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FORUM ARTICLE

Hemoglobin Oxidation and Heme in Atherosclerosis—Link Between Intraplaque Hemorrhage and Progression of Complicated Atherosclerotic Lesion

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Abstract

Significance: Solid evidence has demonstrated the pathophysiologic role of hemoglobin (Hb) oxidation and heme in the remodeling of arterial tissue in complicated atherosclerotic lesions with hemorrhage. Oxidation of Hb to ferric and ferryl states and subsequent accumulation of ferryl hemoglobin, heme released from globin, peptides derived from fragmentation of globin result in cellular activation and dysfunction, as well as polarization of resident cells and macrophages—all of which drive plaque formation and instability.
Recent Advances: Oxidized Hb and heme are endogenous pro-atherogenic agonists that target vascular endothelial cells, smooth muscle cells, neutrophils, and macrophages. Rearrangement of the actin cytoskeleton, disruption of intercellular integrity, activation of proinflammatory genes involving nuclear factor kappa B, the c-Jun N-terminal kinase, and the p38 mitogen-activated protein kinase signal transduction pathways are key events leading to endothelial dysfunction. Polarization of macrophages involves cellular responses toward inflammation, calcification, and angiogenesis. Pathways affected include gene changes associated with phosphoinositide 3-kinase signaling, lipid transport, tissue remodeling, and vascularization. This review focuses on the maladaptive cellular responses to Hb oxidation and its products, together with oxidation of low-density lipoprotein and plaque lipid material implicated in the progression of the complicated atherosclerotic lesion, and provides insights into the potential adaptive molecular mechanisms.
Critical Issues: Identification of molecular targets and novel therapeutic applications are needed to prevent remodeling of vascular tissue in the hemorrhaged complicated atherosclerotic lesion.
Future Directions: Future research should focus on the red blood cell infiltration of early lesions as a source of extracellular Hb-bearing danger signals. *Antioxid. Redox Signal.* 00, 000–000.

Keywords: hemoglobin, heme, vascular diseases

Introduction

Atherosclerotic plaques, especially hemorrhaged lesions, contribute to a considerable extent to deaths, according to the World Health Organization. The American Heart Association classification system categorizes atherosclerotic plaques according to histopathologic characteristics rather than functional or clinical significance. This scheme delineates six progressive stages of lesion development: type I (initial lesion), type II (fatty streak), type III (pre-atheroma), type IV (atheroma), type V (fibroatheroma), and type VI (complicated lesion) (Stary et al., 1995). Among these, plaques considered most vulnerable typically demonstrate type IV or type V histologic features (Mehta and Shah, 2006).

The prototypical "vulnerable plaque" is characterized by distinct morphological features, including a thin fibrous cap,

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marked macrophage infiltration, relative depletion of smooth muscle cells, and a large lipid-rich necrotic core often accompanied by calcified nodules. These structural alterations are thought to result from repeated cycles of plaque rupture and healing, contributing to progressive luminal narrowing and the development of high-grade coronary stenosis (Virmani et al., 2000).

Type VI lesions, designated as complicated plaques, are defined by surface disruption, intraplaque hematoma or hemorrhage, and superimposed thrombosis (Stary et al., 1995). The complicated atherosclerotic lesion is characterized by ruptures of the neovascularized vessel derived from the vasa vasorum (Barger et al., 1984; Michel et al., 2014; Moreno et al., 2006) and also by ruptures on the plaque surface and/or hemorrhage into the plaque (Moreno, 2010).

It has been demonstrated that the interaction between plaque material and red blood cells (RBCs) within the lesion initiates RBC lysis. Once released from RBCs, free hemoglobin (Hb) is subjected to rapid oxidative modifications (Nagy et al., 2010). Approximately half a century ago, it was discovered that following the oxidation of Hb to methemoglobin (metHb, Fe^{3+}), heme exchange can occur both among the globin subunits and between Hb and albumin (Bunn and Jandl, 1966; Bunn and Jandl, 1968). Heme moieties released from metHb in ruptured RBCs have been found to translocate to the endothelium (Balla et al., 1993).

The elucidation of heme synthesis and catabolism laid the foundation for the earliest therapeutic application of heme in the treatment of porphyria (Lamon et al., 1978). This advancement also led to the first documented vascular complication—venous phlebitis—following the intravenous administration of hematin, thereby suggesting a potential role of heme in the development of vascular pathologies (Dhar et al., 1978; Simionatto et al., 1988). This adverse effect was significantly mitigated by the development of the heme arginate complex (Herrick et al., 1989; Mustajoki et al., 1986). The therapeutic use of heme in human medicine prompted studies on heme catabolism, ultimately leading to the discovery of the heme oxygenase (HO) enzyme. However, these early observations did not immediately highlight the broader pathophysiological significance of heme and heme oxygenase (HO) in other human diseases (Keyse and Tyrrell, 1989; Tenhunen et al., 1969).

Later studies showed that heme's ambivalent nature gives it unique biological properties: acutely, it sensitizes cells to free radicals, while chronically, it activates the protective HO–ferritin system (Balla et al., 1992a; Balla et al., 1991b; Balla et al., 1993; Cermak et al., 1993). Both the pro-oxidant and protective effects of heme have been demonstrated in cell cultures, organ injury models, and whole organisms (Balla et al., 1995; Nath et al., 1992). The discovery of Hb- and heme-binding proteins, haptoglobin (Hp) and hemopexin (Hpx), with high affinity for removing free heme and Hb from the extracellular space underscores the physiological importance of the heme/Hb alarm system (Muller-Eberhard and Cleve, 1963; Smith and Morgan, 1978; Tenhunen et al., 1970).

The discovery of heme binding to toll-like receptor 4 (TLR4) initiated extensive research into its proinflammatory effects in various cell lines and animal models (Belcher et al., 2014; Figueiredo et al., 2007). At the same time, an anti-inflammatory pathway was identified involving carbon monoxide (CO), a by-product of HO activity (Otterbein et al., 2000). Heme catabolism also produces bilirubin, an important endogenous antioxidant. Bilirubin efficiently scavenges peroxyl radicals *in vitro* supporting its potentially beneficial role as a physiological antioxidant (Stocker et al., 1987a; Stocker et al., 1987b). These findings underscored the dual—or "Janus-faced"—nature of heme. CO acts as an endogenous gaseous signaling molecule with diverse functions (Cárnio et al., 2025), including inhibition of heme release from heme proteins (Bunn and Jandl, 1966), prevention of organ rejection in xenotransplantation (Sato et al., 2001), and various other protective effects (Bauer et al., 2025; Motterlini and Otterbein, 2010).

Evidence shows that upregulation of heme oxygenase-1 (HO-1) is protective against multiple pathological processes associated with atherosclerosis. Pharmacological induction or viral-mediated overexpression of HO-1 markedly attenuated atherogenesis in hypercholesterolemic animal models (Ishikawa et al., 2001a; Ishikawa et al., 2001b), whereas genetic deletion of HO-1 in apolipoprotein E (ApoE)–deficient ($ApoE^{-/-}$) mice accelerated atherosclerotic progression and increased lesion burden (Yet et al., 2003). In a vulnerable plaque model in $ApoE^{-/-}$ mice, HO-1 expression was markedly increased in vulnerable compared with stable atherosclerotic lesions. Pharmacological induction of HO-1 with cobalt protoporphyrin attenuated the progression toward a vulnerable plaque phenotype, as evidenced by reduced necrotic core formation and intraplaque lipid accumulation, together with increased fibrous cap thickness and vascular smooth muscle cell (VSMC) content. Conversely, inhibition of HO-1 using zinc protoporphyrin exacerbated plaque vulnerability. Notably, plaque stabilization was most pronounced following adenoviral HO-1 overexpression compared with sham virus–treated controls, confirming that the protective effects on plaque stability were specifically attributable to HO-1 (Cheng et al., 2009).

Several clinical trials have investigated pharmacological induction of HO-1 in atherosclerosis and related cardiovascular diseases. Probucol and its derivatives inhibit experimental atherosclerosis in an HO-1–dependent manner (Wu et al., 2006); however, clinical outcomes have been inconsistent, with both beneficial (Byrne et al., 2010; Sawayama et al., 2006) and neutral effects reported (Ko et al., 2014), and safety concerns have limited its clinical use (Stocker, 2009). Although translation to clinical application remains limited, targeting the heme–HO-1 axis represents a promising avenue for future studies aimed at reducing vascular inflammation and plaque progression (Ayer et al., 2016).

Although the induction of HO-1 constitutes a key adaptive response with antioxidant and anti-inflammatory properties that are generally protective in atherosclerosis, it is insufficient on its own to counteract the pathological effects of free heme within atherosclerotic plaques and the adjacent endothelium. In the context of atherosclerosis, heme and iron overload act as potent catalysts for vascular damage. Free heme released from lysing erythrocytes can saturate the available HO-1. Hemorrhaged atherosclerotic lesions contain oxidized forms of Hb and are characterized by high heme content (100 μmol/mg protein) (Gáll et al., 2018; Pethő et al., 2021a). When intracellular or extracellular heme levels exceed HO's capacity to degrade heme, excess non-degraded heme can accumulate. In addition, heme degradation by HO-1 potentially creates the pro-oxidant by-product iron. Research revealed a causal link between excessive iron

and the progression of atherosclerotic lesions (Vinchi et al., 2020; Xiao et al., 2020). Research indicates a significant link between metal accumulation and the severity of arterial disease. Studies have shown that iron levels are statistically elevated within the intima of atherosclerotic lesions compared to healthy tissue, with iron accumulation correlating positively with cholesterol levels (Stadler et al., 2004).

Recent spatial mapping further clarifies these findings by showing that iron is primarily localized within intraplaque hemorrhages (IPHs). Furthermore, symptomatic carotid plaques exhibit significantly higher concentrations of vanadium, iron, copper, molybdenum, and cadmium within their core areas compared to asymptomatic plaques (Huang et al., 2025).

While HO-1 is robustly expressed in response to IPH, it remains unclear whether its enzymatic throughput is sufficient to neutralize the pro-oxidant burden of recurrent bleeding. The persistence of iron deposits and lipid oxidation in HO-1–positive regions suggests a potential functional exhaustion or a mismatch between heme release and catabolic capacity.

In this study, we focus on the nature of arterial wall hemorrhage to demonstrate that cell-free Hb and free heme play significant pathophysiological roles in atherosclerosis.

Hemorrhage and Hb Fate in the Atherosclerotic Plaque

Hemorrhage, particularly IPH, primarily results from the rupture of neovascularized vessels within the vessel wall and atherosclerotic plaques. This pathological neovascularization predominantly originates from the vasa vasorum circulation system, a specialized network of microvessels that supplies the arterial wall (Barger et al., 1984; Michel et al., 2014; Moreno et al., 2006). The extravasation and subsequent lysis of RBCs result in the accumulation of cell-free Hb in the plaque core, where Hb undergoes redox-driven structural transitions.

IPH and RBC extravasation

In RBCs, Hb, a tetramer protein consisting of two α and two β subunits with a heme prosthetic group on each globin chain, is protected from the extracellular space. RBCs possess an efficient antioxidant system that maintains Hb in its functional ferrous (Fe^{2+}) state, essential for oxygen binding. This system comprises both enzymatic components—such as superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, metHb reductase—and non-enzymatic antioxidants (vitamin C and E) (Daraghmeh and Karaman, 2024; Nagababu et al., 2003).

All types of plaque hemorrhages and hemolysis are initiated by the oxidative environment of atherosclerotic lesions (Nagy et al., 2010), which contains lipid peroxidation products such as lipid hydroperoxides, aldehydes, and carbonyl compounds (Li et al., 2006). Once released from RBCs, free Hb undergoes rapid oxidative modifications, transitioning from ferrous form (Fe^{2+}) to metHb (Fe^{3+}), and subsequently to the highly reactive ferryl state (ferryl hemoglobin [ferrylHb], $Fe^{4+} = O^{2-}$). This oxidation cascade ultimately results in the release of free heme into the extracellular space, where it contributes to complex pathophysiological processes. Importantly, the completion of this cascade is not necessary or guaranteed, and the yield of the ferryl species depends on the concentration of the peroxy species present. The transition of Hb through various oxidative states follows a complex pseudoperoxidase cycle where the formation of highly reactive ferryl species depends heavily on the availability of peroxides (Goldman et al., 1998; Ratanasopa et al., 2015).

While excess peroxide drives the formation of ferryl Hb, low levels often result in the reaction stalling at the metHb state or undergoing rapid autoreduction (Ratanasopa et al., 2015). Crucially, the oxidative cascade does not need to reach completion for heme dissociation to occur. In fact, metHb is the primary driver of heme loss, releasing its heme group $\sim$5-fold faster than the ferryl form and 3.4-fold faster than the stable ferrous form (Ratanasopa et al., 2015). This is because the ferryl state often creates covalent heme–protein cross-links that trap the heme, whereas the conformational distortions in metHb weaken globin–heme interactions without securing them, making metHb the leading source of toxic "labile" heme in clinical hemolytic conditions (Kassa et al., 2016; Potor et al., 2013).

IPH is associated with plaque vulnerability and disruption. A study with 29 subjects (14 cases with IPH and 15 controls with comparably sized plaques without IPH at baseline) showed that hemorrhage within carotid atherosclerotic plaques was associated with accelerated plaque progression (Takaya et al., 2005). The study concluded that recurrent intraplaque bleeding may promote atherosclerotic progression by enlarging the lipid core, increasing overall plaque volume, and introducing additional factors that contribute to plaque instability (Takaya et al., 2005).

In the Plaque At RISK study, IPH detected by magnetic resonance imaging in carotid plaques was significantly associated with disrupted plaque surfaces (defined as ulceration or fissured fibrous caps) compared to plaques without IPH, independent of stenosis severity (van Dijk et al., 2015). This suggests that hemorrhage significantly contributes to plaque instability.

FerrylHb plays a pivotal role in inflammation and tissue degeneration in ruptured abdominal aortic aneurysms (AAA), a disease pathology linked to atherosclerosis (Ding et al., 2025).

Fate of Hb in the hemorrhagic plaque

Natural, endogenous protective mechanisms exist against this extracellular free Hb and heme, emphasizing the importance of Hb–heme stress in vascular disorders. Extracellular free Hb is sequestered by Hp, whereas heme is efficiently bound to plasma Hpx (Muller-Eberhard et al., 1968) and α1-microglobulin (A1M), the latter of which is also present in most tissues, including blood vessel walls (Akerström et al., 2007; Berggård et al., 1998). Intracellularly, cytoplasmic heme is degraded by HOs, and the released iron is oxidized and sequestered by ferritin. This tightly regulated system functions as an effective endogenous defense mechanism against heme-induced stress. Ferritin exhibits remarkable protective properties, as it also attenuates calcification in VSMCs and valvular interstitial cells (VICs) (Sikura et al., 2019; Zarjou et al., 2009).

