C57BL/6 background and fed with custom diets containing low or high plant sterols. Abcg5−/− and Abcg8−/− mice present hematological parameters comparable with those of wild-type mice when fed a low-sterol diet (<0.01% weight/weight), but develop a profound macrothrombocytopenia and hemolytic anemia, accompanied with prolonged bleeding time, when fed a high-sterol diet (1% weight/weight) for 6 weeks. Thus, the observations confirm that the severity of sitosterolemia and its hematological presentation, Mediterranean stomatocytosis/ macrothrombocytopenia, depends on both genetic and dietary components.

By flow cytometry, using the fluorescent sterol dye filipin and gas chromatography–mass spectrometry, Kanaji et al demonstrate that plant sterols incorporate directly into the platelet membrane of Abcg5−/− mice fed a high-sterol diet. Sterol incorporation has disruptive effects on lipid asymmetry in Abcg8−/− mice fed a high-sterol diet, promoting the generation of platelet-derived microparticles with increased surface-bound fibrinogen despite markedly reduced surface expression of its receptor, the integrin αIIbβ3. Platelets isolated from Abcg8−/− mice fed a high-sterol diet have impaired responsiveness to agonist-induced activation and adhere poorly to von Willebrand factor and type I collagen at high shear rates.

Biochemical analysis shows that platelets isolated from Abcg8−/− mice fed a high-sterol diet have increased activation of the calcium-activated protease calpain, resulting in loss of the cytoskeletal and scaffold protein filamin A (FlaA) and in shedding of the von Willebrand factor receptor GPIbα subunit from the platelet surface. Plasma samples from sitosterolemia patients have increased levels of glycocalcin, the carbohydrate-rich portion of GPIbα that can be cleaved by calpain, corroborating the observations in Abcg5−/− and Abcg8−/− mice. Previous studies have shown that deficiency in the GPIbα-FlaA linkage results in macrothrombocytopenia, poor platelet adhesion, and prolonged bleeding time.

Abcg8−/− mice fed a high-sterol diet have increased numbers of bone marrow megakaryocytes compared with Abcg8−/− mice fed a low-sterol diet, consistent with the previous mouse models. Isolated bone marrow megakaryocytes differentiate normally, but have slightly decreased expression of calpain, GPIbα, and FlnA, suggesting that megakaryocytes, similar to platelets, are activated by sterol accumulation.

In conclusion, the study by Kanaji et al demonstrates that the bleeding abnormalities and macrothrombocytopenia associated with sitosterolemia are due to direct plant sterol incorporation into the platelet membrane, resulting in platelet hyperactivation, reduced αIIbβ3 surface expression, loss of the GPIbα-FlaA linkage, microparticle formation, and ultimately poor hemostatic functions (see figure). Many questions remain unanswered. For example, how does sterol accumulation activate calpain? What are the cellular mechanisms responsible for thrombocytopenia? Sitosterolemia is significantly underdiagnosed because it is influenced by both genetic and dietary components, which is particularly relevant in Western societies that consume a high-sterol diet. Whether the observations by Kanaji et al are relevant to coronary artery disease and atherosclerotic disease remains to be elucidated.

Conflict-of-interest disclosure: The author declares no competing financial interests.

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Comment on Belcher et al, page 2757

HO-1 and CO: fighters vs sickle cell disease?

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In this issue of Blood, Belcher et al report on the use of pegylated hemoglobin saturated with carbon monoxide (CO) to inhibit vaso-occlusive events in mouse models of sickle cell disease (SCD).

It has been more than 60 years since sickle cell anemia (SCA) was first characterized at the molecular level by Linus Pauling. SCA occurs when thymine is substituted for adenine in the 6th codon of the beta-globin gene, resulting in a substitution of the hydrophilic aminoacid glutamic acid by the hydrophobic amino acid valine (Hb S).

