | INTRODUCTION

The complicated pathophysiology of sickle cell disease (SCD, all sickle genotypes except sickle trait) includes a multitude of vascular aberrancies. In general, these are derived from the disease's two dominant pathogenic vectors that result from proximate abnormalities of the sickle erythrocyte: vasoocclusion-induced ischemia–reperfusion injury (I/R)[1] and hemolysis.[2] Although it is fashionable to view these as discrete processes having divergent clinical consequences, our view is that, for the most part, both hemolysis and I/R contribute to all clinical manifestations, albeit in varying ways and proportions.[1]

Since the endothelium comprises the homeostatically-dynamic, biological interface between blood and tissue, it is not surprising that endothelial cell abnormalities are a prominent pathobiological feature of SCD. A note on terminology to avoid confusion: we emphasize that use of "endothelial cell dysfunction" (ECD) herein refers specifically to *dysfunction of the endothelial cell itself*. Thus, this is distinct from the two known special examples of ECD mimicry due to effects outside of the endothelial cell (hemolytic consumption of NO and oxidative smooth muscle cell dysfunction).

Sickle cell disease exhibits each of the potential categorical manifestations of ECD: permeability barrier dysfunction, assumption of a pro-adhesive/pro-inflammatory phenotype, acquisition of a procoagulant phenotype, and vasoregulatory dysfunction. This review focuses solely upon the latter – specifically, the characteristic maladaptive dysfunction of the vasodilatory enzyme endothelial nitric oxide synthase (eNOS). Dysfunction of eNOS is evident in both humans with SCD[3–5] and sickle transgenic mice.[6]

TABLE 1 Known inducers of eNOS dysfunction present in SCD

	Seen in hemolysis	Seen in I/R
↑ arginase	+	+
↑ asymmetric dimethyl arginine	+	+
↑ complement activation	+	+
↑ glycocalyx degradation	+	+
↑ free fatty acids	−	+
↑ inflammatory mediators	+	+
↑ microparticles	+	+
↑ oxidized LDL	+	+
↑ oxygen radicals	+	+
↑ TLR4 ligands (HMGB1, heme)	+	+

Abbreviations: HMGB1, high mobility group box 1; LDL, low density lipoproteins;TLR4, Toll-like receptor 4.

After briefly summarizing eNOS itself, we individually discuss each of the 10 identified features of SCD that have been shown in other and experimental contexts to induce eNOS dysfunction (Table 1). We conclude with some thoughts about the implications of the data reviewed herein for therapeutics of SCD.

2 | ENDOTHELIAL NITRIC OXIDE SYNTHASE

The functional eNOS dimer is a complicated enzyme[7] having binding domains for the metabolic substrate L-arginine (arginine) and six necessary cofactors: tetrahydrobiopterin (BH_4), calmodulin, heme, NADPH (reduced nicotinamide adenine dinucleotide phosphate), flavin adenine nucleotide, and flavin mononucleotide. It is influenced by post-translational modifications, for example, several possible sites of phosphorylation and S-glutathionylation. Furthermore, the interaction of eNOS with caveolin affects function, as does its cellular location, for example, caveolar or cytoplasmic. And oxidation can adversely impact the necessary dimerization of the enzyme.

Under normal circumstances, the enzyme's product is NO. However, if endothelial cell arginine or tetrahydrobiopterin (BH_4) are deficient, or a steric inhibitor such as asymmetric dimethylarginine (ADMA) is elevated, eNOS becomes "uncoupled" and produces superoxide instead of NO (Figure 1).

3 | ECD-PROMOTING FACTORS

The following promoters of eNOS dysfunction, extant in the specific SCD context, are known consequences of I/R and/or hemolysis, as identified in experimental and/or clinical literatures (Table 1). Since there is no way at present to rank the relative importance of these different promotors on endothelial biology in SCD, we discuss them in alphabetical order. In the discussion of each individual stressor, we identify its biological context, document its hemolytic and/or I/R

origin in general, indicate its presence in SCD, and describe its specific relevance to ECD in the SCD context.

3.1 | Arginase, asymmetric dimethylarginine

Arginine availability to eNOS is determined by the endothelial cell's cytoplasmic arginine concentration, availability of required cofactors, and level of any steric eNOS inhibitors such as ADMA.[8,9]

3.1.1 | Plasma arginase

In SCD, the increased plasma arginase derives mostly from sickle RBC via intravascular hemolysis, and this lowers plasma arginine.[10] However, it does seem likely that arginase release from activated leucocytes, as seen in sepsis, also contributes somewhat in SCD. Also, I/R can be another source of plasma arginase, in which case it is released from the affected tissue.[11]

3.1.2 | Endothelial arginase

Of more direct relevance, the activity of arginase *within* endothelial cells can be increased, thereby depleting endothelial arginine. This can be induced by inflammatory signaling, so presumably as a consequence of both I/R and hemolysis. Indeed, several factors that are elevated in SCD are experimentally documented enhancers of endothelial arginase activity, for example: TNF (tumor necrosis factor),[12] oxidized low-density lipoproteins (oxLDL),[13] and thrombin.[14] However, the status of endothelial arginase activity has not been studied in SCD.