A range of conditions—including tissue injury, inherited hemolytic syndromes, sepsis, surgical trauma, brain hemorrhages, atherosclerosis with plaque rupture, kidney diseases

with hematuria, rhabdomyolysis, hemolytic uremic syndrome, diabetic vasculopathies, and retinopathy of prematurity—highlight the pathophysiological significance of free Hb and heme in human pathologies (Gáll et al., 2019; Michel and Martin-Ventura, 2020).

Excessive levels of free Hb and heme can exceed the binding capacities of endogenous scavenger proteins such as Hp and Hpx, leading to tissue damage (Vallelian et al., 2022; Vissa et al., 2023).

A key event in atherosclerosis onset and progression is the oxidative modification of low-density lipoprotein (LDL) within the arterial wall (Pirillo et al., 2013). Both free Hb and heme contribute to the oxidative modification of LDL, enhancing its atherogenic potential.

Oxidative chemistry of Hb in atherosclerotic plaques

Hb can undergo spontaneous autooxidation, during which the ferrous iron (Fe^{2+}) is oxidized to the ferric state (Fe^{3+}), forming metHb and generating a superoxide anion radical ($O_2\bullet^-$). The superoxide radical can undergo dismutation, either spontaneously or *via* enzymatic catalysis, leading to the formation of hydrogen peroxide (H_2O_2). While the primary pathway for H_2O_2 generation is enzymatic superoxide dismutation, H_2O_2 may also arise from the two-electron oxidation of molecular oxygen catalyzed by various oxidases, including xanthine oxidase, glucose oxidase, and amino acid oxidase, among others. Additionally, both superoxide and H_2O_2 can be generated through non-enzymatic mechanisms, such as electron leakage from the mitochondrial electron transport chain (Wong et al., 2017).

Hb has been proposed as a potential Fenton's reagent, capable of reacting with H_2O_2 to generate hydroxyl radicals (•OH). This reaction involves the oxidation of ferrous iron within Hb, following the classical Fenton chemistry mechanism: $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \bullet OH + OH^-$. Sadrzadeh et al. (1984) demonstrated that the Fenton-like reaction involving Hb and H_2O_2 is inhibited by the pre-oxidation of Hb, as well as by the presence of the H_2O_2-scavenging enzyme catalase or the Hb-binding protein Hp, but not by superoxide dismutase).

Subsequent studies have expanded on this finding, indicating that high concentrations of H_2O_2 can degrade metHb, releasing free iron. This iron then reacts with H_2O_2 through Fenton chemistry, generating highly reactive •OH (Gutteridge, 1986; Puppo and Halliwell, 1988).

Substantial evidence, derived from both *in vivo* and *in vitro* studies, supports the role of Hb as a pseudoperoxidase. This activity can be summarized as follows (Alayash and Wilson, 2022; Jia et al., 2007; Kassa et al., 2015; Patel et al., 1996): Hb engages in a self-propagating redox cycle upon interaction with H_2O_2, generating a series of redox-active intermediates. Ferrous Hb ($HbFe^{2+}$) undergoes a two-electron oxidation upon reacting with H_2O_2 or lipid hydroperoxides (LOOH), resulting in the formation of ferryl Hb ($HbFe^{4+} = O^{2-}$) (Giulivi and Cadenas, 1998; Giulivi and Davies, 1994). In contrast, when metHb ($HbFe^{3+}$) reacts with H_2O_2, it produces a ferryl Hb radical species [$Hb\bullet^+(Fe^{4+} = O^{2-})$]. Furthermore, compelling evidence demonstrates that the free radicals detected in frozen blood samples are identical to those generated through the oxidation of purified metHb by H_2O_2 (Svistunenko et al., 2002).

The high-valence ferryl ($Fe^{4+} = O$) species reacts with specific amino acids *via* intramolecular electron transfer in the α (Tyr-24, His-20, Tyr-42) and β (Tyr-35, Tyr-130, Cys-93) globin chains to form protein radicals (Deterding et al., 2004; Lardinois et al., 2008; McArthur and Davies, 1993; Ramirez et al., 2003; Svistunenko et al., 2002).

Termination of globin-centered radical species leads to the formation of covalent globin–globin crosslinks, resulting in the generation of Hb dimers, tetramers, and higher-order multimers. FerrylHb can be reduced back to metHb by various reducing agents. This reduction may occur through direct interaction with the heme pocket or *via* intramolecular electron transfer involving specific amino acid residues, notably tyrosine at position α42 (McArthur and Davies, 1993; Reeder et al., 2008).

The spectral fingerprints of the transient ferrylHb can be captured by a spectrophotometric method (Meng and Alayash, 2017).

The footprints of these complex Hb oxidation sequences can be followed during the progression of human atherosclerosis (Nagy et al., 2010). The protein oxidation marker dityrosine accumulates in complicated lesions of the aorta or its primary branches, accompanied by the formation of covalently cross-linked Hb (dimer, tetramer, polymer), a hallmark of ferrylHb (Nagy et al., 2010). Oxidation hotspots in Hb (β1Cys93; β1Cys112; β2Cys112; α1Cys104) were identified, and the formation of the Tyr36 radical in the β chain was revealed in complicated atherosclerotic lesions of carotid arteries (Potor et al., 2021). An analytical approach can be employed to quantify the oxidative states of heme-iron in Hb within complex atherosclerotic plaques of the carotid arteries, revealing that $\sim$55% of the total Hb exists as ferrylHb, 39% as metHb, and only 1.4% as ferrousHb (Potor et al., 2021). Furthermore, Hb oxidation was found to generate globin-derived peptide fragments, leading to their accumulation in such lesions (Posta et al., 2020). The mechanisms of Hb oxidation are summarized in Figure 1.

Cellular Targets of Hb and Heme

Following IPH and Hb oxidation, vascular and immune cells within the atherosclerotic lesion are directly exposed to increasing concentrations of Hb and heme. Endothelial cells (ECs), monocytes/macrophages, and VSMCs represent primary targets of Hb and heme, triggering diverse biological effects leading to vascular maladaptation and dysfunction.

This section introduces the major cellular players in vascular diseases and examines how their responses to Hb and heme integrate vascular remodeling.

Endothelial activation and barrier disruption

Vascular endothelium forms a single layer on the luminal surface of blood vessels. ECs play a significant role in cardiovascular integrity due to their key functions in maintaining vascular homeostasis by regulating vascular tone, permeability, blood flow, coagulation and inflammation (Shireman and Pearce, 1996).

Oxidized Hb species, particularly free heme and ferrylHb, exert a range of detrimental effects on ECs, compromising endothelial integrity (Balla et al., 1991b; Nagy et al., 2010; Potor et al., 2021). This includes the initiation of a cascade

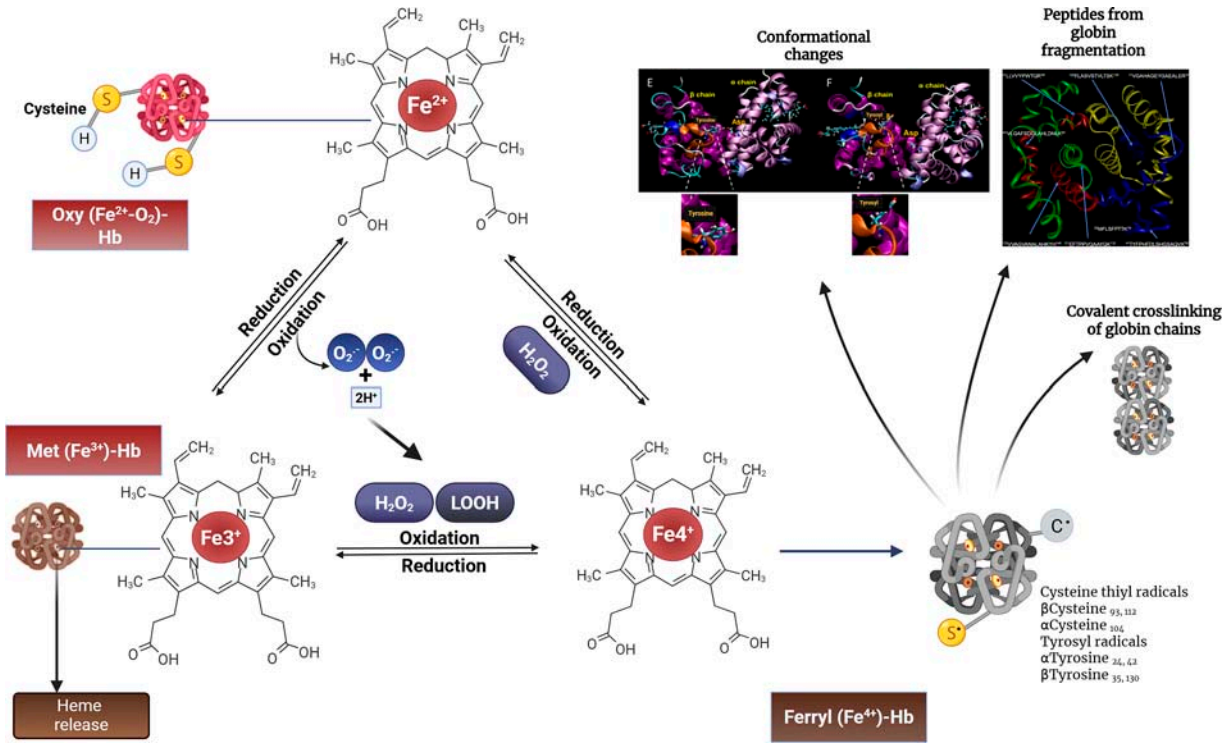

FIG. 1. Mechanisms of hemoglobin oxidation. Hemoglobin (Hb) can undergo spontaneous autooxidation, during which the ferrous iron (Fe^{2+}) is oxidized to the ferric state (Fe^{3+}), forming methemoglobin (metHb) and generating a superoxide anion radical ($O_2\bullet^-$). The superoxide radical can undergo dismutation, either spontaneously or *via* enzymatic catalysis, leading to the formation of hydrogen peroxide (H_2O_2). FerrousHb ($HbFe^{2+}$) undergoes a two-electron oxidation upon reacting with H_2O_2 or lipid hydroperoxides (LOOH), resulting in the formation of ferrylHb [$HbFe^{4+}$]. MetHb ($HbFe^{3+}$) reacts with H_2O_2 or LOOH, producing a ferrylHb radical species [$Hb\bullet^+(Fe^{4+} = O^{2-})$], that can be damaging to the Hb itself by migrating to cysteine-93 of the β chain and other "hotspot" amino acids in the α (Tyr-24, His-20, Tyr-42) and β (Tyr-35, Tyr-130, Cys-93) subunits. FerrylHb can be reduced back to metHb by various reducing agents. This reduction may occur through direct interaction with the heme pocket or *via* intramolecular electron transfer. Termination of globin-centered radical species leads to the formation of covalent globin-globin cross-links and conformational changes resulting in the generation of Hb dimers, tetramers, higher-order multimers, and globin-derived peptides. See Supplementary Video for the full dynamic process for conformational changes. Conformational changes reproduced from Potor et al. (2021), licensed under CC BY-NC 4.0. Peptides from globin fragmentation reproduced from Posta et al. (2020), licensed under CC BY-NC 4.0. Image created using BioRender. Gall, T. (2026) https://BioRender.com/hojg7sv.

of harmful stimuli, including inflammation and oxidative stress, which collectively contribute to endothelial dysfunction and differentiation of invading monocytes into proinflammatory macrophage phenotype.

The expression of adhesion molecules, including intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and E-selectin, plays a pivotal role in the pathogenesis of atherosclerosis. These molecules facilitate the recruitment of inflammatory cells to the endothelial surface, which is a critical step in the initiation and progression of plaque formation within the vessel wall (Davies et al., 1993).

Inflammatory stimuli, such as lipopolysaccharide (LPS), are well-established inducers of adhesion molecules expression, including ICAM-1, VCAM-1, and E-selectin. Similarly, heme has been shown to induce the expression of these adhesion molecules in ECs, thereby facilitating the adhesion not only monocytes but polymorphonuclear leukocytes (PMNs) to the endothelial surface (Wagener et al., 1997) (Fig. 2). In addition to heme, ferrylHb and Hb-derived peptides function as potent agonists in the induction of adhesion molecule expression through the activation of the nuclear factor kappa B (NF-κB) signaling pathway (Silva et al., 2009). This activation leads to the reorganization of the actin cytoskeleton, compromising the integrity of the EC monolayer. This response is mediated through the activation of c-Jun N-terminal kinase and p38 mitogen-activated protein kinase (MAPK) signaling cascades (Silva et al., 2009). ECs exposed to ferrylHb and Hb-derived peptides also exhibit increased permeability and enhanced monocyte adhesion (Potor et al., 2013). Both ferrylHb and globin-derived peptides induce intercellular gap formation together with decreased junctional resistance in the endothelium (Posta et al., 2020). While many mechanistic insights come from *in vitro* models, clinical observations highlight how the depletion of Hp correlates with junctional damage and barrier dysfunction. Clinical associations have been found between Hp polymorphisms and microvascular dysfunction. Individuals with the Hp2-2 genotype have a reduced capacity to clear Hb, leading to higher oxidative stress and a significantly elevated risk of cardiovascular and endothelial injury compared to those with the Hp1-1 genotype (Blackburn et al., 2018; MacKellar and Vigerust, 2016).

