Mechanisms of vascular instability in a transgenic mouse model of sickle cell disease

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Nath, K. A., V. Shah, J. J. Haggard, A. J. Croatt, L. A. Smith, R. P. Hebbel, and Z. S. Katusic. Mechanisms of vascular instability in a transgenic mouse model of sickle cell disease. Am J Physiol Regulatory Integrative Comp Physiol 279: R1949–R1955, 2000.—We investigated a transgenic mouse model of sickle cell disease, homozygous for deletion of mouse β-globin and containing transgenes for human βα and βα–globins linked to the transgene for human α-globin. In these mice, basal cGMP production in aortic rings is increased, whereas relaxation to an endothelium-dependent vasodilator, A-23187, is impaired. In contrast, aortic expression of endothelial nitric oxide synthase (NOS) is unaltered in sickle mice, whereas expression of inducible NOS is not detected in either group; plasma nitrate/nitrite concentrations and NOS activity are similar in both groups. Increased cGMP may reflect the stimulatory effect of peroxides (an activator of guanylate cyclase), because lipid peroxidation is increased in aortae and in plasma in sickle mice. Despite increased vascular cGMP levels in sickle mice, conscious systolic blood pressure is comparable to that of aged-matched controls; sickle mice, however, evince a greater rise in systolic blood pressure in response to nitro-L-arginine methyl ester, an inhibitor of NOS. Systemic concentrations of the vasoconstrictive oxidative product 8-isoprostanone are increased in sickle mice. We conclude that vascular responses are altered in this transgenic sickle mouse and are accompanied by increased lipid peroxidation and production of cGMP; we suggest that oxidant-inducible vasoconstrictor systems such as isoprostanes may oppose nitric oxide-dependent and nitric oxide-independent mechanisms of vasodilatation in this transgenic sickle mouse. Destabilization of the vascular balance in the sickle vasculature by clinically relevant states may predispose to vasoocclusive disease.

Vasoocclusive disease underlies chronic organ dysfunction and acute, painful crises that typify sickle cell disease (5, 15, 21). Such vasoocclusive disease, recently highlighted in a transgenic knockout sickle mouse expressing exclusively human sickle hemoglobin (6, 28, 36), involves numerous mechanisms: sickling of red blood cells, enhanced endothelial adhesiveness of red blood cells, abnormal red cell rheology, procoagulant and proinflammatory processes, activation of the endothelium, the release and/or elaboration of vasoactive species, and neurohumoral responses (5, 9, 15, 21). Studies of the microcirculation in rodents using human sickle cells indicate that a key event in the initiation of vasoocclusive disease is the adherence of young, non-dense, sickle cells to the venular endothelium and the subsequent ensnaring of dense, and less pliant, sickle cells; the injured venular endothelium releases vasoconstrictive substances that narrow the distal vasculature; retrograde entrapment of cells then ensues, thereby effecting progressive vascular occlusion (21).

The view that vasoactive processes may contribute to the initiation and/or maintenance of vasoocclusive disease is supported by the following lines of evidence: circulating levels of vasoactive species, such as endothelin and prostanoids, are increased in sickle patients, especially during crisis (11, 13); postocclusive reactive hyperemia is diminished in sickle patients (23); periodic microcirculatory flow, which possibly reflects dysregulation of vascular tone, is described in patients with sickle cell disease (35).

Whether vascular responses are intrinsically altered in sickle cell disease cannot be assessed by the currently available literature. Uncovering the existence of abnormal responses innate to the vasculature in sickle cell disease is not feasible with approaches currently employed either in patients or in extant disease models; in either setting, alterations in hemodynamic parameters that may be construed as reflective of intrinsically abnormal vascular responses may, in fact, include contributions from, or alterations in, “nonvascular” processes such as sickling of red blood cells or other intravascular processes.

To directly examine the vasculature in sickle cell disease, we combined one approach that has afforded new insights in the pathogenesis of sickle cell disease, the use of the transgenic sickle mouse (25), with another approach that has not to date been utilized in these transgenic models, namely, the study of vascular...
rings isolated from such animals (19). This latter technique is more feasible, and thus more commonly utilized, in rats and larger animals; it allows the study of vascular responses to defined agonists and perturbations relevant to the in vivo setting without the confounding effect of sickling and other intravascular processes.