3.1.3 | Asymmetric dimethyl arginine

This naturally occurring metabolite of arginine is formed excessively under I/R conditions, as evidenced by increased plasma levels.[15] So, ADMA is probably elevated by hemolysis since this has been observed in severe malaria,[16] and in SCD ADMA level correlates with plasma LDH (lactate dehydrogenase).[17]

3.1.4 | ECD impact of arginine/ADMA disturbance

When endothelial cell arginine is insufficient, eNOS uncouples.[7,9] It is believed, however, that the critical determinant of eNOS status is not arginine alone, but rather the endothelial ADMA:arginine ratio. When this is abnormally elevated, it causes ADMA to compete with arginine for association with eNOS,[7] and it also is believed to inhibit endothelial import of arginine from plasma.[8] Consistent with the ADMA:arginine ratio being critical, in non-sickle experimental I/R models supplementation with arginine improves eNOS function in face of high plasma ADMA.[8]
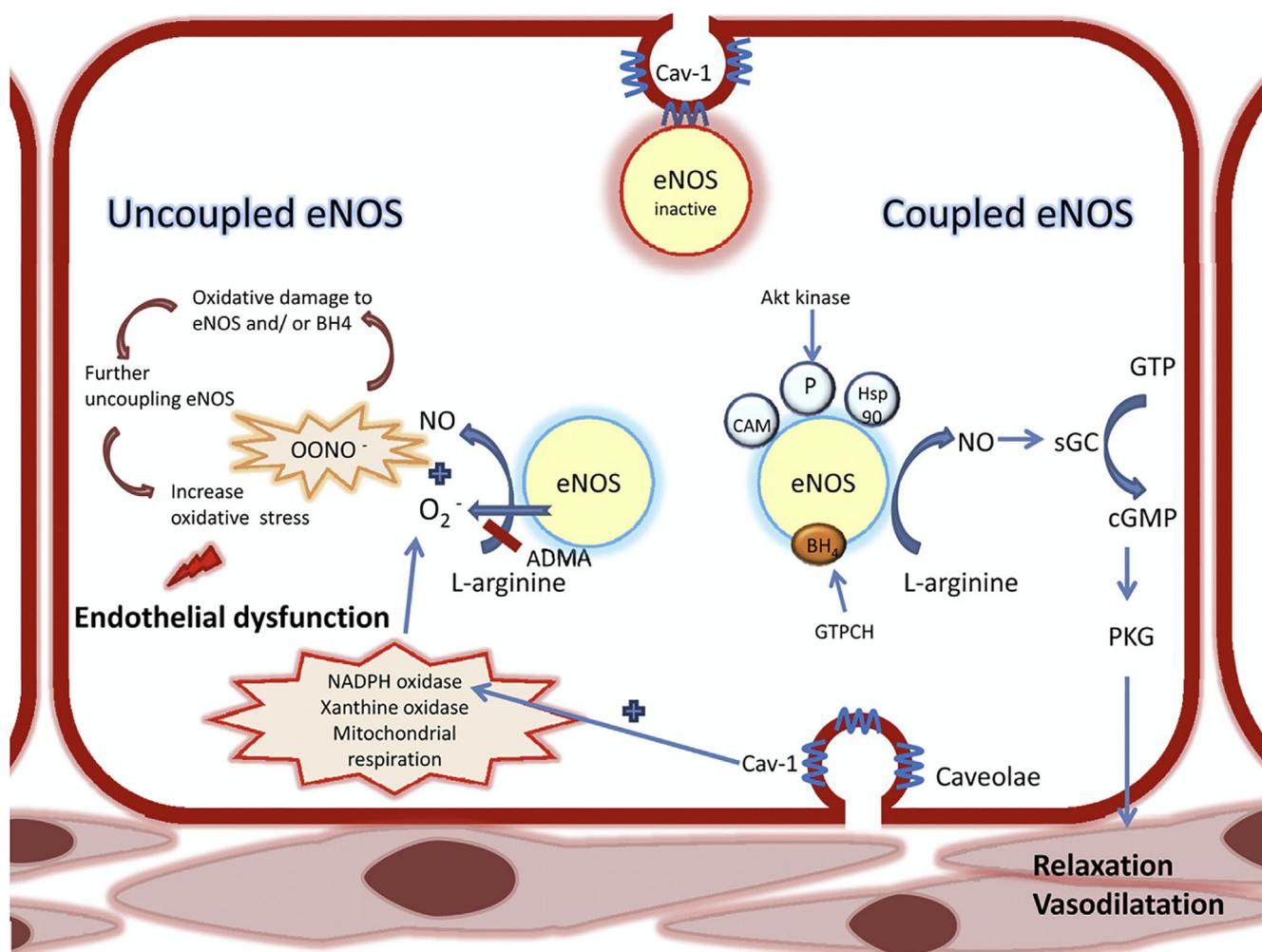
FIGURE 1 Central role of endothelial NO synthase (eNOS) uncoupling in the pathogenesis of endothelial dysfunction. eNOS is localized at the plasma membrane caveolae. In endothelial cells, eNOS is inactive when it is bonded with caveolin 1 (cav-1). When it becomes active, eNOS disassociates from cav-1 and binds with calmodulin (CAM) and heat shock protein 90 (Hsp90) and together with phosphorylation of serine sites (e.g., Ser[1177]). The functional eNOS protein is a dimer (so-called coupled eNOS). Tetrahydrobiopterin (BH4), an essential cofactor of eNOS, is necessary for optimal eNOS activity. BH4 facilitates NADPH-derived electron transferring from the eNOS reductase to the oxygenase domain to convert L-arginine to NO and L-citrulline. NO plays a major role in relaxation of smooth muscle surrounding arterioles and maintaining vascular function by inhibition of vasoconstriction, platelet aggregation, leukocyte adhesion, and cell proliferation through the cGMP-dependent downstream signaling cascade. Interaction between L-arginine and asymmetric dimethylarginine (ADMA; endogenous competitive inhibitor of NOS) is likely direct competition for eNOS. When availability of L-arginine or BH4 levels are inadequate, eNOS becomes unstable and uncoupled, leading to subsequently less NO production and more superoxide generation. Moreover, interaction between NO and superoxide leads to formation of peroxynitrite, a potent oxidant, which further oxidizes BH4, resulting in eNOS uncoupling as a vicious cycle, with subsequent endothelial dysfunction. Figure is reproduced with permission from Ref.[21]