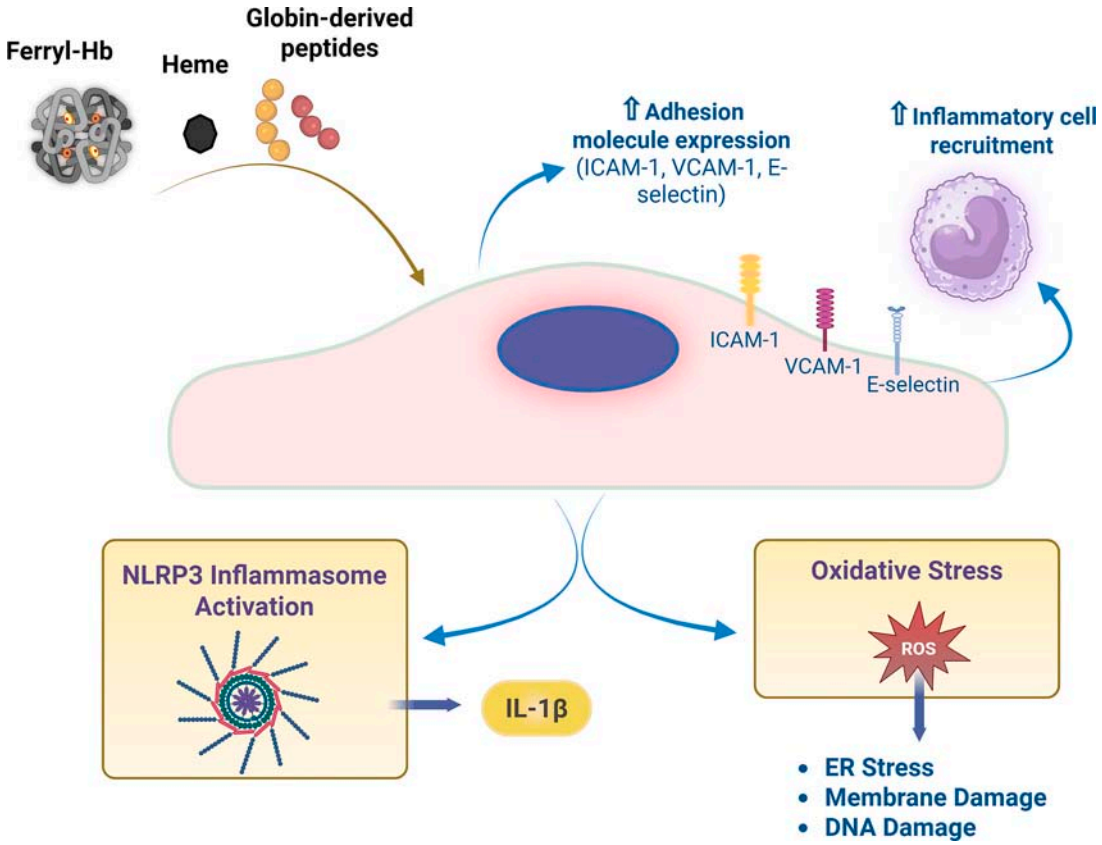

FIG. 2. Heme and ferryl-hemoglobin as proinflammatory and oxidative stimuli. Ferryl-hemoglobin (ferryl-Hb) and heme released upon Hb oxidation induce the expression of endothelial adhesion molecules such as intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and E-selectin, promoting the recruitment of inflammatory cells to the endothelium. Additionally, heme, ferryl-Hb, and globin-derived peptides catalyze the activation of the NLR pyrin domain containing 3 (NLRP3) inflammasome, leading to interleukin-1β (IL-1β) secretion. Hb oxidation also generates reactive oxygen species, which contribute to endoplasmic reticulum (ER) stress, membrane, and DNA damage, ultimately resulting in endothelial dysfunction. Image created using BioRender. Gall, T. (2026) https://BioRender.com/e4oyjjd.

Overall, increased adhesion molecule expression on ECs plays a crucial role in the cellular responses to heme, ferrylHb, and Hb-derived peptides, contributing to the inflammation and endothelial dysfunction.

Heme and oxidized Hb as pro-inflammatory stimuli

Heme, released from RBCs, functions as a potent pro-inflammatory stimulus and is recognized as a damage-associated molecular pattern (DAMP). Emerging evidence indicates that heme contributes to inflammatory responses through its interaction with innate immune receptors and its ability to generate reactive oxygen species (ROS).

Pathogen-associated pattern recognition receptors, including toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), are capable of recognizing both PAMPs and endogenous danger-associated molecular patterns, such as heme.

Heme represents one of the most extensively characterized Hb-derived DAMPs, having effects on various immune and non-immune cell populations. In hemolytic disorders such as hereditary spherocytosis and sickle cell anemia, erythrocyte lysis leads to the release of free heme, which in turn drives persistent inflammation and tissue injury. This process triggers a deleterious cycle wherein inflammation and tissue damage promote further hemolysis, thereby exacerbating disease progression (Gozzelino et al., 2010; Martins and Knapp, 2018; Soares and Bozza, 2016). Ruptured plaques are associated with excessive lysis of RBCs, and the subsequent release of heme and Hb further contributes to a vicious circle, increasing inflammation and tissue damage within atherosclerotic plaques.

Endothelial cells. The activation of NLR pyrin domain containing 3 (NLRP3) inflammasome can be triggered by a range of DAMPs. Within atherosclerotic lesions, cholesterol crystals, calcium phosphate crystals, and oxidized LDL (OxLDL) have special significance (Karasawa and Takahashi, 2017). Emerging evidence shows the importance of inflammation and NLRP3 activation in the progression of atherosclerosis.

Exposure to heme markedly induces the upregulation of NLRP3 expression and promotes the production of mature interleukin-1β (IL-1β) in human umbilical vein endothelial cells (HUVECs). This pro-inflammatory response is significantly potentiated by prior priming with LPS, which enhances heme-driven IL-1β secretion (Erdei et al., 2018). Importantly, the capacity of free heme to activate the NLRP3 inflammasome depends on the integrity of its

molecular structure, as neither protoporphyrin IX nor free iron alone provokes IL-1β secretion (Erdei et al., 2018).

While free heme is often characterized as a potent DAMP, its pro-inflammatory effects are largely context dependent. In clinical scenarios involving sterile hemolysis or Hb-based oxygen carriers, the expected systemic inflammatory response is frequently absent (Vallelian et al., 2018). This suggests that heme acts as a weak stimulus, requiring a secondary insult—such as LPS priming—to manifest a robust innate immune response.

Hb oxidation occurs in ruptured atherosclerotic lesions and human brain hemorrhages, resulting in the accumulation of globin-derived peptides and ferrylHb (Posta et al., 2020). Interestingly, these globin-derived peptides do not bind either to Hp or albumin. In response to Hb-derived peptides, increased expression of tumor necrosis factor-α (TNF-α) and activation of both NLRP3 and caspase-1 are detected in ECs, followed by increased generation of IL-1β and nuclear translocation of NF-κb (Posta et al., 2020). These effects can mechanistically be distinct from free heme signaling and likely involve peptide-mediated cellular stress responses rather than direct heme-dependent inflammasome activation.

Posta et al. (2020) demonstrated that ferrylHb facilitates the nuclear translocation of NF-κB within ECs. This process induces the expression of the NLRP3 inflammasome, ultimately leading to elevated secretion of the proinflammatory cytokine IL-1β (Posta et al., 2020). Furthermore, in HUVECs primed with LPS, ferrylHb has been identified as the specific Hb species responsible for the upregulation of IL-1β messenger RNA (mRNA) levels, a characteristic not observed with other Hb variants (Erdei et al., 2018).

It should be noted that much of the available evidence has been generated using HUVECs. While these models provide useful mechanistic insights, they may not fully reflect the characteristics of arterial ECs in the context of atherosclerosis. Accordingly, extrapolation of these findings to *in vivo* disease settings should be made with appropriate consideration.

Clinical data on inflammasome activation primarily derive from sickle cell disease (SCD) patients. In SCD, excess inflammation serves as a critical driver of clinical complications and is directly correlated with patient mortality (van Beers et al., 2015). Evidence indicates that inflammatory activation in both ECs and hematopoietic cells contributes substantially to these adverse outcomes. Supporting mechanistic data have also been obtained from experimental models: In SCD mice, heme binds to TLR4 on ECs in a manner analogous to LPS, thereby activating downstream NF-κB signaling and promoting transcription of pro-inflammatory genes. This results in the upregulation of adhesion molecules, cytokines/chemokines, and procoagulant factors, ultimately facilitating leukocyte recruitment and vascular inflammation (Belcher et al., 2014).

The activation of the NLRP3 inflammasome in the vascular environment appears to be, at least partly, associated with hemolysis. The capacity of free heme to trigger IL-1β secretion is strictly dependent on its molecular integrity; neither protoporphyrin IX nor free iron alone is sufficient (Erdei et al., 2018). However, during Hb oxidation in atherosclerotic lesions or hemorrhages, the inflammatory milieu is further complicated by the release of ferrylHb and globin-derived peptides. These degradation products, which bypass traditional scavenger proteins like Hp, serve as independent activators of NLRP3 and NF-κB (Posta et al., 2020), suggesting that Hb breakdown promotes endothelial dysfunction through multiple, structurally diverse ligands.

Overall, the interaction between heme/Hb and the NLRP3 inflammasome in ECs has been shown to trigger the release of pro-inflammatory cytokines like IL-1β, contributing to vascular dysfunction and exacerbating endothelial injury (Fig. 2).

Macrophages. Macrophages are also key players in plaque development. Heme, but not iron-free porphyrins, activates the NLRP3 inflammasome in LPS-primed macrophages, resulting in IL-1β processing and contributing to mortality in murine models of hemolysis (Dutra et al., 2014). Interestingly, heme treatment of unprimed macrophages elicits only a modest increase in IL-1β secretion, which is significantly enhanced following LPS priming. In human primary macrophages, heme activates caspase-1 *via* an inflammasome-dependent pathway, whereas caspase-4 and caspase-5 activation occurs independently of canonical inflammasomes. Notably, both caspase-4 and caspase-5 were essential for heme-induced IL-1β release, with caspase-4 identified as the primary mediator of heme-induced cell death (Bolívar et al., 2021).

Others have found that not only NLRP3 but also NLR pyrin domain containing 12 (NLRP12) mediates inflammasome and PANoptosome activation in response to heme combined with PAMPs, thereby driving inflammatory cell death known as PANoptosis. The expression of NLRP12 is induced through toll-like receptor-2 and -4 (TLR-2/TLR-4)-dependent signaling pathways. Given its central role in this process, NLRP12 and associated signaling molecules represent promising therapeutic targets for the treatment of hemolytic and inflammatory diseases (Sundaram et al., 2023).

It has been demonstrated that heme, unlike its analogs or biosynthetic precursors, induces TNF-α secretion by macrophages through a mechanism dependent on myeloid differentiation primary response 88, TLR-4, and cluster of differentiation 14 (CD14) (Fig.ueiredo et al., 2007). FerrylHb, but not Hb or metHb, also induces active IL-1β production in LPS-primed macrophages *via* an NLRP3 inflammasome-dependent mechanism (Nyakundi et al., 2019).

Chronic hemolysis, a hallmark of SCD, leads to significantly elevated plasma concentrations of heme (Muller-Eberhard et al., 1968). Peripheral blood mononuclear cells from SCD patients show elevated mRNA expression of NLRP3, caspase-1, IL-1β, and interleukin-18 (IL-18) (Pitanga et al., 2016). Monocytes in these patients express higher baseline levels of IL-1β and exhibit an exaggerated release of the cytokine upon exposure to heme (Belcher et al., 2000; Bolívar et al., 2021).

Overall, these findings indicate that both heme and ferrylHb catalyze inflammasome activation in macrophages, which is likely involved in the pathology of ruptured plaques.

Vascular smooth muscle cells. VSMCs play central roles throughout atherosclerosis. They migrate from the media into early lesions, contributing to lesion expansion

while also generating an extracellular matrix–rich fibrous cap that overlies the necrotic core.

Early growth response-1 (Egr-1) is a pivotal transcriptional regulator linking oxidative stress to inflammatory and thrombotic signaling in vascular disease. Sustained Egr-1 expression is a hallmark of atherosclerotic lesions in both humans and experimental models, where it drives the expression of pro-inflammatory and pro-thrombotic genes. Genetic deletion of Egr-1 in hyperlipidemic mice markedly attenuates atherosclerosis, reducing lesion size and complexity at the aortic root and suppressing inflammatory gene transcripts. These findings establish Egr-1 as a central amplifier of vascular inflammation during atherogenesis (Blaschke et al., 2004; Harja et al., 2004).

Heme, released during hemolysis and enriched in regions of disturbed blood flow, emerges as an upstream inflammatory stimulus in the vascular wall. In VSMCs, heme robustly induces Egr-1 expression through ROS-dependent activation of ERK1/2–Elk-1 and NF-κB pathways, positioning Egr-1 as a downstream effector of heme-driven oxidative stress (Hasan and Schafer, 2008). This signaling cascade links mechanical and hemolytic stress to transcriptional inflammatory responses within the vessel wall.

Beyond Egr-1 induction, free heme directly promotes inflammatory VSMC phenotypic switching. Heme stimulates nicotinamide adenine dinucleotide phosphate (NADPH) oxidase–dependent ROS generation, activating redox-sensitive MAPK and NF-κB signaling pathways that drive VSMC proliferation and migration—key processes in lesion development. Importantly, heme also induces HO-1, which acts as an intrinsic anti-inflammatory and antioxidant counter-regulatory mechanism. Inhibition of HO activity exacerbates ROS production and VSMC proliferation, whereas heme metabolites (CO and biliverdin) blunt these effects, highlighting a critical balance between pro- and anti-inflammatory heme signaling (Moraes et al., 2012).

Inflammatory activation of VSMCs by heme further extends to innate immune pathways. Heme exposure induces the expression of IL-1β and ICAM-1, promoting leukocyte recruitment and vascular inflammation. Notably, heme also upregulates NLRP3, a core component of the inflammasome, implicating heme as a trigger of inflammasome priming in vascular cells and linking hemolysis-derived signals to IL-1β–driven inflammatory amplification (Ding et al., 2026).

The interaction between heme and VSMCs is reflected by the induction of endoplasmic reticulum (ER stress) in atherosclerotic lesions (Gáll et al., 2018).

Collectively, these studies support a model in which free heme acts as a potent pro-inflammatory danger signal in the vasculature, driving ROS-dependent activation of Egr-1, NF-κB, and inflammasome pathways in VSMCs. Egr-1 emerges as a key transcriptional node integrating oxidative stress with inflammatory gene expression, while HO-1 provides a crucial endogenous brake on heme-induced vascular inflammation. This axis represents an important mechanistic link between hemolysis and vascular inflammation.

Immune modulatory effect of heme and Hb

The pathogenesis, progression, and complications of atherosclerosis are profoundly influenced by immune cells, the cytokines they secrete, and the complex interactions between these cellular components. Furthermore, studies on atherosclerosis have identified distinct immune cell populations that both facilitate and inhibit the formation of lesions. Notably, different subtypes of the same immune cell lineage can exhibit divergent pro-inflammatory or anti-inflammatory effects, contributing to the disease's dynamic immune response. The interplay between Hb, heme, and immune cells underscores a complex mechanism by which hemolysis and oxidative stress contribute to the pathogenesis of atherosclerosis.