In our studies, we initially focused on the nitric oxide system in light of the dependency on the nitric oxide synthase (NOS) system in preserving tissue perfusion, especially in the presence of vasoconstrictors (12), and the possibility that metabolites of nitric oxide may be altered during sickle crises (34). Additionally, in a transgenic sickle mouse (7), upregulation of endothelial NOS (eNOS) and inducible NOS (iNOS) occurs in the kidney (1), the latter representing an organ prone to the vascular complications of sickle cell disease (37).

We thus performed physiological and biochemical studies of aortae isolated from a transgenic mouse model of sickle cell disease, focusing initially on the nitric oxide system and correlating these findings with vascular responses observed in vivo.

METHODS

Transgenic mouse model of sickle cell disease. This model is homozygous for deletion of mouse β-globin and contains transgenes for human βS and βS-antiles globins linked to the transgene for human α-globin (8). Studies were conducted in aged-matched control and sickle mice comprising similar numbers of male and female mice. Although the various studies of aortic rings, in aggregate, involved mice that ranged in age from 0.5 to 2.0 yr, each study was undertaken in similarly aged control and transgenic mice.

Studies of aortic rings. Experiments were performed on 3-mm-long aortic rings from mice that had been anesthetized with ketamine (50 mg/kg ip) as detailed in our previous studies (19, 20, 44). Rings were studied in modified Krebs-Ringer bicarbonate solution (control solution) of the following composition (mM): 118.3 NaCl, 4.7 KCl, 2.5 CaCl2, 1.2 MgSO4, 1.2 KH2PO4, 25.0 NaHCO3, 0.026 calcium EDTA, and 11.1 glucose. In certain rings, the endothelium was removed mechanically. Each ring was connected to an isometric force transducer and suspended in an organ chamber.

Aortic rings were immersed in control MEM and were incubated for another 30 min in MEM containing indomethacin (10−5 M) and IBMX (10−4 M) to inhibit the production of prostanooids and the degradation of cyclic nucleotides by phosphodiesterases, respectively. Then, all rings were removed from the solution and frozen in liquid nitrogen.

Western analysis. Western analysis was performed as previously described (43) by use of antibodies against eNOS (monoclonal eNOS antibody; catalog no. N30020, Transduction Laboratories, Lexington, KY) and iNOS (polyclonal rabbit iNOS antibody; catalog no. PA3–030A, Affinity Bioreagents, Golden, CO).

Plasma concentrations of nitrate/nitrite. These measurements were performed using the Nitrate/Nitrite Colorimetric Assay Kit (Cayman Chemical, Ann Arbor, MI).

NO activity. To provide sufficient tissue, 16 control and 16 transgenic mice were utilized in which the aortae from 4 mice from either group were separately pooled. The conversion of 3H-labeled L-arginine to 3H-labeled L-citrulline was used to determine NO activity, as previously described (38). Aortic tissue was homogenized in a lysis buffer, and samples were incubated with a buffer containing 1 mM NADPH, 3 mM tetrahydrobiopterin (BH4), 100 nM calmodulin, 2.5 mM CaCl2, 50 mM L-valine, 10 mM L-arginine, and 0.2 mM L-[3H]arginine at 37°C. To determine NO activity, duplicate samples were incubated in the presence and absence of nitro-L-arginine methyl ester (L-NAME; 1 mM) or vehicle for 20 min. The reaction was terminated by a cold stop buffer, and the reaction mixture was passed over a column containing Dowex AG 50Wx-8 resin into a vial and analyzed using a liquid scintillation counter. Radiolabeled counts per minute of L-citrulline generated were measured.

Lipid peroxidation. Lipid peroxidation was determined using the thiobarbituric acid assay, or TBARS, as previously described (26).

Systolic blood pressure. Systolic blood pressure was determined in awake, restrained mice using the Mouse Tail Blood Pressure System (Kent Scientific, Litchfield, CT). In additional groups of mice, systolic blood pressure was determined before and 5 h after L-NAME (50 mg/kg body wt ip) (10).

Plasma concentrations of 8-isoprostane. The 8-Isoprostane Enzyme Immunoassay Kit (Cayman Chemical) was employed in the measurement of plasma concentrations of total and free 8-isoprostane.

Statistics. Results are presented as means ± SE. For statistical analyses, the Student t-test or the Mann-Whitney test was used, as appropriate. Results are considered significant for P < 0.05.