3.1.5 | The sickle context

Arterial rings removed from sickle transgenic mice manifest deficient acetylcholine-induced, eNOS-dependent vasorelaxation that is mitigated if the mice had been treated with an inhibitor of both the plasma and endothelial arginases.[18] However, a very small study of SCD subjects supplemented with arginine found no benefit for flow-mediated dilation, the common clinical test for eNOS functionality (most commonly performed by using Doppler to assess post-occlusion hyperemia at the brachial artery[19]). A study of sickle mice found arginine supplementation to blunt oxidant stress.[20] A larger study did find

that arginine supplementation can reduce vasoocclusive crisis frequency. Given available data, we expect the endothelial cell's intracellular AMDA: arginine ratio to be elevated in SCD subjects.

3.1.6 | The arginine paradox

A positive endothelial response to increasing plasma arginine can reflect "arginine paradox". This term refers to the fact that exogenous arginine supplementation boosts endothelial eNOS activity— even when plasma and endothelial arginine levels are normal. In fact,

endothelial arginine is normally abundant, from 10 to 30 times higher than the arginine Km for isolated eNOS.[8,9] So, a beneficial endothelial response to infused arginine does not prove that deficient *plasma* arginine was a constraint on eNOS function.

3.2 | Complement

The complement (C) cascades are part of the innate immune system, normally functioning to damage and clear organisms or cells (Figure 2). Pathogenic complement activation can be triggered by both I/R[22,23] and hemolysis.[24]

3.2.1 | The sickle context

The complement system is activated in SCD.[25,26] This occurs through assembly of alternative pathway C3 and C5 convertases on the phosphatidylserine-rich outer membrane of sickle RBCs and the microparticles they release.[27] It also is partially heme dependent.[25] A study of SCD subjects identified not only an increased level of activation fragment C5a but also evident microvascular deposition of C5b-9 in small vessels of skin biopsies.[28] Treatment of SCD

patients with hydroxyurea produces substantial reductions in complement activation.[25]

### 3.2.2 | ECD impact of complement activation

The complement terminal attack complex impairs endothelial-dependent vasorelaxation, possibly by uncoupling eNOS.[29–31] Inflammatory consequences of complement activation in SCD are relevant; for example, recombinant C5a induces inflammation and vaso-occlusion in sickle mice.[26] In hemolytic processes, TLR4 signaling can apparently activate complement in an endothelial and P-selectin dependent manner.[32] Interestingly, factor H (a complement control protein) prevents the P-selectin mediated adhesion of sickle red cells to endothelial cells.