Chemotactic and recruitment effects of heme and Hb. A substantial body of evidence indicates that both heme and ferrylHb promote leukocyte infiltration (Graça-Souza et al., 2002; Porto et al., 2007; Silva et al., 2009). Furthermore, heme also stimulates neutrophils, leading to an increase in ROS and interleukin-8 (IL-8) production (Graça-Souza et al., 2002; Porto et al., 2007). The activation of neutrophils triggers the release of neutrophil extracellular traps (NETs) as part of the immune response to microbial infection. Heme plays a pivotal role in enhancing NET release, particularly in the context of SCD (Chen et al., 2014). Interestingly, heme-induced NET formation depends on ROS production but is independent of TLR-4 activation. Additionally, the presence of iron within the porphyrin ring is crucial for the initiation of NET formation (Ohbuchi et al., 2017).

NET formation plays an important role in atherosclerotic plaque development. Neutrophils are recruited into plaques in response to inflammatory signals and cholesterol crystals, which activate the NLRP3 inflammasome and drive further neutrophil infiltration and NET formation (NETosis) (Karasawa and Takahashi, 2025). Inside the plaque, neutrophils release enzymes such as matrix metalloproteinases, elastase, and myeloperoxidase, which degrade the extracellular matrix and weaken the fibrous cap, making plaques more prone to rupture (Ionita et al., 2010). Neutrophils also generate ROS, contributing to the oxidative milieu and further tissue damage within the plaque (Hosokawa et al., 2011). Given that heme promotes neutrophil activation, neutrophils are one of the central players in the progression and complications of atherosclerosis.

Hb oxidation and macrophage polarization. Leukocyte recruitment and the expression of pro-inflammatory cytokines are key features of early atherogenesis. Solid evidence shows that inflammation drives all stages of atherosclerosis (Libby et al., 2002).

Macrophages exhibit diverse phenotypes, such as pro-inflammatory (M1) and alternatively activated (M2), each playing distinct roles in atherosclerosis progression (Moore and Tabas, 2011). Factors like heme, Hb, and inorganic iron influence macrophage polarization (Boyle et al., 2009; Finn et al., 2012; Soares and Hamza, 2016). The detection of ferrylHb within atherosclerotic plaques suggests its potential involvement in the pathogenesis of the disease (Potor et al., 2021). FerrylHb is internalized by macrophages through cluster of differentiation 163 (CD163) receptor–mediated endocytosis and subsequently directed to lysosomes. This intracellular trafficking promotes pro-atherogenic macrophage polarization, which contributes to the inflammatory environment observed in human atherosclerotic plaques.

Macrophage exposure to ferrylHb induces the upregulation of genes involved in proinflammatory signaling pathways, including IL-1β, TNF-α, IL-8, and interleukin-6 (IL-6), consistent with the development of a pro-inflammatory macrophage phenotype. Concurrently, ferrylHb exposure leads to the downregulation of anti-inflammatory markers such as cluster of differentiation 209 and interleukin 27 receptor subunit alpha, further reinforcing this phenotypic shift. Notably, a 39% overlap in differentially expressed genes between ferrylHb-exposed human macrophages and those found in complicated atherosclerotic lesions suggests not only the presence but the potential role for ferrylHb in the progression of atherosclerosis (Potor et al., 2021). The pro-atherogenic response of macrophages to ferrylHb also includes the promotion of calcification, angiogenesis, apoptosis, and tissue remodeling (Potor et al., 2021).

Analysis of hemorrhaged human atherosclerotic plaques identified a distinct macrophage subset termed hemorrhage-associated macrophages (HA-mac), characterized by high CD163 and low HLA-DR expression. Although these cells accumulated iron, they exhibited paradoxically reduced oxidative damage, as indicated by lower 8-oxo-guanosine levels. *In vitro* exposure of monocytes to HbHp complexes recapitulated the HA-mac phenotype, producing $CD163^{high}$/$HLA\text{-}DR^{low}$ cells with enhanced Hb clearance, reduced H_2O_2 and ROS production, diminished oxidative stress, and improved survival. Neutralization of interleukin-10 (IL-10) prevented HA-mac differentiation, demonstrating an autocrine IL-10–dependent mechanism. Non-linear dynamic modeling further revealed that an IL-10/CD163-positive feedback loop drives commitment to a discrete HA-mac lineage, with simulations predicting an all-or-none phenotypic switch at threshold HbHp levels—an effect confirmed experimentally. Collectively, these findings describe a novel atheroprotective macrophage subpopulation induced by IPH and suggest that therapeutic induction of the HA-mac phenotype may offer cardioprotective potential in atherosclerosis (Boyle et al., 2009).

FerrylHb has been shown to inhibit the osteoclastic differentiation of macrophages within hemorrhaged atherosclerotic plaques *via* halting osteoclastogenic signaling pathways downstream of receptor activator of nuclear factor kappa-B, decreasing osteoclastic genes expression such as nuclear factor of activated T cells, cytoplasmic 1, calcitonin receptor, dendritic cell–specific transmembrane protein, implicating its role in tissue remodeling and calcification during atherosclerotic lesion progression (Zavaczki et al., 2020). This finding indicates that ferrylHb influences not only macrophage polarization but also their differentiation pathways, with potential implications for tissue remodeling and calcification processes in atherosclerotic lesions.

Overall, ferrylHb-induced macrophage phenotype switch plays a role in plaque progression by triggering pro-inflammatory polarization, calcification, apoptosis, angiogenesis, and tissue remodeling in ruptured hemorrhaged plaques (Fig. 3).

Oxidized Hb links IPH to immune-driven aneurysm progression. FerrylHb has recently emerged as a key driver of inflammation and tissue degeneration in ruptured AAA. Two-electron oxidation of Hb in hemorrhaged AAA results in the formation of ferrylHb, characterized by oxidation of βCys93, αCys104, and βCys112 residues, and represents the terminal oxidative product of Hb–leukocyte interactions. FerrylHb is internalized by neutrophils and macrophages *via* CD163-mediated endocytosis, triggering peptidylarginine deiminase 4 (PAD4)–dependent NETosis, release of elastase and myeloperoxidase, and subsequent extracellular matrix degradation. AAA tissues show increased CD163 expression in macrophages and neutrophils, inducible by ferrylHb through PAD4 signaling, suggesting a feed-forward inflammatory loop. Transcriptomic analysis of ruptured AAA reveals a distinct gene expression profile, with 43% overlap (884 genes) shared with ferrylHb-stimulated macrophages, including inflammatory, angiogenic, and tissue remodeling pathways. Collectively, these findings implicate ferrylHb-driven innate immune activation as a key contributor to AAA progression (Ding et al., 2025). Functionally, ferrylHb polarizes neutrophils toward a highly pro-inflammatory phenotype, marked by increased inflammatory gene expression, degranulation, elastase and myeloperoxidase release, and NETosis. In parallel, ferrylHb-activated macrophages promote inflammation, apoptosis, calcification, and pathological tissue remodeling—hallmarks of AAA progression (Ding et al., 2025). Consistent with these findings, AAA tissues exhibit significantly elevated total heme levels, reflecting enhanced heme release from oxidized Hb (Ding et al., 2026). A strong positive correlation between metHb and total heme content across human and murine samples further supports a tight coupling between Hb oxidation and heme accumulation in hemorrhagic aneurysms (Ding et al., 2025; Ding et al., 2026). Moreover, AAA lesions show upregulation of hemolysis- and iron-handling genes (*e.g.*, HO-1 and ferritin heavy chain), alongside increased expression of inflammatory cytokines and apoptotic markers. Collectively, these data define an Hb–heme–immune axis that drives inflammatory and degenerative remodeling in both hemorrhaged atherosclerotic plaques and ruptured AAA (Ding et al., 2025; Ding et al., 2026).

Heme-driven oxidative stress in atherosclerosis

Heme exposure of ECs aggravates their damage induced by H_2O_2 and activated PMNs (Balla et al., 1991b). This damage is inhibited by hydrophobic oxygen radical scavenger/iron chelator U74500A and heme-binding protein Hpx, supporting that EC lysis is dependent on lipid peroxidation catalyzed by heme-iron. Redox-active iron has been demonstrated to effectively amplify intracellular ROS generation (Halliwell and Gutteridge, 1984). Heme can also induce PMN activation (Graça-Souza et al., 2002). Heme exposure of phospholipid liposomes (Gutteridge and Smith, 1988), purified DNA (Aft and Mueller, 1983), and purified protein (Aft and Mueller, 1984) was shown to lead to lipid peroxidation, DNA strand scission, and protein degradation. Oxidative damage of mitochondrial DNA occurs in the liver of rats during intravenous heme treatment (Suliman et al., 2002). The administration of free heme to VSMCss has been demonstrated to induce oxidative modifications of proteins and activate the unfolded protein response, leading to ER stress (Gáll et al., 2018). These supports that heme induces extensive oxidative modification of various macromolecules.

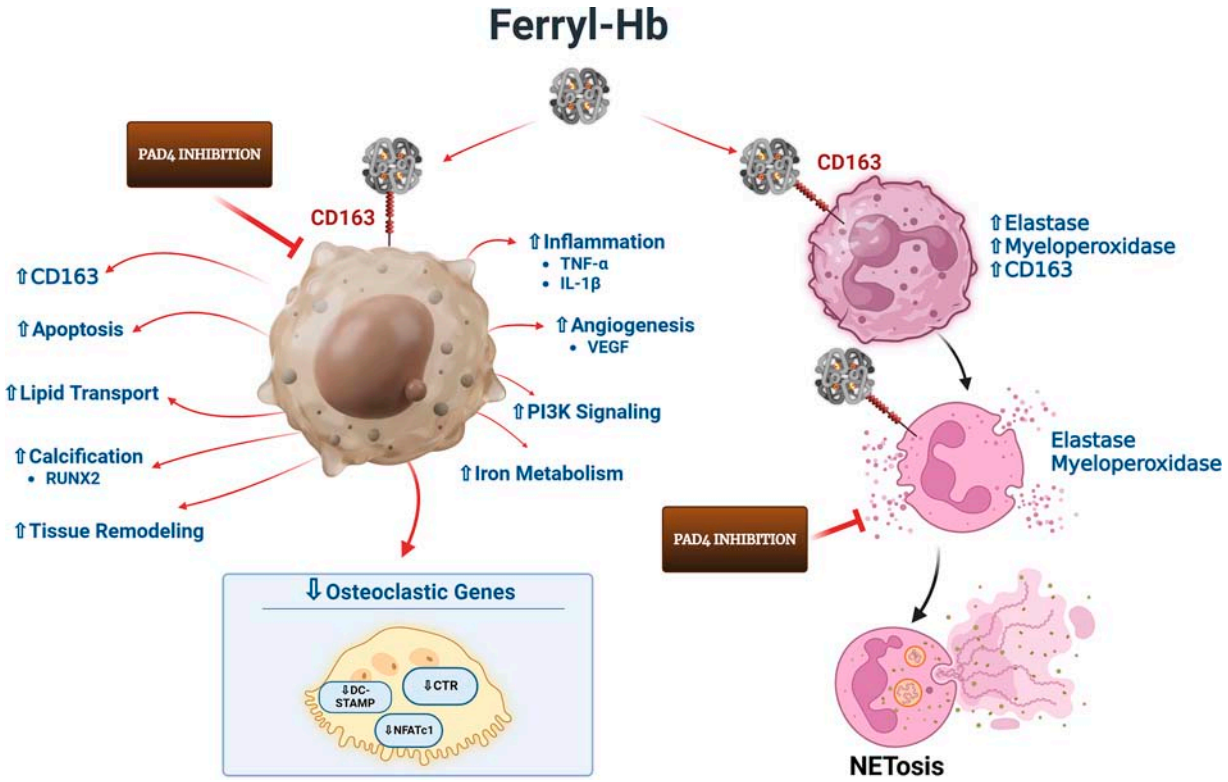

FIG. 3. Hemoglobin oxidation induces the polarization of macrophages towards a proatherogenic phenotype and activates neutrophil granulocytes. Ferryl-hemoglobin (ferryl-Hb) is internalized by macrophages *via* cluster of differentiation 163 (CD163) receptor-mediated endocytosis and trafficked to lysosomes, where it promotes pro-atherogenic macrophage polarization. This process contributes to the inflammatory milieu characteristic of human atherosclerotic plaques. Exposure to ferryl-Hb impacts macrophage phenotypic switch, influencing the expression of genes associated with inflammation (tumor necrosis factor-α [TNF-α]), interleukin-1β (IL-1β), angiogenesis (vascular endothelial growth factor [VEGF], tissue remodeling, iron metabolism, apotosis (caspase-3 [CASP3]), PI3K signaling, lipid transport, and calcification (runt-related transcription factor 2 [RUNX2]). Additionally, ferryl-Hb inhibits osteoclastic differentiation of macrophages within hemorrhaged plaques by decreasing osteoclastic gene expression (nuclear factor of activated T cells, nuclear factor of activated T cells, cytoplasmic 1 [NFATc1], calcitonin receptor [CTR], dendritic cell–specific transmembrane protein [DC-STAMP]), implicating its role in tissue remodeling and calcification during atherosclerotic lesion progression is internalized by neutrophils and macrophages *via* CD163. FerrylHb activates peptidylarginine deiminase 4 (PAD4), promoting histone citrullination and NETosis, accompanied by the release of elastase and myeloperoxidase. PAD4 signaling also contributes to upregulation of CD163 expression, reinforcing ferrylHb uptake and amplifying inflammatory and extracellular matrix–degrading responses that drive AAA progression. Image created using BioRender. Gall, T. (2026) https://BioRender.com/iku19ue.

Oxidative DNA damage plays an important role in atherosclerosis. ROS-induced oxidative DNA damage and the expression of DNA repair enzymes are elevated in atherosclerotic plaques (Martinet et al., 2002). Others have found that coronary artery disease in humans is characterized by an increase in DNA damage that is positively correlated with the severity of the atherosclerotic disease (Botto et al., 2001). In human atherosclerotic tissues as well as in ApoE$^{-/-}$ mice, mitochondrial DNA damage correlates with the extent of atherosclerosis and also precedes atherogenesis in young mice (Ballinger et al., 2002).