SCD includes SCA and the compound heterozygous sickle hemoglobinopathies. It is one of the most common monogenic diseases worldwide, responsible for over 80% of the significant hemoglobinopathies in the world. Remarkable progress has been achieved in the care of individuals with SCD, including the use of hydroxycarbamide (hydroxyurea), yet the only definite therapy remains limited to stem cell transplantation.
and the overall life expectancy is still quite low. Development of new therapies is therefore required.

The work of Belcher et al in this issue explores the therapeutic potential of CO in 2 mouse models of SCD, first shown to reduce the degree of sickling of erythrocytes in a human subject in 1963. The investigators had previously shown that inhaled CO reduced vascular stasis in transgenic sickle mice expressing β^s hemoglobin. They now extend their previous findings by using a pegylated form of hemoglobin (polyethylene glycol-conjugated human Hb) saturated with CO gas, termed MP4CO. Upon administration, MP4CO releases CO that rapidly equilibrates with erythrocyte Hb. MP4CO was shown to be efficacious in limiting vascular stasis induced by hypoxia in NY1DD transgenic sickle mice and in preventing cardiorespiratory collapse induced by hemin in heterozygous HbAS-Townes mouse, models for SCD and SCD trait, respectively.

This work is quite important, not only because it describes a novel therapeutic modality in experimental mice that could have the potential for clinical applications in humans but also because of new insights into the mechanism(s) of action, which may open the window to explore additional therapeutic strategies aimed at different targets. The pathogenesis of SCD is primarily determined by polymerization of Hb S during its deoxygenation, resulting in sickling of the erythrocytes that become rigid, irregularly shaped, and unstable, with subsequent intravascular hemolysis, blood cell adhesion, vaso-occlusion, and hypoxia-reoxygenation (H/R) injury. Reactive oxygen species can be generated at various points, especially during the reoxygenation phase when the hypoxic and Fe^{2+} milieu of MP4CO-induced bene

cial effects are mediated by HO-1 upregulation. These findings are consistent with previous reports from the same group in which HO-1 delivery by the Sleeping Beauty transposon system or administration of bilirubin or CO, HO-1 enzymatic byproducts, resulted in decreased vascular stasis in sickle cell transgenic mice. It is possible, however, that Sn-PP could have induced nonspecific pharmacological effects, collateral to HO-1 inhibition. Therefore, full demonstration that MP4CO-mediated beneficial effects are HO-1 mediated will require the use of HO-1 null mice.

The investigative team also showed that HO-1 induction was likely mediated by activation of the Nrfr2 transcription factor because there were increased levels of Nrfr2 protein in the nucleus. Nrfr2 is regarded as a master regulator of the antioxidant defense, comprising a large number of antioxidant genes and phase 2 detoxifying enzymes, in addition to HO-1. It is possible that Nrfr2 activation may have led to upregulation of various antioxidant genes, in addition to HO-1, that could have contributed to the MP4CO-induced beneficial effects. Although the findings shown by the authors are suggestive of an involvement of Nrfr2, future work with Nrfr2 null mice is required to determine whether MP4CO effects and HO-1 induction were entirely Nrfr2 dependent.

Fully dissecting the involvement and concerted actions of HO-1 and Nrfr2 on MP4CO-induced effects in the vasculature are needed because it has been shown that they could exert opposite actions, beneficial vs deleterious, in other vascular inflammatory processes such as atherosclerosis.

The present work invites the continued exploration of the therapeutic potential of MP4CO, which will need to be carefully titrated to avoid CO toxicity and the dreaded reduction in the capacity of erythrocytes to deliver oxygen to the tissues. In addition, the identification of convergent beneficial actions from Nrfr2, HO-1, and CO into a synchronized axis that feeds onto itself, as the end byproduct (CO) is capable of inducing Nrfr2 activation and HO-1 upregulation, offers an opportunity to design additional therapeutic interventions that can be aimed at the different levels of this axis.

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