3.3 | Endothelial glycocalyx thinning

The endothelial glycocalyx (EGX) covers the luminal surface of all endothelial cells, and the spaces between them, as a 200–1000 nm thick layer immediately adjacent to the plasma membrane (Figure 3).[34] It is composed of a complex variety of glycoproteins
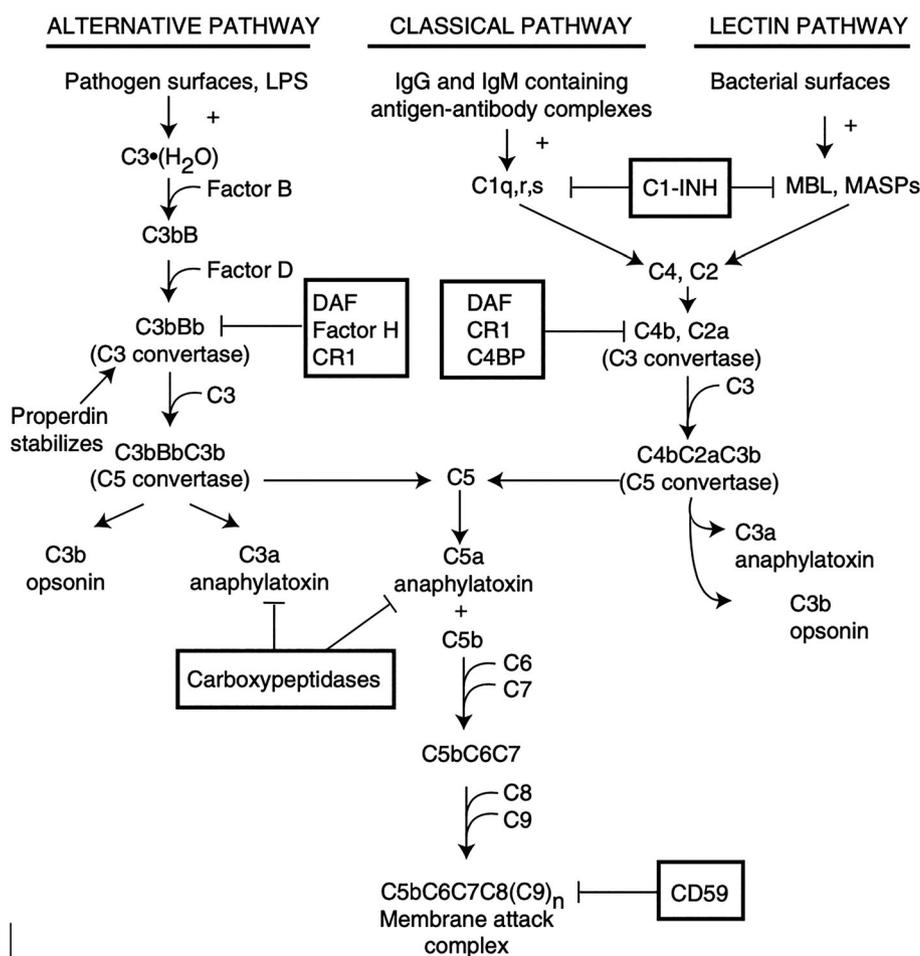
FIGURE 2 Basic structure of the complement system. C, complement; C4BP, C4b binding protein; CR, complement receptor; DAF, decay accelerating factor; INH, inhibitor; LPS, endotoxin. Reproduced with permission from Figure 1 from Ref. 33
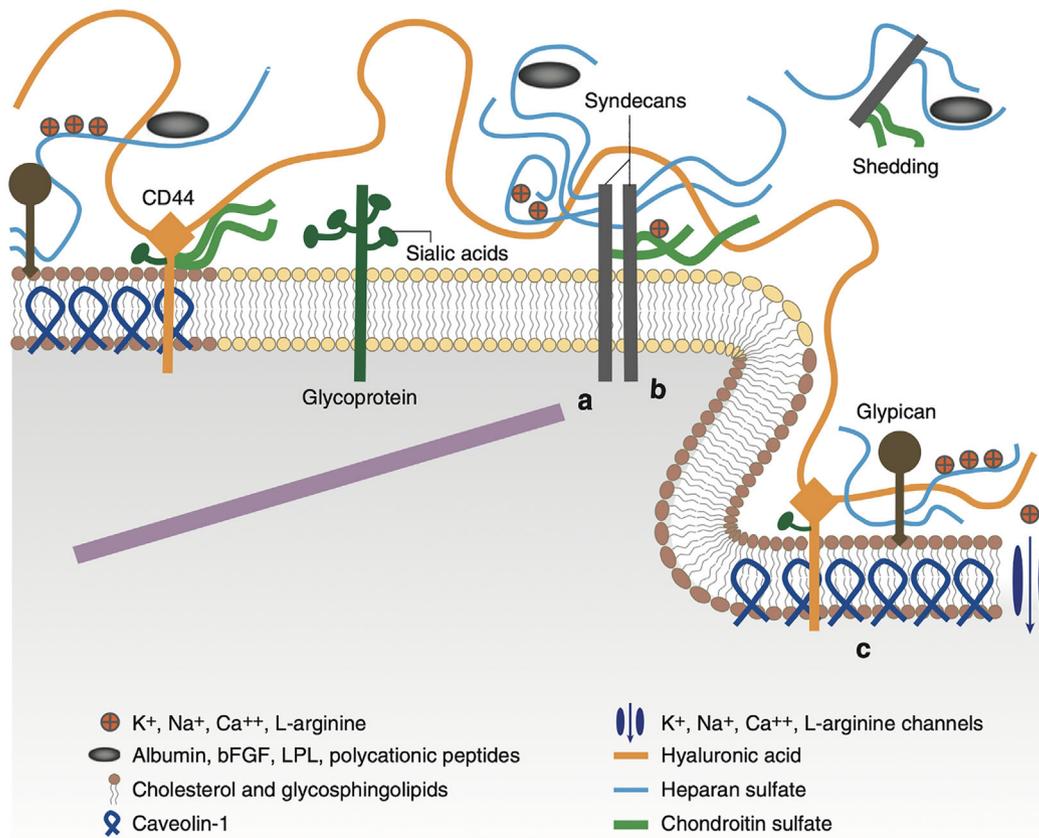
FIGURE 3 The endothelial glycocalyx (EGX) basic features. bFGF, basic fibroblast growth factor. LPL, lipoprotein lipase. Figure reproduced with permission from Figure 1 from Ref. 50

carrying sialic acid and proteoglycans decorated with glycosaminoglycan chains having negatively charged sulfates (∼70% heparan sulfate). Other components include membrane-anchored syndecans and glycosylphosphatidylinositol-anchored glypican. Also, embedded within it are long strands of high molecular weight hyaluronan, either unanchored or anchored to CD44. Normally, the integrins, the selectins and CAMs, and the outer aspects of membrane channels and receptors are mostly buried within the EGX. Biophysically, this EGX is a hydrated, negatively charged, porous gel—the "pores" of which normally are sized so as to prevent solutes larger than albumin from passing to subendothelial space. Thus, despite being an external feature, the EGX is not only attached but is also a functionally an integral part of the endothelial cell.