Oxidative stress and ER stress coexist in several human pathologies (Cao and Kaufman, 2014). Oxidative modification of proteins also plays a pivotal role in atherosclerosis by the oxidative modification of LDL, triggering ER stress, which contributes to inflammation and cell death (Gáll et al., 2019). Hemorrhage exacerbates ER stress in human atherosclerotic plaques compared to healthy controls or non-hemorrhagic atheromas (Pethő et al., 2021b). In cultured ECs, free heme induces ER stress, but pretreatment with heme arginate confers resistance to this stress. Carbon monoxide–releasing molecules (CORMs), rather than bilirubin, also mitigate ER stress through heme oxygenase-1 (HO-1) induction. Furthermore, depletion of HO-1 exacerbates heme-induced cell death, a process that cannot be counteracted by typical cell death inhibitors. This study suggests that heme-induced ER stress and associated cell death may play a role in the pathogenesis of diseases involving hemorrhage and hemolysis, including atherosclerosis (Pethő et al., 2021b).

Unstable plaques are associated with increased ER stress, which in turn triggers apoptosis in macrophages and smooth muscle cells, contributing to plaque vulnerability (Myoishi et al., 2007). Inhibitors of ER stress, such as 4-phenylbutyric acid and tauroursodeoxycholic acid, have been shown to alleviate atherosclerosis, suggesting its potential pathogenic role in vascular diseases (Erbay et al., 2009; Sun et al., 2018); however, cross-talk mechanisms are still uncovered.

LDL and Plaque Lipids as Indirect Heme Mediators

LDL particle exhibiting the oxidative modification pattern has been implicated in the pathogenesis of atherosclerosis (Balla et al., 1991a; Pirillo et al., 2013; Tsimikas and Witztum, 2024). Search for the causes of this *in vivo* modification has been the center of LDL research in order to find preventive and therapeutic interventions. It was revealed that LDL undergoes an oxidative modification during its incubation with ECs (and with a number of other cell types) (Hessler et al., 1983; Steinbrecher et al., 1984), and autooxidation was also shown to occur (Esterbauer et al., 1987). An extremely simple procedure, prolonged incubation of isolated LDL in room air resulting in minimally modified LDL, has been shown to induce certain inflammatory responses in vascular cells (Liao et al., 1991). Search for identifying catalyst of oxidation suggested copper, as modification of isolated LDL can be initiated by copper (II) chloride at micromolar concentration (Quehenberger et al., 1988). Contrary to copper, iron, another transition metal, is present in atherosclerotic lesions; however, LDL particles have a unique characteristic, elemental iron cannot catalyze their oxidation. In the early 90s, heme was demonstrated to act as a possible mediator of LDL oxidation and endothelial injury (Balla et al., 1991a). Heme not only binds to LDL particles but also sensitizes ECs to ROS (Balla et al., 1990; Balla et al., 1991b; Balla et al., 1992b).

Free radical–mediated oxidative cleavage of the protoporphyrin IX ring leads to iron release and increased elemental iron content in LDL particles (Balla et al., 1991a).

Heme and Hb: Pro-oxidant drivers of lipid peroxidation and plaque progression

The oxidation of LDL and the subsequent lipid peroxidation are pivotal to the development of atherosclerosis. The process of lipid peroxidation of polyunsaturated fatty acids in cell membranes and lipoproteins generates a variety of oxidized lipids and reactive carbonyl species, which in turn modulate gene expression and promote further inflammation and plaque progression (Hennig and Chow, 1988; Witztum and Steinberg, 1991; Zhong et al., 2019).

Heme-initiated LDL oxidation *in vitro* is assessed by measuring conjugated dienes, lipid hydroperoxides, and thiobarbituric acid reactive substances (TBARS) as lipid oxidation assay readouts, with TBARS representing an assay-derived marker rather than a physiological product. Heme-modified LDL particles exhibit increased electrophoretic mobility due to the loss of reactive amino groups of apolipoprotein B-100 (Apo B-100), and fragmentation of Apo B-100 also occurs. During the course of LDL oxidation, degradation of the porphyrin ring occurs, and iron is trapped within the LDL particle as the footprint of heme presence. A broad scale of oxidatively modified LDL can be observed depending upon the exposure time and the amount of heme associated with LDL particles (Balla et al., 1991a). Heme-catalyzed LDL oxidation is suitable to assess oxidative resistance of LDL based on heme degradation, the loss of absorbance at 504 nm (Balla et al., 1991a; Ujhelyi et al., 2006; Ujhelyi et al., 1998). Heme arginate is significantly less catalytic (Balla et al., 2000). HO-1 deficiency in humans is characterized by early onset of atherosclerosis and intravascular hemolysis in childhood (Yachie et al., 1999). Formation of metHb and translocation of heme from globin to LDL particles was found to occur with a subsequent oxidative modification of LDL in the circulation (Jeney et al., 2002). Degradation of the porphyrin ring and the presence of heme-derived iron within LDL particles were observed in such a disease state.

Heme was demonstrated to catalyze oxidation of atheroma lipid, and heme moieties of oxidized Hb after liberation also induce lipid peroxidation. During the interaction of atherosclerotic plaque, lipid with Hb results in metHb and ferrylHb generation with a concomitant lipid oxidation, leading to a vicious circle (Nagy et al., 2010).

Myeloperoxidase and other heme proteins from vascular inflammatory cells and resident vessel wall cells can also be the source of heme, but comparing their amount to the Hb measured inside the plaques is much less. Oxidative damage by myeloperoxidase has been proposed to deprive high-density lipoprotein in human atherosclerotic tissue (Shao et al., 2012).

In summary, the interplay between heme and LDL oxidation serves as a critical driver in the pathogenesis of atherosclerosis. Whether originating from intravascular hemolysis or the degradation of Hb within the plaque itself, the liberation of heme triggers a cascade of lipid peroxidation and apolipoprotein modifications.

Mechanisms of OxLDL-induced endothelial dysfunction

Activation of ECs and NLRP3 inflammasome pathway. The interaction of OxLDL with scavenger receptors, including lectin-like OxLDL receptor-1 (LOX-1) and cluster of differentiation 36 (CD36), on ECs initiates intracellular signaling pathways, notably involving the activation of MAPKs and NF-κB signaling cascade (Davies et al., 1993). This activation induces EC activation, characterized by the upregulation of adhesion molecules such as VCAM-1, ICAM-1, and E-selectin. These adhesion proteins play a critical role in mediating the recruitment and activation of circulating monocytes and other leukocytes to the endothelial surface, a key inflammatory process contributing to the pathogenesis of atherosclerosis. In addition to this, OxLDL activates NLRP3 inflammasome, triggering pro-inflammatory cytokine release and cell death, contributing to endothelial dysfunction and the development of atherosclerosis (Hang et al., 2020; Wu et al., 2020) (Fig. 4).

Increased oxidative stress. OxLDL induces both NADPH oxidase activity and mitochondrial dysfunction in ECs, leading to increased ROS production (Fig. 4). OxLDL has been demonstrated to stimulate ECs to generate superoxide anions (O_2^-), with NADPH oxidase (NOX) identified as a potential enzymatic source of this ROS production. Furthermore, the study reported that OxLDL-induced proliferation of HUVECs was inhibited by diphenyleneiodonium (DPI), a known NOX inhibitor, thereby implicating NADPH oxidase in the proliferative response elicited by OxLDL (Heinloth et al., 2000). However, the inhibition of OxLDL-induced proliferation of ECs by DPI has been interpreted as evidence for the involvement of NOX in mediating the proliferative response. While this finding supports a role for NOX-derived ROS, it is important to note that DPI is a non-selective inhibitor of flavoproteins. In addition to NOX, DPI

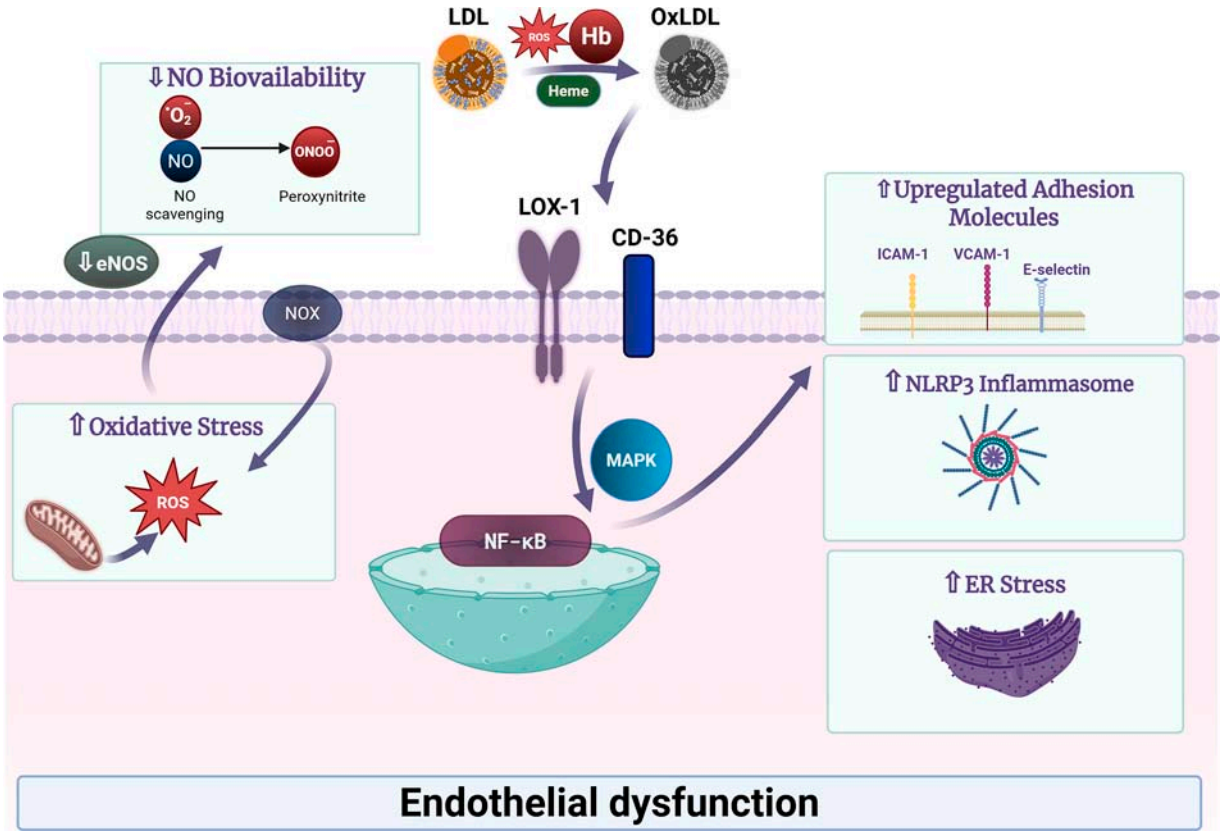

FIG. 4. Endothelial dysfunction provoked by heme induces the oxidation of low-density lipoprotein. Hemoglobin (Hb), heme, and reactive oxygen species (ROS) drive the oxidation of low-density lipoprotein (LDL), forming oxidized LDL (OxLDL). OxLDL interacts with scavenger receptors such as lectin-like oxidized LDL receptor-1 (LOX-1) and cluster of differentiation 36 (CD36) on endothelial cells (ECs), activating intracellular signaling pathways including MAPKs and NF-κB. This results in the upregulation of adhesion molecules (intracellular adhesion molecule-1, ICAM-1; vascular cell adhesion molecule-1, VCAM-1; E-selectin) and formation of the NLR pyrin domain containing 3 (NLRP3) inflammasome. OxLDL enhances oxidative stress *via* nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activation and mitochondrial dysfunction and induces cell death through mechanisms including endoplasmic reticulum (ER) stress. Additionally, OxLDL decreases nitric oxide (NO) bioavailability *via* different mechanisms. This includes the reaction of OxLDL-derived superoxide with NO to form reactive peroxynitrite and the downregulation of endothelial NO synthase (eNOS). MAPKs, mitogen-activated protein kinase; NF-κB, nuclear factor kappa B. Image created using BioRender. Gall, T. (2026) https://BioRender.com/m692fn0.

effectively inhibits other enzymatic sources of ROS and reactive nitrogen species (RNS), including nitric oxide synthases (NOS) (Stuehr et al., 1991). Therefore, although NOX likely plays a predominant role in OxLDL-driven signaling, the inhibitory effects of DPI cannot be attributed exclusively to NOX activity.

OxLDL has been shown to significantly reduce oxygen consumption and the activities of mitochondrial complexes I–IV in porcine aortic ECs. OxLDL also lowers the NAD^+/NADH ratio and increases both intracellular and extracellular ROS (Roy Chowdhury et al., 2010).

Mitochondria play a central role in OxLDL-triggered oxidative responses in ECs. It has been shown that mitochondria are a key source of OxLDL-induced ROS/RNS in ECs, while cells lacking functional mitochondria (rho0 cells) showed reduced ROS/RNS production in response to OxLDL, supporting, at least partly, the mitochondrial origin of OxLDL-driven oxidative damage (Zmijewski et al., 2005).

Disrupting mitochondrial homeostasis involves significant oxidative stress, decreased mitochondrial membrane potential, reduced ATP production, and mitochondrial dynamics disorders, specifically increased fission mediated by proteins like dynamin-related protein-1 and mitochondrial fission factor (Cui et al., 2022; Li et al., 2021; Liu et al., 2023). Therapeutic agents like atorvastatin and melatonin, as well as regulatory molecules such as miR-199b-5p, have shown potential in inhibiting these effects and restoring mitochondrial integrity (Cui et al., 2022; Li et al., 2021; Liu et al., 2023).

Direct scavenging of NO and decreased NO biovailability. Reduced nitric oxide (NO) leads to impaired vasodilation, a hallmark of endothelial dysfunction. NO, synthesized by ECs, is fundamental to the regulation of vascular homeostasis and the preservation of endothelial function. As a potent vasodilator, NO facilitates vascular relaxation, inhibits platelet aggregation and adhesion, and suppresses leukocyte migration—mechanisms that collectively contribute to the prevention of atherosclerosis (Gliozzi et al., 2019).

OxLDL disrupts the molecular machinery of endothelial nitric oxide synthase (eNOS) through several distinct mechanisms.

eNOS activity is dependent on its localization within caveolae. Exposure to oxLDL depletes cholesterol from these membrane domains, leading to the redistribution of both caveolin-1 and eNOS. This translocation results in the suppression of acetylcholine-induced eNOS activation (Blair et al., 1999) (Fig. 4).

OxLDL has been shown to alter eNOS phosphorylation. OxLDL impairs protein kinase C alpha activity, leading to altered eNOS phosphorylation at Thr495, disruption of the eNOS signaling complex, and increased production of ROS (Fleming et al., 2005). OxLDL also increases arginase II activity in ECs, which competes with eNOS for L-arginine, reducing substrate availability for NO production (Ryoo et al., 2006).