RESULTS

Because of previous evidence indicating induction of eNOS and iNOS in the kidney in a transgenic sickle mouse (1, 7), we first examined whether the second messenger of nitric oxide, cGMP, is increased in aortic rings. As demonstrated in Fig. 1, cGMP in sickle mice
was increased approximately threefold.

Such increments in cGMP led us to determine whether alterations in vascular behavior, specifically endothelium-dependent relaxation, also exist in this model. We studied vascular responses in aortic rings in vitro. Relaxation was studied in response to A-23187, a calcium ionophore that stimulates endothelial generation of nitric oxide, and in response to the nitric oxide donor DEA-NONOate; such studies were undertaken in intact aortic rings and after endothelial denudation of aortic rings. Percent relaxation of aortic rings from sickle mice, compared with control rings, was blunted in response to A-23187, and significantly so at concentrations of 10^{-6} M and 3 \times 10^{-6} M (Fig. 2). These differential effects in response to A-23187 in control and sickle aortic rings were not observed in aortic rings denuded of endothelium (data not shown).

A-23187 may exert other effects besides those related to endothelial generation of nitric oxide; we thus utilized an additional approach that employed the nitric oxide-generating agent DEA-NONOate. The relaxation response to the nitric oxide donor DEA-NONOate was also blunted: percent relaxation of rings from sickle mice compared with control rings was significantly lower in response to DEA-NONOate, at 10^{-5} M and 3 \times 10^{-9} M (Fig. 3, left). These differences were not apparent when aortic rings were studied after the denudation of the endothelium (Fig. 3, right).

The enhanced generation of cGMP by aortic rings from sickle mice in conjunction with a blunted vascular response to endothelium-dependent and nitric oxide-dependent vasodilators led us to examine expression of NOS. Expression of eNOS was not significantly altered in sickle mice (Fig. 4); we did not detect expression of iNOS in either group (Fig. 4). Indirect measures such as plasma nitrate/nitrite were comparable in control and sickle mice [27.7 \pm 3.4 vs. 32.8 \pm 2.9 \mu M, n = 6 in each group, P = nonsignificant (NS)]. NOS activity, on the basis of the conversion of arginine to citrulline, was not significantly different in control and sickle mice (74 \pm 14 vs. 72 \pm 7 pmol-mg protein^{-1}.min^{-1}, n = 4 in each group, P = NS).

The comparability in NOS activity in conjunction with increased cGMP content led us to consider mechanisms responsible for such effects. Because peroxides can stimulate guanylate cyclase (2, 42, 45), we measured peroxide content in plasma and aortic tissues in sickle mice. As demonstrated, plasma levels of lipid peroxides (Fig. 5, left) and aortic content of lipid peroxides (Fig. 5, right) were increased in sickle mice.

Because sickle mice exhibit increased vascular production of cGMP, we measured systolic blood pressure so as to determine the net effect on systemic hemodynamics. As shown in Fig. 6, left, systolic blood pressures measured in conscious mice were not significantly different in control and sickle mice. In additional studies, L-NAME was administered to sickle and control mice in vivo; sickle mice evinced a greater increment in systolic blood pressure compared with controls (Fig. 6, right).

These findings underscore the vasorelaxant contribution of nitric oxide in the maintenance of systemic blood pressure in the basal state in sickle mice.

We considered whether 8-isoprostane, a potent, oxidant-inducible vasoconstrictor species (32), is increased in sickle mice. We thus measured plasma concentrations of total and free 8-isoprostane. Although plasma concentrations of total 8-isoprostane were not significantly altered (345.3 \pm 20.3 vs. 390.1 \pm 20.7 pg/ml, n = 6 in each group, P = NS), plasma concentrations of free 8-isoprostane were significantly increased in sickle mice.

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**Fig. 2.** %Relaxation response to A-23187 in control and sickle aortic rings with endothelium. Data are expressed as % of maximal relaxation induced by papaverine [3 \times 10^{-4} M; 100% = 1.70 \pm 0.24 g (n = 6) and 1.14 \pm 0.35 g (n = 6) for control and sickle aortic rings, respectively; these papaverine-related effects are not significantly different between control and sickle mice]. The – symbols on the y-axis = relaxation, + on the y-axis = contraction. *P < 0.005.