The remainder of the overall endothelial surface layer is composed of an overlying mat of proteins deposited on top of the EGX. This is an "interactome" that includes >200 proteins having heparan sulfate binding capability.[34] Examples include: anti-thrombin III, heparin cofactor II, von Willebrand factor, tissue factor pathway inhibitor, C1 esterase inhibitor, extracellular superoxide dismutase, xanthine oxidase, inflammatory chemokines, and albumin. A number of these proteins are functionally optimized via association with EGX.[34]

Even normally, EGX thickness is not uniform; rather, it varies in different parts of the vasculature. Of particular relevance to interpretations of experimentation, the EGX thickness on cultured human

umbilical vein endothelial cells was found to be only one-twentieth of the EGX thickness of the parent umbilical vein endothelium from which they came.[35] This suggests it would be optimal if in vitro observations of endothelial functions are confirmed in a whole-animal model.

3.3.1 | EGX degradation

Pathologic thinning of the EGX is a hallmark feature of I/R specifically and inflammation generally.[36] Thinning is accomplished by a variety of blood- and endothelial-derived inflammatory "sheddases", for example, heparanase, proteases, and hyaluronidase.[37] We find no reports of hemolysis causing EGX degradation. However, it is likely that hemolysis does so, for example., via its TLR4 signaling impact of increasing TNF and ROS, both of which promote EGX shedding.

3.3.2 | The SCD context

Given the complex, robust inflammatory milieu of SCD, it would seem virtually certain that EGX thinning is a feature of its endothelial biology. However, the EGX has been largely ignored in this disease.

A single imaging study revealed evidence for abnormal EGX thinning in sickle patients.[38] The elevation of natriuretic peptides in SCD[39] may be implicated, since A,B, and N peptides are each implicated in EGX shedding.[40]

3.3.3 | ECD Impact of EGX thinning

Known consequences of abnormal EGX thinning include increased barrier porosity and permeability, abnormal exposure of adhesion molecules, compromise of anti-thrombotic capacity, and loss of endothelial mechanosensing of shear stress.[32] Indeed, EGX integrity is a critical endothelial component enabling sensing of wall shear stress – it is known to be required for normal shear stress-induced eNOS activation.[41] Conversely, the replacement of any shed EGX is dependent upon the endothelial cell's eNOS/NO-dependent EGX husbandry. Thus, EGX structural components are in a dynamic equilibrium between their mechanosensing-triggered assembly and their degradative loss by shedding.[34,42] Thus, it seems highly likely that compromise of endothelial mechanosensing due to EGX thinning is a major contributor to eNOS dysfunction in the SCD context, given the prominence of its degradation as a feature of both I/R and other inflammatory pathobiologies.[34]

3.4 | Free fatty acids

We find no evidence that hemolysis per se increases free fatty acid (FFA) levels. But in the context of I/R, enhanced activity of phospholipase A2 (PLA2) can liberate FFA, a specific example being oleic acid.[43]

3.4.1 | The sickle context

In SCD, PLA2 is increased, even more so in association with acute chest syndrome.[44] Consequently, levels of some FFA are elevated in SCD; among these, in fact, is oleic acid.[45] We emphasize this because, decades ago, oleic acid injection was a classical way to experimentally induce an acute respiratory distress syndrome.[46] Thus, it is not surprising that in SCD, fat embolism is associated with acute chest syndrome (ACS), and that the latter tends to occur early in vasoocclusive crises.[47]

### 3.4.2 | ECD impact of FFA

Free fatty acids can promote ECD.[48] For example, in cultured endothelial cells, oleic acid downregulates eNOS expression and decreases its activity by interfering with the AMPK/PI3K/Akt/eNOS pathway of activation.[49] Free fatty acids also can, promote induction of the NLRP3 inflammasome, stimulate endothelial NADPH oxidase, and activate TLR4 signaling.[48] Each of these occurrences is known to directly or indirectly compromise eNOS function.

3.5 | Inflammatory mediators

Stemming from both hemolysis and I/R pathobiology, the complex inflammatory vascular milieu of SCD includes a great variety of perturbative substances.[1,2] This includes: cytokines and chemokines, vasoactive mediators and metabolites, growth factors, reactive oxygen species, activated complement proteins, activated coagulation and fibrinolysis components, lipid-sourced mediators, and others. Within such general categories reside many factors that, for example, initiate release and/or activation of EGX sheddases[34,37] (see EGX subsection) or activate NADPH oxidase, a common pathway to ECD (see ROS subsection).

3.6 | Microparticles (MP)

Microparticles are sub-micron sized vesicles that can variably retain some membrane features and cytoplasmic components of their cells of origin (Figure 4). Thereby, they can be a mode of intercellular information trafficking. Microparticles are present normally, but they are released in far greater numbers by platelets, leucocytes, and endothelial cells as a feature of either activation or dysfunction. Red blood cells release them in various hemolytic disorders.[51] Thus, MP are pathologically present in a great variety of vascular disorders.[52] Microparticles also are released in experimental and naturally occurring I/R contexts, the parent cell source depending upon the affected tissue; for eample, liver MP in hepatic I/R,[53] endothelial MP in stroke I/R.[54]
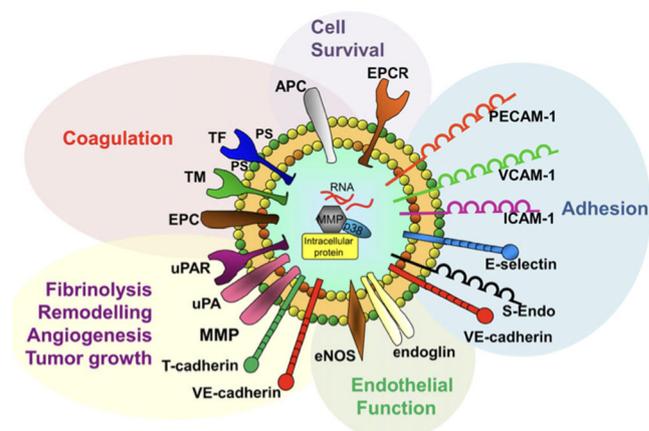
FIGURE 4 An endothelial-derived microparticle (EMP) showing accompanying functionalities reported in literature. Not all EMP display each moiety shown here. eNOS, endothelial nitric oxide synthase; EPC, endothelial protein C; EPCR, endothelial protein C receptor; ICAM-1, intercellular cell adhesion molecule −1; MMP, matrix metalloprotease; PECAM-1, platelet endothelial cell adhesion molecule – 1; S-Endo (aka, P1H12, melanoma cell adhesion molecule); TF, tissue factor; TM, thrombomodulin; uPA, urokinase plasminogen activator; uPAR, uPA receptor; VCAM-1, vascular cell adhesion molecule – 1. Reproduced with permission from Figure 1 from Ref. 86