In summary, the detrimental impact of OxLDL on vascular health extends far beyond simple lipid accumulation, manifesting as a multi-pronged assault on the molecular machinery of the endothelium. By physically displacing eNOS from caveolar domains, disrupting critical phosphorylation signaling pathways, and diverting essential substrates through arginase II activation, OxLDL effectively transitions the ECs from a protective state to a pro-atherogenic one. These biochemical disruptions culminate in a state of chronic NO deficiency, reinforcing the cycle of oxidative stress and inflammation that drives the progression of cardiovascular disease.

EC death. OxLDL promotes EC death through multiple mechanisms (Fig. 4). OxLDL-induced EC death reveals a complex landscape where multiple pathways can be activated depending on the cellular context. While Dimmeler et al. (1997) demonstrate that oxLDL triggers a classic apoptotic suicide pathway in ECs mediated by ROS and the activation of CPP32-like proteases (caspase-3), Pörn-Ares et al. (2003) identify an alternative, calpain-mediated signaling pathway in human dermal microvascular cell line (HMEC-1) cells. In the latter model, oxLDL induces cell death through the activation of μ-calpain and the cleavage of the pro-apoptotic protein Bid, notably occurring in the absence of caspase-3 activation; in fact, caspase-3 was found to be inactivated *via* polyubiquitination (Pörn-Ares et al., 2003). Despite these differing enzymatic drivers—caspase-dependent *versus* calpain-dependent—both studies conclude that oxLDL-induced injury leads to the formation of nucleosomal DNA fragments characteristic of apoptosis, providing a critical mechanistic link to the progression of atherosclerosis (Dimmeler et al., 1997; Pörn-Ares et al., 2003).

OxLDL triggers apoptotic cell death *via* the overexpression of LOX-1 and subsequent attenuation of protective autophagic response mediated by Beclin-1 and microtubule-associated protein 1 light chain 3 (Mollace et al., 2015).

Others have found that ER stress activating pro-apoptotic pathways characterized by the induction of proapoptotic proteins C/EBP homologous protein (CHOP) and BCL2 apoptosis regulator (Bcl-2), as well as caspase-12 activity, play an important role in EC apoptosis. By blocking OxLDL-induced expression of CHOP and Bcl-2 and activation of caspase-12 activity, EC apoptosis is attenuated (Hong et al., 2014). Additionally, inhibitors targeting ER stress pathways and Nox-4 knockdown attenuated ER stress marker expression and EC apoptosis. These results indicate that LOX-1 mediates OxLDL-induced EC apoptosis possibly through the ER stress signaling pathway (Fig. 4).

Oxidized LDL as a downstream effector of heme and Hb in macrophages and VSMCs

Heme-driven oxidative modifications provide a mechanistic link between RBC damage, IPH, and redox-dependent inflammatory signaling in macrophages and VSMCs.

Macrophages are highly responsive to oxLDL generated in heme-rich environments. OxLDL is internalized primarily through scavenger receptors such as CD36, scavenger receptor A, and lectin-like oxidized LDL receptor-1 (LOX-1), leading to excessive lipid accumulation and foam cell formation (Chistiakov et al., 2017; Moore et al., 2013). Heme and Hb further exacerbate this process by increasing intracellular oxidative stress, overwhelming antioxidant defenses, and amplifying redox-sensitive signaling pathways (Gáll et al., 2019; Gáll et al., 2022). Uptake of OxLDL promotes NADPH oxidase activation and mitochondrial dysfunction, resulting in ROS production and activation of NF-κB and inflammasome signaling (Lee et al., 2010; Sun et al., 2024). Uptake of OxLDL drives the release of pro-inflammatory cytokines and chemokines, sustaining chronic inflammation within atherosclerotic lesions (Chistiakov et al., 2017; Sun et al., 2024). In parallel, heme-induced oxidative stress impairs macrophage efferocytosis and promotes apoptosis, contributing to necrotic core formation in advanced plaques (Tabas, 2009).

VSMCs are similarly affected by OxLDL generated downstream of heme and Hb oxidation. OxLDL induces a phenotypic switch from a contractile to a synthetic or inflammatory state, characterized by enhanced proliferation, migration, and extracellular matrix remodeling (Bennett et al., 2016; Chen et al., 2023).

Traditionally viewed as plaque stabilizing due to their role in forming the fibrous cap, reduced VSMC content—often assessed by loss of contractile markers—has been associated with plaque vulnerability. However, lineage-tracing and transcriptomic studies have revealed that VSMCs constitute a larger and more heterogeneous plaque population than previously recognized, with functions that may also be detrimental. During atherosclerosis, VSMCs downregulate contractile genes and undergo phenotypic switching into diverse states, including macrophage-like, foam cell–like, osteochondrogenic-like, and myofibroblast-like phenotypes. This plasticity occurs across distinct plaque regions and strongly influences plaque development and stability (Grootaert and Bennett, 2021).

The contribution of VSMCs to plaque foam cell populations was historically underestimated due to reliance on marker-based cell identification. Lineage-tracing studies using VSMC-specific CreERT2 systems have demonstrated that a substantial proportion of foam cells in advanced lesions originate from VSMCs that have undergone phenotypic switching (Bennett et al., 2016; Shankman et al., 2015).

Therapeutic Approaches

Here, we provide a brief overview of potential therapeutic approaches targeting the underlying mechanisms of vascular

diseases such as heme/Hb metabolism, oxidative stress, inflammation, and lipid oxidation. There is an enormous amount of scientific data proving that hemorrhage, vessel wall, and plaque bleeding are not innocent bystanders of the pathophysiology of atherosclerosis, which is why our focus is heme-/Hb-centered prevention and therapy.

N-acetylcysteine

N-Acetylcysteine (NAC) is a derivative of the amino acid L-cysteine and is widely recognized in pharmacology for its role as a potent antioxidant and a mucolytic agent. NAC acts as an antioxidant through and as a reduced glutathione (GSH) precursor. By providing cysteine, NAC helps replenish GSH stores, especially during oxidative stress or acetaminophen toxicity. NAC also acts as an antioxidant by direct radical scavenging: the free thiol (-SH) group on the NAC molecule can interact directly with ROS such as (•OH) and H_2O_2 (Aldini et al., 2018).

NAC has been shown to inhibit the development of atherosclerosis in multiple animal models. NAC reduces foam cell formation—an early marker of atherosclerosis—by lowering ROS levels and downregulating CD36 expression in the presence of oxidized LDL. Microarray analysis revealed that NAC upregulated ApoE involved in lipid efflux and antioxidant genes, while downregulating genes related to oxidative stress in human acute monocytic leukemia THP-1 cells (Sung et al., 2012). NAC reduces atheroma progression in a murine model of uremia-enhanced atherosclerosis, probably *via* a decrease in oxidative stress (Ivanovski et al., 2005). Early and appropriate administration of NAC may effectively mitigate inflammation and the progression of atherosclerosis in aging LDL receptor$^{-/-}$ mice without severe hyperlipidemia, while preserving the M2 macrophage population and enhancing CD146 expression (Zhu et al., 2022).

Early studies indicated that NAC does not inhibit LDL oxidation (Kleinveld et al., 1992), later studies have shown that NAC lowers LDL oxidation (Rattan and Arad, 1998). NAC treatment also significantly reduced serum levels of OxLDL in patients with coronary artery disease and hyperlipidemia, without affecting total LDL levels. Furthermore, a decrease in ROS production and a reduction in atherosclerotic plaque formation have also been observed in hyperlipidemic LDL receptor knockout mice treated with NAC (Cui et al., 2015). Further well-designed clinical trials are necessary to conclusively determine NAC's role in modulating LDL oxidation in humans.

Hb/heme scavengers

Natural defense systems that mitigate the toxicity induced by Hb and heme consist of both extracellular and intracellular components. The extracellular mechanisms include proteins such as Hp, Hpx, and A1M, while the intracellular response primarily involves the HO-1/ferritin system.

Haptoglobin. Hp confers structural stabilization of Hb and mitigates its oxidative interactions with lipoproteins, thereby providing substantial protection against Hb-induced vascular toxicity. The canonical pathway of Hp function involves CD163-mediated internalization of Hp–Hb complexes from plasma (Graversen et al., 2002). Through these mechanisms, Hp modulates deleterious pseudo-peroxidase activity of Hb, potentially redirecting it toward an antioxidative role under conditions of oxidative stress (Cooper et al., 2013; Schaer et al., 2013).

The binding of Hb to Hp attenuates both ferryl iron formation and free radical reactivity of Hb in response to peroxide exposure (Cooper et al., 2013). Hb/Hp complex mitigates Hb-driven LDL peroxidation, while Hp significantly delays the EC damage provoked by Hb-driven LDL oxidation (Schaer et al., 2013). Additionally, this interaction inhibits oxidative fragmentation of the globin chains, further contributing to the protective role of Hp (Posta et al., 2020). In a sheep model, Hp administration into the subarachnoid space also prevents Hb-induced cerebral vasospasm, supporting its therapeutic potential in human pathologies associated with hemorrhage (Hugelshofer et al., 2019).

Under conditions of excessive hemolysis, elevated Hb concentrations can deplete endogenous Hp levels, leading to increased tissue damage. Administration of exogenous Hp may facilitate enhanced clearance of free Hb during hemolytic episodes and mitigate complications associated with circulating Hb. Hp improves survival and reduces inflammation following transfusion of stored RBCs after hemorrhagic shock in mice while also preventing kidney injury and hemoglobinuria by retaining free Hb in the plasma (Graw et al., 2016). Hp treatment reduces heme/iron toxicity in the kidneys following hemolysis in a murine model of SCD (Chintagari et al., 2015). A recent systematic review examining the therapeutic application of Hp in hemolysis has concluded that exogenous Hp administration is associated with reduced Hb levels and elevated circulating Hp during hemolytic episodes. Furthermore, Hp infusion is linked to a decreased risk of acute kidney injury (AKI) after hemolysis. Importantly, no significant adverse events have been reported following exogenous Hp administration across a broad range of hemolytic etiologies (Baldetti et al., 2024). Nonetheless, human studies investigating the potential therapeutic application of Hp in hemolytic diseases remain limited, underscoring the need for further research in this area.

Hemopexin. Hpx is a plasma glycoprotein that binds and inactivates heme and consists of a single 60-kDa polypeptide chain (Balla Lab invest 1991). The detoxification of heme is predominantly mediated by Hpx *via* LDL receptor-related protein 1 (LRP1)–dependent endocytosis in the liver, which facilitates subsequent heme degradation. Heme scavenging represents a critical mechanism through which Hpx confers protection against oxidative stress and associated inflammatory disorders. Moreover, Hpx may play a novel protective role in mitigating heme- and oxidative stress–induced inflammatory conditions, such as atherosclerosis (Mehta and Reddy, 2015).

Hpx prevents heme-iron accumulation within the cardiovascular system, thereby reducing the generation of ROS, the upregulation of adhesion molecules, and the oxidative inactivation of NOS and NO (Vinchi et al., 2013). Hpx treatment is a promising novel therapy to protect against heme-induced cardiovascular dysfunction in hemolytic disorders. While Hpx has shown promise in preclinical studies, its safety and efficacy in humans require further investigation through clinical trials.

Alpha-1-microglobulin. A1M is a small (26 kDa) protein belonging to the lipocalin family (Akerstrom et al., 2000). It is primarily synthesized in the liver and interacts with various proteins, including immunoglobulin A (IgA), albumin, and prothrombin (Berggård et al., 1997). A1M is an efficient radical scavenger and heme-binding protein (Akerström et al., 2007; Rutardottir et al., 2016). A1M is abundantly expressed in human carotid artery plaques, and its expression is upregulated in ECs, smooth muscle cells, and macrophages in response to both heme and ferrylHb (Pethő et al., 2021a). A1M inhibits heme-induced oxidative modification of LDLs and plaque lipids isolated from human atherosclerotic plaques and mitigates oxidized LDL-driven cell death (Pethő et al., 2021a). Administration of exogenous A1M exhibits neuroprotective effects in a preterm rabbit pup model of intraventricular hemorrhage (Romantsik et al., 2019). RMC-035, a recombinant variant of endogenous human A1M, is under development for the prevention of AKI in patients undergoing cardiac surgery. Multiple intravenous administrations of RMC-035 have been well tolerated in this patient population (Weiss et al., 2023), suggesting its potential therapeutic application in hemolytic diseases.

In a randomized double-blind placebo-controlled phase 2a study with 177 patients (RMC-035: 89, placebo: 88) undergoing open-chest cardiac surgery at high risk for AKI, the most frequently reported treatment-emergent adverse events for RMC-035 were chills (30.3%), nausea (21.3%), and anemia (20.2%). In that proof-of-concept study, RMC-035 did not reduce AKI 72 h after cardiac surgery. However, RMC-035 was associated with improved secondary renal outcomes (Zarbock et al., 2024).

Although A1M has strong heme-binding capacity, its quantitative importance *in vivo* is questionable because Hpx and albumin are present in much higher plasma concentrations and bind heme more avidly (Zager, 2017). In addition, recombinant A1M has a very short plasma half-life due to rapid glomerular filtration, unlike Hpx and albumin, which largely remain intravascular, making sustained heme scavenging by recombinant A1M unlikely (Åkerström and Gram, 2014). These necessitates further clinical trials on the potential use of A1M in heme-related diseses.

Given that A1M significantly reduces oxidative modifications of Hb and plaque lipids, its application may extend beyond renal protection to attenuate oxidative damage and toxicity driven by oxidized Hb and lipids in hemolytic diseases. Future research should focus on clinical trials assessing the efficacy of A1M in diverse hemolytic conditions, as well as elucidating its mechanistic roles in mitigating oxidative stress and inflammation in hemolysis-associated pathologies.

Carbon monoxide

Besides NO and hydrogen sulfide (H_2S), carbon monoxide (CO) is a classical endogenous messenger gas molecule gaining attention in vascular disorders.

CO is a poisonous gas that inhibits the release of oxygen from Hb; however, at very low concentrations, CO can have signaling roles and therapeutic effects (Bansal et al., 2024; Kinoshita et al., 2020). CO, which is a by-product of heme catabolism by HO-1 and HO-2, plays an essential role acting as an endogenous gasotransmitter (Ryter et al., 2006). Over the past decades, CO has been generated a lot of interest due to its therapeutic potential in inflammatory diseases, SCD, and atherosclerosis (Belcher et al., 2018; Otterbein et al., 2003; Vyas et al., 2023).

HBI-002 is an orally administered liquid CO drug designed to achieve peak carboxyhemoglobin (COHb) levels ≤10%. Preclinical studies in murine SCD models showed reduced vascular stasis, improved hematologic markers, increased HO-1 and nuclear factor erythroid 2–related factor 2 (Nrf2), and decreased NF-κB activation, supporting potential clinical benefit (Belcher et al., 2018). A phase 1 open-label study in 20 healthy adults demonstrated dose-dependent increases in COHb, with targeted COHb levels up to 10% achievable. COHb returned to baseline within 24 h without accumulation. No serious adverse events (SAEs) occurred; only mild (Grade 1), transient adverse events were reported, and no clinically significant laboratory abnormalities were observed. An ongoing phase 2a open-label study in adults with SCD has reported interim data from six subjects. Daily dosing (1.6 mg/kg then 2.7 mg/kg) achieved target COHb ranges (4%–7% low dose; 7%–10% high dose). No SAEs were reported, and HBI-002–related adverse events were generally mild. Two subjects experienced PICC line–related complications (infection and thrombosis), consistent with known risks. Overall, HBI-002 was well tolerated with manageable safety findings, supporting continued clinical development (Diaz et al., 2025).

While CORMs were initially developed to provide a controlled alternative to CO gas inhalation, recent evidence suggests that classical metal/borane-carbonyl complexes (specifically CORM-2, CORM-3, CORM-A1, and CORM-401) might be unreliable tools for *in vivo* studies (Bauer et al., 2024; Bauer et al., 2023; Yang et al., 2024). Their limitations stem from unpredictable release kinetics, inherent chemical reactivity, and biological effects that occur independently of CO. The primary requirement for a CO donor is the predictable delivery of CO; however, classical CORMs are highly sensitive to their microenvironment. For example, the CO-release yield and rate of CORM-401 are significantly altered by the presence of endogenous reagents such as thiols, peroxides, and dithionite (Bauer et al., 2023). Similarly, CORM-2 and CORM-3 often fail to release significant CO unless in the presence of strong nucleophiles, with CORM-2 frequently undergoing a water–gas shift reaction to release CO_2 instead of CO (Yang et al., 2024). These "idiosyncratic" release patterns make it nearly impossible to determine the actual dose of CO delivered to tissues *in vivo*.

A major concern in CO research is the attribution of biological effects to CO when they may actually be caused by the CO-delivery vehicle itself. While CORM-2 and CORM-3 significantly induce HO-1 expression, CO gas itself (even at concentrations up to 5%) fails to do so in various cell lines (Yang et al., 2024). This suggests that the HO-1 induction frequently cited as a CO mechanism is actually a CO-independent effect of the metal complex. In many experimental settings, the biological findings observed with CORMs are not replicated when using CO gas or CO-saturated solutions (Yang et al., 2024). This discrepancy suggests that data on the classical metal/borane-carbonyl complexes (specifically CORM-2, CORM-3, CORM-A1, and

CORM-401) can have limitations in investigating the *in vivo* effects of CORMs.

Hydrogen sulfide

H_2S was traditionally regarded as a toxic gas; however, it is now recognized as an important physiological mediator involved in a broad range of biological processes. These include the regulation of synaptic transmission, vascular tone, inflammatory responses, gene transcription, and angiogenesis (Kimura, 2014). The side effects of H_2S exposure are heterogeneous and largely determined by the concentration encountered (Arnold et al., 1985). At relatively low levels, exposure is often benign and is characteristically associated with a recognizable "rotten egg" smell (Arnold et al., 1985). Mild exposure may produce vague, nonspecific complaints such as headache, nausea, and vomiting, which frequently resolve quickly once the individual is removed from the source (Haouzi et al., 2016; Reiffenstein et al., 1992).

High concentrations, particularly those exceeding 1000 ppm, are associated with profound toxicity, including sudden collapse, seizures, central nervous system depression, cardiovascular instability, and respiratory failure (Ng et al., 2019).

Endogenous H_2S is synthesized in humans through both enzymatic and non-enzymatic pathways. Enzymatic production primarily involves the enzymes cystathionine β-synthase and cystathionine γ-lyase (CSE), which generate H_2S predominantly from L-cysteine (Wang, 2002). A third enzyme, 3-mercaptopyruvate sulfurtransferase, contributes to H_2S production by catalyzing the conversion of 3-mercaptopyruvate—formed from L-cysteine and α-ketoglutarate *via* the action of cysteine aminotransferase—into H_2S (Kimura et al., 2015). Non-enzymatic production of H_2S can occur through the reduction of thiosulfate (Olson et al., 2013) or from sulfur-containing compounds present in certain dietary sources, such as garlic (Benavides et al., 2007).

Multiple lines of evidence support the protective role of H_2S in vascular diseases, particularly through its ability to mitigate the oxidation of LDL and Hb. In ApoE$^{-/-}$ mice, H_2S administration reduces atherogenic diet–induced atherosclerotic plaque formation (Potor et al., 2018; Wang et al., 2009). Exogenous H_2S inhibited heme and Hb-mediated lipid oxidation derived from human complicated lesions. H_2S significantly attenuated Hb oxidation, thereby preventing the formation of ferrylHb derivatives. By disrupting Hb–lipid interactions, sulfide reduced the pro-oxidant activity of oxidized Hb, leading to diminished expression of endothelial adhesion molecules and preservation of endothelial integrity (Potor et al., 2018). In addition, CSE expression is predominantly upregulated in macrophages, foam cells, and myofibroblasts within human atherosclerotic lesions obtained from carotid artery specimens of patients. A comparable expression pattern was observed in aortic lesions of ApoE$^{-/-}$ mice maintained on a high-fat diet (Potor et al., 2018). Others have found that H_2S may protect against atherosclerosis by reducing harmful LOOHs to less reactive compounds,

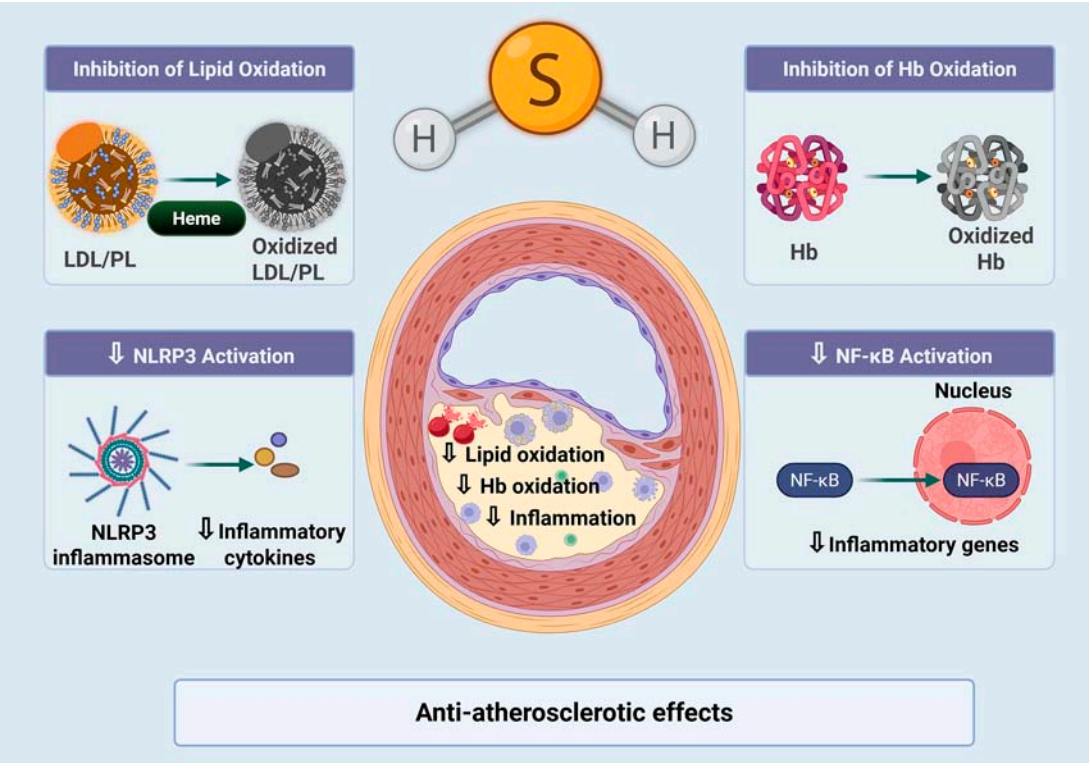

FIG. 5. Mechanisms of hydrogen sulfide (H_2S)–mediated protectivity in atherosclerosis. H_2S exerts multifaceted protective effects in atherosclerosis by directly inhibiting the oxidation of LDL and Hb. H_2S suppresses Hb oxidation and heme-catalyzed oxidative modification of LDL and plaque lipids. Additionally, H_2S reduces NF-κB activation and NLRP3 inflammasome formation, thereby decreasing the inflammatory response. Image created using BioRender. Gall, T. (2026) https://BioRender.com/75uurny.

neutralizing the damaging effects of OxLDL (Muellner et al., 2009).

Reaction of H_2S with Hb forms sulfHb (or sulfheme), resulting in the covalent incorporation of a sulfur atom into the porphyrin ring of the heme group (Docherty et al., 2020; Pietri et al., 2011). SulfHb is often considered to have lower prooxidant activity compared to Methb because the sulfur-carbon bond is stable and irreversible (Saeedi et al., 2015; Soderstrom et al., 2023). Furthermore, the presence of sulfur in one or two subunits of the Hb tetramer can decrease the oxygen affinity of the remaining unmodified subunits, leading to a rightward shift in the oxygen dissociation curve and contributing to clinical cyanosis (Soderstrom et al., 2023).

EC-specific knockout of CSE increases expression of the adhesion molecule CD62E and enhances monocyte adhesion, even without inflammatory stimuli. Although CSE expression is upregulated in both murine and human atherosclerotic lesions, levels of H_2S were reduced within the atheromas and in systemic circulation due to phosphorylation-dependent inhibition of CSE, promoting endothelial dysfunction and atherosclerosis (Bibli et al., 2019).

Beyond its direct inhibitory effects on the oxidation of LDL and Hb, H_2S mediates indirect anti-atherosclerotic actions by mitigating oxidative stress and inflammatory responses. H_2S serves as a robust anti-inflammatory agent across diverse cell types, predominantly through suppression of the TLR-4/NF-κB signaling pathway(Sun et al., 2019) and suppression of NLRP3 inflammasome activation (Wu et al., 2019).

In a murine model of atherosclerosis, GYY4137 reduced plaque size and foam cell accumulation while lowering pro-inflammatory cytokines, likely through downregulation of TLR-4 (Zheng et al., 2020). In a diabetes-accelerated atherosclerosis model, GYY4137 similarly decreased plaque development, suppressed NLRP3 inflammasome activation, and diminished expression of adhesion molecules ICAM-1 and VCAM-1, highlighting its potential to mitigate inflammation and vascular injury. (Zheng et al., 2019). Additionally, H_2S-mediated S-sulfhydration of Kelch-like ECH-associated protein at cysteine 151 promotes the nuclear translocation of Nrf2, leading to the upregulation of HO-1 expression. This process reduces oxidative stress and ultimately attenuates

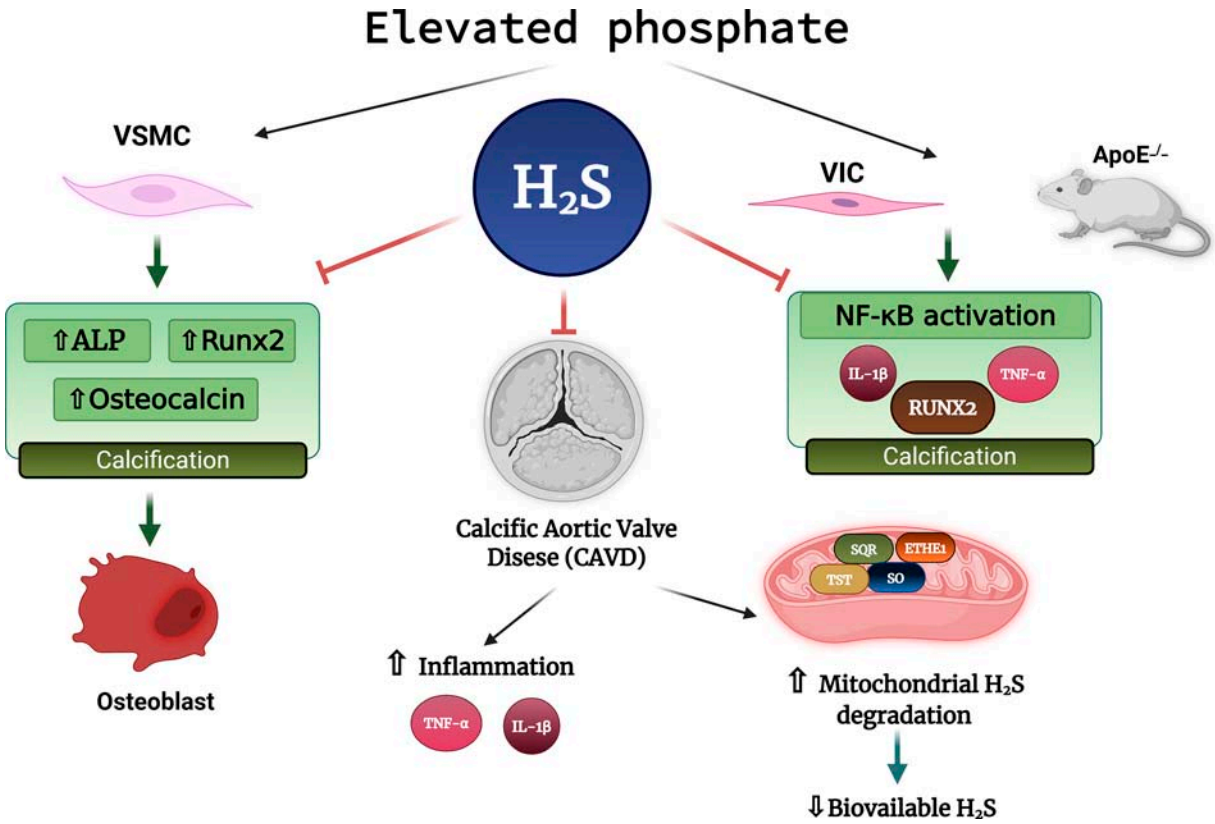

FIG. 6. H_2S attenuates vascular and valvular calcification through anti-osteogenic and anti-inflammatory mechanisms. In vascular smooth muscle cells (VSMCs), H_2S reduces extracellular matrix calcium deposition and downregulates key osteogenic markers—alkaline phosphatase, osteocalcin, and RUNX2—induced by calcifying stimuli such as elevated phosphate levels. Similarly, in valvular interstitial cells (VICs), H_2S-releasing compounds suppress inflammation and calcification. In ApoE$^{-/-}$ mice, H_2S inhibits aortic valve inflammation by blocking NF-κB nuclear translocation and reducing IL-1β and TNF-α expression, thereby limiting osteogenic differentiation *via* RUNX2 regulation. In patients with calcific aortic valve disease (CAVD), decreased bioavailable H_2S coincides with elevated IL-1β and TNF-α levels. This is accompanied by upregulation of mitochondrial H_2S-catabolizing enzymes (sulfide quinone oxidoreductase [SQR], persulfide dioxygenase [ETHE1], sulfite oxidase [SO], and thiosulfate sulfurtransferase [TST]), suggesting increased H_2S degradation. H_2S administration, such as AP39, a mitochondria-targeted H_2S donor, restores anti-calcific and anti-inflammatory responses in VICs and VSMCs, underscoring the therapeutic potential of H_2S vascular/valvular calcification. Image created using BioRender. Gall, T. (2026) https://BioRender.com/x5wtp94.

diabetes-accelerated atherosclerosis in murine models in mice (Xie et al., 2016).