3.6.1 | The sickle context

Sickle cell disease plasma contains abnormally increased MP from RBC, platelets, leucocytes, and endothelial cells, those from RBC being the most abundant.[55–57] Their numbers increase during a vaso-occlusive crisis. The RBC MP probably are released from sickle RBC as a response to sickling/unsickling,[58] aided by Hb spicule-mediated deformation of the plasma membrane[59] and by thiol oxidation of membrane proteins.[60] In SCD up to half of total plasma cell-free heme is accounted for by hemoglobin trapped within RBC MP.[55]

3.6.2 | ECD impact of MP

Sickle RBC-derived, heme laden MP adhere to and transfer heme into endothelium, resulting in oxidant stress, impaired acetylcholine-dependent NO-mediated vasorelaxation, and even vaso-occlusion.[61] In the sickle mouse, RBC MP also increased endothelial adhesion molecule expression, reportedly via a TLR4 signaling dependent mechanism.[62] Interestingly, *endothelial*-derived MP in various experimental contexts similarly impair NO-dependent vasorelaxation,[63–65] indicating that the adverse impact of MP need not be a function of contained heme per se. One study found that endothelial-derived MP induced permeabalization and acute lung injury.[64]

3.7 | Oxidized low density lipoproteins (oxLDL)

Oxidized low density lipoproteins can promote hemolysis,[66] and hemolysis likely promotes oxLDL formation, because cell-free heme readily partitions into and oxidizes LDL.[67–69] In an experimental intestinal I/R model, oxLDL were found to accumulate not only locally in intestine but also in liver and lung, indicating vascular distribution.[70] Furthermore, impact synergy can occur because oxLDL effects on endothelial cells are mediated in part by LOX-1 (oxidized LDL receptor 1), the expression of which is augmented in I/R.[71] Signaling from oxLDL can also occur via CD36 on platelets and monocytes/ macrophages, so perhaps also by CD36 positive microvascular endothelial cells.[72]

3.7.1 | The sickle context

Cell-free HbS is a source of liberated free heme.[73] The LDL of SCD subjects are oxidizable by heme abnormally easily.[69] Thus, plasma levels of oxLDL are described to be *proportionately* elevated in both SCD subjects[74,75] and sickle mice.[76]

3.7.2 | ECD impact of OxLDL

These oxLDL adversely impact eNOS in several ways. They downregulate eNOS in a TLR4-dependent manner,[77] and they inhibit acetylcholine-induced activation of eNOS by interfering with the eNOS/caveolae interaction.[78] Note, OxLDL induce endothelial

NADPH oxidase[79] and diminish eNOS activity. In addition, oxLDL triggers LOX-1 signaling[80] that, in turn, leads to multiple aberrancies, including endothelial vasoregulatory dysfunction.[81] Another impact of oxLDL includes decreasing arginine transport into endothelial cells and induction of endothelial arginase.[82] Also, oxLDL are a possible ligand for TLR4 signaling (see TLR4 sub-section).[82]

3.8 | Reactive oxygen species (ROS)

Both I/R[83] and hemolysis[84] generate ROS via multiple mechanisms. Sources probably include hemoglobin S auto-oxidation, NADPH oxidase induction, xanthine oxidase, myeloperoxidase, mitochondrial dysfunction, and uncoupled eNOS.

3.8.1 | The sickle context

Sickle cell disease subjects exhibit biological footprints of excess oxidant activity.[85] In the sickle mouse, endothelial ROS can be generated to excess (Figure 5).[87–89] Many known features of SCD biology are inducers of NADPH oxidase activity, including: TNF, thrombin, ROS, endothelin I, angiotensin II, vascular endothelial growth factor, interleukin-1, oxLDL, and signaling by Toll-like receptor 4 (TLR4). Concurrently, individuals with SCD tend to be somewhat deficient in antioxidants.[85]

### 3.8.2 | ECD impact of ROS

The NADPH oxidase generation of superoxide is believed to be a dominant ROS source within endothelial cells in vascular diseases generally.[90] Also, NADPH oxidase activation can lead to eNOS dysfunction by exerting multiple effects. In particular, it causes (probably via peroxynitrite) the oxidation of BH4 and GTP cyclohydrolase I, thereby dropping the BH4:eNOS ratio to < 1 so that the enzyme uncouples and itself generates superoxide.[91]

3.9 | TLR4 signaling

TLR4 is a damage-pattern recognition receptor that is functionally expressed on monocytes/macrophages, neutrophils, immune cells, platelets, endothelial cells, and smooth muscle cells, among others. Upon its engagement with an appropriate ligand, TLR4 produces inflammatory signaling causing ROS generation, NF-κB activation, inflammasome enhancement, and release of inflammatory cytokines and chemokines.

3.9.1 | The sickle context

TLR4 signaling has not been measured in SCD subjects per se, but it is elevated in sickle mice.[92,93] Known TLR4 activators that are elevated in SCD include ligands resulting from both hemolysis (e.g., heme[92–94])
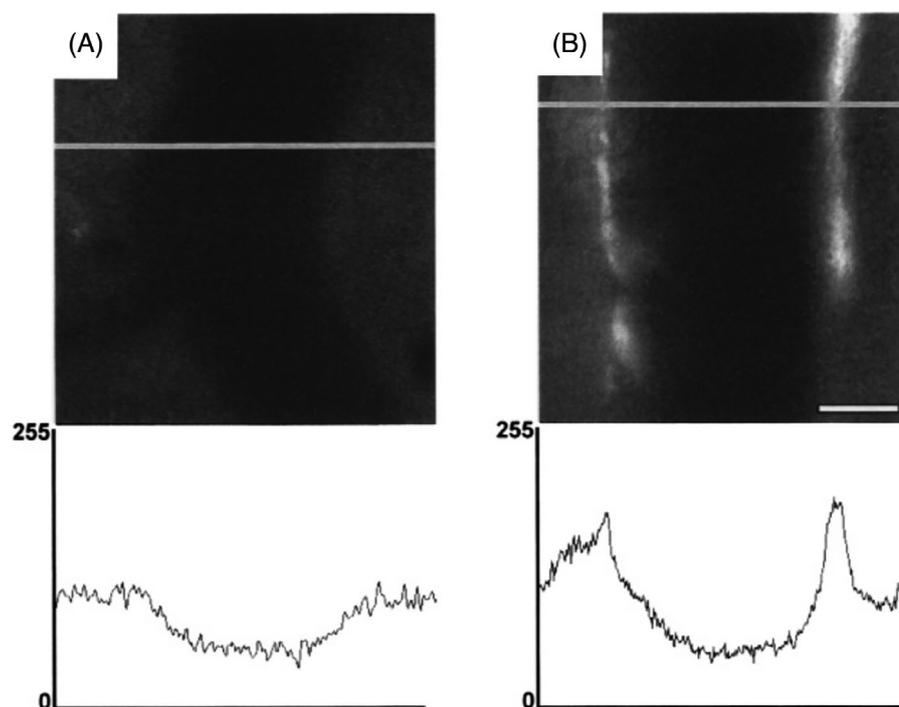
FIGURE 5 Oxidant generation by endothelium in situ. In sickle mice, cremaster venular segments were monitored for oxidant generation, as detected by fluorescence from DHR (dihydrorhodamine 123). The vessel runs vertically, the lines of signal indicating oxidant generation by endothelial cells in the walls of the single vessel. Bar indicates 10 μm. (A) Normoxia control. (B) After 3 hours of hypoxia followed by 30 min of normoxia, the DHR signal appears confirming increased oxidant generation. (Such exposure to hypoxia followed by room air induces a clear I/R state that converts endothelium of the mild-phenotype sickle mouse to replicate that of an *unmanipulated* severe-phenotype mouse.) Reproduced with permission from Figure 1 from Ref. 87

and I/R (e.g., high mobility group box 1, HMGB1[95,96]). Both heme[2] and HMGB1[97] are elevated in plasma of both humans with SCA and sickle mice. Other TLR4 signaling ligands that may be relevant in SCD can include the FFA palmitate,[80] RBC-derived microparticles, hyaluronan and heparan sulfate from EGX degradation,[98] abnormal shear stress patterns,[99] and oxidized LDL.[98]

3.9.2 | ECD impact of TLR4 signaling

A study using mouse endothelial cells demonstrated that HMGB1/ TLR4 signaling adversely impairs acetylcholine stimulated, eNOS-dependent vasorelaxation.[100] A study of murine macrophages documented that TLR4 signaling by HMGB1 and hemoglobin can be synergistic,[101] as it can be for heme and endotoxin.[94]

We speculate that HMGB1 is far more important in SCD than is currently recognized. In cardiology it has been specifically implicated in promotion of all stages of the inflammatory arteriopathy of atherosclerosis.[102] Perhaps it plays similar role in the inflammatory arteriopathy of SCD. We elsewhere have proposed a unique mechanism by which elevated HMGB1 can compromise endothelial mechanosensing of shear stress,[103] a mechanism directly relevant to the SCD context.

4 | VASOCONSTRICTORS

An inflammatory milieu can include a variety of vasoconstrictors and these, of course, can impact endothelial vasoregulation. A general discussion of all endothelial-derived vasoregulatory molecules is beyond

the scope of this eNOS-focused review. However, we do note that SCD subjects are reported to have elevated levels of the vasoconstrictors endothelin-1 (ET-1),[104] angiopoietin-2 (Ang-2)[105] and thromboxane A2. Also reported is a depressed level of the vasodilator prostacyclin, although platelet activating factor and prostaglandin E2 levels are reportedly elevated. Interestingly, exposure of endothelial cells to sickled RBC boosts their ET-1 production.[106] Also notable is that two vasoconstrictors, ET-1 and Ang-2, are stored in Weibel-Palade bodies. Since Weibel-Palade activation is normally inhibited by NO, the NO deficient status of endothelium in SCD likely promote endothelial vasoconstrictor release.