H_2S exerts multifaceted protective effects in atherosclerosis by directly inhibiting LDL and Hb oxidation. Through these mechanisms, H_2S reduces plaque formation, suppresses pro-inflammatory cytokine production, inhibits inflammasome activation, and decreases adhesion molecule expression, collectively mitigating vascular injury and disease progression (Fig. 5). Future research should focus on elucidating the precise molecular targets and pathways of H_2S in human atherosclerosis, optimizing the delivery and dosing of H_2S donors.

H_2S has been demonstrated to inhibit both VSMC calcification and their transdifferentiation into osteoblast-like cells (Zavaczki et al., 2011). Specifically, H_2S was found to reduce calcium deposition within the extracellular matrix and downregulate the expression of key osteogenic markers, including alkaline phosphatase, osteocalcin, and runt-related transcription factor 2 (RUNX2), triggered by calcifying stimuli such as high phosphate (Fig. 6) (Zavaczki et al., 2011).

Similarly, H_2S-releasing compounds exert anti-inflammatory and anti-calcific effects on VICs. In ApoE$^{-/-}$ mice, H_2S attenuates inflammation and calcification within the aortic valve. Mechanistically, H_2S suppresses the nuclear translocation of NF-κB and the subsequent expression of pro-inflammatory cytokines, including IL-1β and TNF-α. Under pro-calcific conditions, NF-κB mediates the activation and nuclear translocation of the osteogenic transcription factor RUNX2, suggesting a mechanistic link between inflammation and calcification, orchestrated through the transcriptional regulation by NF-κB and Runx2 (Fig. 6) (Éva Sikura et al., 2021).

In patients with calcific aortic valve disease, aortic valve tissues exhibit reduced levels of bioavailable H_2S alongside elevated pro-inflammatory cytokines, interleukin-1β (IL-1β) and TNF-α, when compared to healthy controls. Mitochondrial enzymes implicated in H_2S catabolism, including sulfide

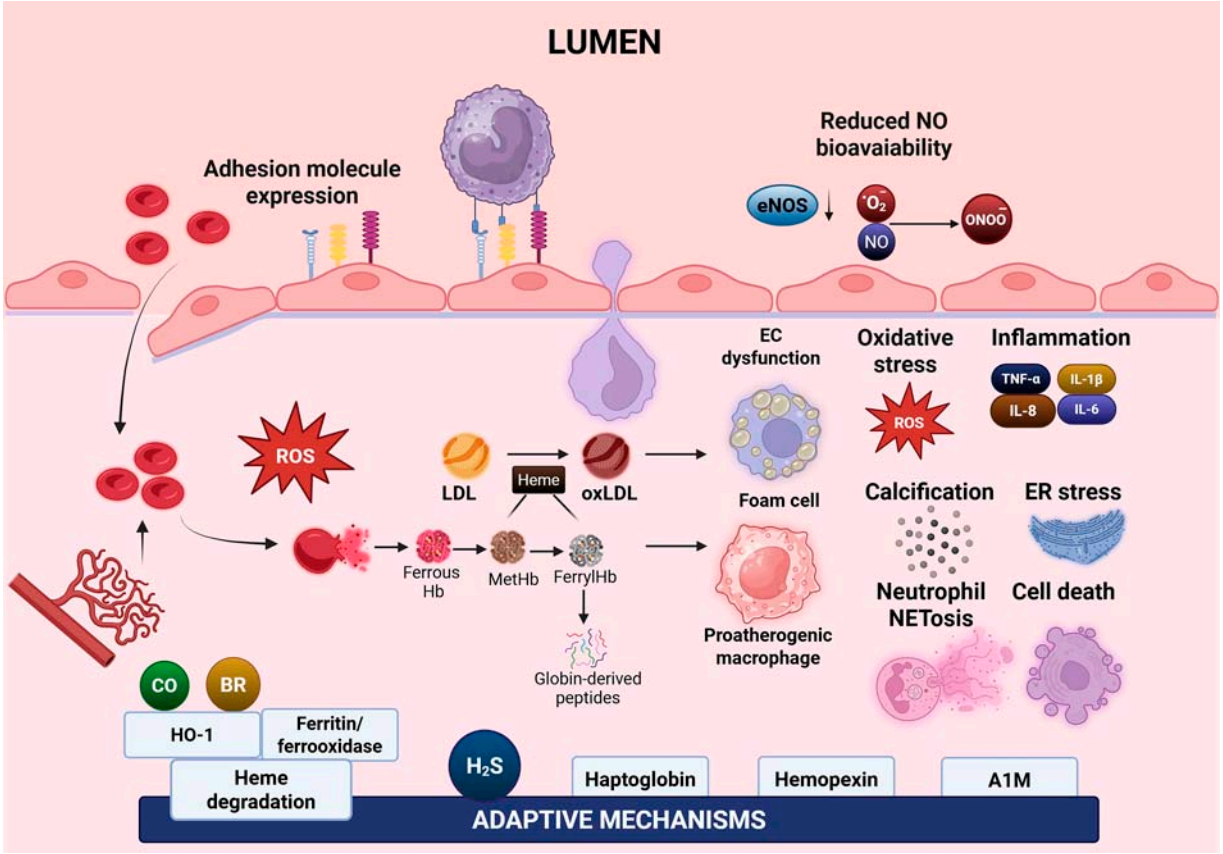

FIG. 7. Pathways of progression of complicated atherosclerotic lesion induced by hemoglobin and heme – adaptive and protective mechanisms. In the highly oxidative environment of atherosclerotic plaques, red blood cells (RBCs) released during hemorrhage from plaque rupture or unmatured, neovascularized capillaries lyse and release Hb. Hb undergoes oxidation, releasing heme and forming metHb, ferryl-Hb, and globin-derived peptides. Both Hb and heme catalyze oxidative modification of LDL. Oxidized Hb, heme, and globin-derived peptides induce inflammatory responses in resident plaque cells. Oxidized Hb, heme, and OxLDL promote adhesion molecule expression on ECs, facilitating mononuclear cell migration into plaques. Ferryl-Hb drives pro-atherogenic macrophage polarization, while OxLDL promotes foam cell formation from infiltrating monocytes. These events trigger oxidative stress, inflammation, calcification, ER stress, cell death, and neutrophil extracellular trap (NET) formation within plaques. Oxidative stress exacerbates endothelial dysfunction by reducing NO bioavailability *via* peroxynitrite formation and decreasing eNOS activity. Adaptive mechanism involve Hb scavenging by haptoglobin, heme scavenging by hemopexin and α1-microglobulin (A1M), heme degradation by the heme oxygenase–ferrtin system generating carbon monoxide (CO) and bilirubin (BR), and endogenous H_2S production. Image created using BioRender. Gall, T. (2026) https://BioRender.com/ftgjbrn.

quinone oxidoreductase (SQR), persulfide dioxygenase, sulfite oxidase (SO), and thiosulfate sulfurtransferase, are upregulated in calcified aortic valves and in VICs exposed to high phosphate, indicating accelerated H_2S degradation. Treatment with AP39, a mitochondria-targeted H_2S donor, mitigates osteoblastic transformation and pro-inflammatory cytokine expression in VICs, highlighting the protective role of H_2S (Fig. 6) (Combi et al., 2023).

Despite the growing research on the effect of H_2S donors as potential therapeutics in animal models, data on their human application are scarce. ATB-346 is a hydrogen sulfide–releasing anti-inflammatory and analgesic drug developed to reduce gastrointestinal (GI) toxicity while maintaining cyclooxygenase (COX) inhibition. In a 2-week, double-blind phase 2B study of 244 healthy volunteers, ATB-346 (250 mg once daily) was compared with naproxen (550 mg twice daily). Both drugs produced equivalent and marked suppression of COX activity (>94%). However, GI outcomes differed substantially: 42% of naproxen-treated subjects developed at least one endoscopically detected ulcer, compared with only 3% of those receiving ATB-346. Naproxen was also associated with a higher number and larger size of ulcers, as well as more frequent GI symptoms, including dyspepsia, abdominal pain, reflux, and nausea. Plasma H_2S levels were significantly higher in the ATB-346 group. Overall, the study demonstrates that ATB-346 provides comparable COX inhibition to naproxen with markedly reduced GI toxicity (Wallace et al., 2020). Further research is needed to evaluate the therapeutic potential and "off-label" effects of H_2S donors as potential therapeutics in human studies.

The discovery of new pathophysiological phenomena in atherosclerosis is crucial for addressing the persistent burden of cardiovascular disease. These therapeutic applications ideally should target underlying mechanisms of vascular diseases such as heme/Hb metabolism, oxidative stress, inflammation, and lipid oxidation to prevent plaque formation and progression. These new approaches may improve patient outcomes and complement existing therapies, contributing to a more comprehensive management of atherosclerosis.

Conclusions and Future Perspectives

Despite excessive research, atherosclerosis has remained a leading cause of death worldwide. Although IPH is usually considered a feature of advanced atherosclerosis, RBC infiltration may also occur at early stages of lesion development due to increased endothelial permeability or leakage from immature microvessels. In the intima, RBCs are exposed to oxidative conditions that promote Hb oxidation and heme release, which enhances lipid peroxidation and contributes to LDL oxidation, leading to endothelial activation and macrophage accumulation. Further studies are needed to define the timing, mechanisms, and pathological relevance of RBC entry in early atherosclerotic lesions. Determining whether these processes are therapeutically targetable may reveal new strategies to limit plaque development. Promising future perspectives in atherosclerosis research should focus on reducing chronic inflammatory response, modulating macrophage polarization and activity, and improving endothelial function (Fig. 7). These efforts with a focus on the pathogenic role of Hb and heme may provide potential therapeutic approaches to prevent plaque formation and reduce the risk of plaque progression and atherosclerotic burden.

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Authors' Contributions

T.G.: Writing—original draft, Writing—review and editing; J.G.B.: Writing—original draft; D.P.: Writing—original draft; G.B.: Writing—original draft, Writing—review and editing, Supervision; J.B.: Writing—original draft, Writing—review and editing, Supervision, Funding acquisition.

Author Disclosure Statement

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Supplementary Material

Supplementary Video

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Abbreviations Used

A1M = α1-microglobulin
AAA = abdominal aortic aneurysm
AKI = acute kidney injury
ApoE = apolipoprotein E
Bcl-2 = BCL2 apoptosis regulator
CD14 = cluster of differentiation 14
CD163 = cluster of differentiation 163
CD36 = cluster of differentiation 36
CHOP = C/EBP homologous protein
CO = carbon monoxide
COHb = carboxyhemoglobin
CORM = carbon monoxide–releasing molecule
COX = cyclooxygenase
CSE = cystathionine γ-lyase
DAMP = damage-associated molecular pattern
DPI = diphenyleneiodonium
EC = endothelial cell
Egr-1 = early growth response-1
eNOS = endothelial nitric oxide synthase
ER = endoplasmic reticulum
FerrylHb = ferryl hemoglobin
GI = gastrointestinal
GSH = glutathione
H_2O_2 = hydrogen peroxide

Abbreviations Used (Cont.)

H_2S = hydrogen sulfide
HA-mac = hemorrhage-associated macrophages
Hb = hemoglobin
HO = heme oxygenase
HO-1 = heme oxygenase-1
Hp = haptoglobin
Hpx = hemopexin
HUVEC = human umbilical vein endothelial cell
ICAM-1 = intracellular adhesion molecule-1
IL-10 = interleukin-10
IL-18 = interleukin-18
IL-1β = interleukin-1β
IL27RA = interleukin 27 receptor subunit alpha
IL-6 = interleukin-6
IL-8 = interleukin-8
IPH = intraplaque hemorrhage
LDL = low-density lipoprotein
LOOH = lipid hydroperoxide
LOX-1 = lectin-like oxidized LDL receptor-1
LPS = lipopolysaccharide
LRP1 = low-density lipoprotein receptor-related protein 1
MAPK = mitogen-activated protein kinase
MetHb = methemoglobin
mRNA = messenger RNA
NAC = *N*-acetylcysteine
NADPH = nicotinamide adenine dinucleotide phosphate
NET = neutrophil extracellular trap
NF-κB = nuclear factor kappa B
NLR = nucleotide-binding oligomerization domain-like receptors
NLRP12 = NLR pyrin domain containing 12
NLRP3 = NLR pyrin domain containing 3
NO = nitric oxide
NOS = nitric oxide synthase
NOX = NADPH oxidase
Nrf2 = nuclear factor erythroid 2-related factor 2
$O_2\bullet^-$ = superoxide anion radical
$\bullet$OH = hydroxyl radical
OxLDL = oxidized LDL
PAD4 = peptidylarginine deiminase 4
PAMP = pathogen-associated molecular pattern
PMN = polymorphonuclear leukocyte
RBC = red blood cell
RNS = reactive nitrogen species
ROS = reactive oxygen species
SAE = serious adverse event
SCD = sickle cell disease
SO = sulfite oxidase
SQR = sulfide quinone oxidoreductase
TBARS = thiobarbituric acid reactive substances
TLR = toll-like receptor
TLR-2 = toll-like receptor-2
TLR-4 = toll-like receptor-4
TNF-α = tumor necrosis factor-α
TST = thiosulfate sulfurtransferase
VCAM-1 = vascular cell adhesion molecule-1
VIC = valvular interstitial cell
VSMC = vascular smooth muscle cell