4.1 | Red cell eNOS

This produces intracellular NO which is involved in complicated relationships between hemoglobin thiols, membrane thiols, nitric oxide, related species, and vascular resistance.[107] Interestingly, RBC eNOS is reported to participate in vascular resistance regulation (e.g., it contributes to depressing blood pressure), but it does not contribute to functional measures of endothelial eNOS activity (acetylcholine-stimulated flow-mediated dilation).[108] Thus, NO from RBC eNOS is believed to decrease vascular resistance by mechanisms different than that of NO from endothelial eNOS, perhaps via NO reaction products. So, in theory, depressed RBC NO, as is said to be the case in SCD, could contribute a vasoconstrictive influence, though it would not mimic eNOS dysfunction per se. Regardless, we speculate that RBC eNOS is of little relevance because most NO generated by it would be readily consumed by the RBC's extremely high intracellular concentration of hemoglobin.

5 | EXAMPLES OF ECD MIMICRY

This review has focused upon the disparate factors in the SCD pathobiological milieu that can cause eNOS dysfunction, that is, dysfunction of the endothelial cell itself. There also are two known examples of ECD mimicry in the SCD context. The distinction between true ECD and ECD mimicry will be important both for interpretation of pathophysiology and if endothelial-related therapeutics are considered.

5.1 | NO consumption by cell-free hemoglobin

The consumption of NO by cell-free oxyhemoglobin residing either in plasma or within MP is well-known and has recently been thoroughly reviewed.[2] By consuming NO outside of the endothelial cell per se, cell-free Hb intercepts the normally unimpeded *transfer* of NO from endothelial cell to smooth muscle cell (SMC). In clinical testing for ECD by assessing flow-mediated dilation, this external NO consumption could mimic a primary NO generation insufficiency resulting from actual eNOS deficiency. We find no evidence that extracellular consumption of NO affects either internal eNOS generation of NO or the endothelial cell's internal NO-mediated functions.

5.2 | Oxidized soluble guanylate cyclase (sGC)

An elegant study of sickle transgenic mice identified a relevant example of smooth muscle cell (SMC) dysfunction: a blunted ability of the SMC to *respond* to NO, a defect caused by abnormal oxidation of the SMC's soluble guanylate cyclase itself.[109] Depending on extent of such modification, it also could possibly mimic the effect on clinical testing of actual eNOS dysfunction.

6 | IMPLICATIONS FOR ENDOTHELIAL-TARGETED THERAPEUTICS

The vast complexity of SCD and the involvement of multiple pathogenic factors leading to eNOS dysfunction (Table 1) create a difficult challenge if endothelial-targeted therapeutics are considered. It is impossible to identify the dominant mechanism underlying eNOS dysfunction. If there even is one, it may differ among individuals, or even vary with time. These challenges could be taken to argue for employing an agent exerting broad salubrious effects upon the endothelial cell.

6.1 | Statins

In this regard, statins benefit the endothelial cell in multiple ways, including reversing eNOS uncoupling.[110] Indeed, a statin has exhibited efficacy in sickle mice (for reducing endothelial activation markers)[111] and in SCD subjects (for reducing pain).[112] Statins have not been tested for ECD impact per se in the specific sickle context, but in atherosclerotic arteriopathy they do exert restorative effects. Indeed, in general medicine rosuvastatin protected against stroke in seemingly-healthy individuals with somewhat elevated CRP but without hyperlipidemia.[113] Statins could perhaps be considered for application in SCD.

7 | CURRENTLY APPROVED DRUGS

Four drugs are currently FDA approved for use in SCD: hydroxyurea, L-glutamine, crizanlizumab, and voxelotor. The former two are known to exert eNOS-sparing effects, whereas no such effects have been reported for the latter two.

7.1 | Hydroxyurea

Hydroxyurea's disparate effects include increasing eNOS protein level[114] and stimulating its activity.[115] A very small study of this drug's effect on a measurement of ECD by flow mediated dilation yielded negative results.[116] Of note, however, the drug can blunt incidence of sickle stroke.[117,118]

7.2 | L-glutamine

L-glutamine exerts pleiotropic effects on endothelial cells, including: improving their redox potential (NAD+/NADH, NADP+/NADPH); boosting glutathione; stimulating heme-oxygenase; promoting synthesis of L-arginine; and fostering NO production.[119] Notably, in a mouse study glutamine ameliorated the defective eNOS function caused by L-Nω-methylarginine, another arginine analogue exerting the same eNOS uncoupling effect as ADMA.[120]

8 | CONCLUSION

Within the exceedingly complex pathobiology of SCD there are 10 identifiable factors that have been shown, largely in other contexts, to adversely impact eNOS function. At present, it is impossible to identify a single, dominant, ECD-inducing agent at which therapy perhaps could be directed. If specific endothelial-sparing therapeutics are considered, this would seem to argue for application of agents known to exert multiple beneficial effects upon the endothelial cell's biology, or for use of a combination of agents. In SCD, observation of abnormal flow-mediated dilation, the clinical test commonly used clinically to detect ECD, could plausibly derive from true eNOS dysfunction (as reviewed herein) or from either of the two noted examples of ECD mimicry.

CONFLICT OF INTEREST

DATA AVAILABILITY STATEMENT

No data were created for this review.

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