**Fig. 3.** %Relaxation response to DEA NONOate in control and sickle aortic rings with (left) or without (right) endothelium. Data are expressed as % of maximal relaxation induced by papaverine [3 \times 10^{-4} M]. At left, 100% = 0.86 \pm 0.16 g (n = 7) and 1.39 \pm 0.21 g (n = 7) for control rings and sickle aortic rings, respectively; these papaverine-related effects are not significantly different between control and sickle mice. At right, 100% = 0.48 \pm 0.07 g (n = 7) and 0.62 \pm 0.14 g (n = 7) for control and sickle aortic rings, respectively; these papaverine-related effects are not significantly different between control and sickle mice. For both panels, – on the y-axis = relaxation; *P < 0.005.
mice (142.5 ± 5.9 vs. 172.8 ± 9.7 pg/ml, n = 6 in each group, P < 0.05).

DISCUSSION

The blunted responses to an endothelium-dependent vasodilator (A-23187) and to a nitric oxide donor (DEA-NONOate) in conjunction with increased vascular levels of cGMP, as observed in our study, are consistent with increased production of nitric oxide. For example, such findings occur in mice that overexpress eNOS (27). Alternatively, such findings may arise in states in which nitric oxide-independent stimulators of guanylate cyclase are present in increased amounts. To differentiate between these possibilities, we examined expression of vascular NOS. Expression of eNOS was unchanged, while in neither group did we observe expression of iNOS. Activity of NOS was unaltered in the vasculature of sickle mice. Thus the increased amounts of cGMP generated by aortic tissue and the blunted response to NO-dependent vasodilators do not reflect upregulation of NOS.

The lack of upregulation of NOS in the sickle vasculature, as described in the present study, contrasts with the upregulation of NOS observed in the kidney in another transgenic sickle mouse model (1). In this latter study, increased expression of eNOS is described in the proximal tubules of sickle mice, and increased expression of iNOS is recognized in the glomeruli and distal tubules of sickle mice; notably, however, the intrarenal vasculature does not exhibit increased expression of either eNOS or iNOS (1). The reasons for these differences with regard to NOS expression are uncertain at the present time, but such considerations as tissue-specific effects (vasculature vs. renal tubules/glomeruli), or genetic differences in these two different transgenic sickle mouse models, may be contributing factors.

Besides nitric oxide, other inducers of guanylate cyclase include peroxides. In this regard, our findings that there are increased amounts of peroxides in plasma and the aortic wall in sickle mice, in the absence of upregulation of NOS, raise the possibility that peroxides may provide the stimulus for guanylate cyclase. In several systems, peroxides stimulate guanylate cyclase activity (2, 42, 45), and the latter is incriminated in the vasodilatation response induced by peroxides in such states (2, 42, 45). Divergent effects of peroxides and oxidant stress on NOS have been described (22, 33). However, in this transgenic sickle model, increased amounts of peroxides in the aortic wall do not influence NOS expression or activity. We suggest that the increased production of cGMP in aortic rings may reflect increased peroxidation in the sickle vasculature. We also point out that other stimuli of guanylate cyclase (e.g., heme oxygenase/carbon monoxide, natriuretic peptides) may contribute to increased production of cGMP in sickle cell disease; examining these other inducers of guanylate cyclase would be of interest in this transgenic model.

The comparability of systolic blood pressure in control and sickle mice in the presence of an upregulated vasodilator species (cGMP) raises the possibility of concomitant expression of vasoconstrictor systems. Relevant to this consideration is the greater increment in systolic blood pressure when an inhibitor of NOS, L-NAME, is administered: by removing, with L-NAME, the vasodilatory component provided by nitric oxide, any preexisting upregulation in vasoconstrictor systems would be unmasked; and, indeed, a fivefold greater rise in systolic blood pressure was observed. The disappearance of the blunted relaxation response to nitric oxide-generating vasodilators after the mechanical removal of the endothelium in isolated arterioles suggests that the endothelium may be the origin of these vasoconstrictor species. Thus it is possible that, in this sickle model, upregulation of a vasodilator system (cGMP) may be accompanied by upregulation in vasoconstrictor systems.

Several vasoconstrictor species may be induced in sickle cell disease, and notably, each of them can be
upregulated by peroxides. Plasma concentrations of endothelin and thromboxanes are increased in sickle patients (11, 13), and the exposure of endothelial cells to sickle erythrocytes increases expression of endothelin-1 (30). Interestingly, oxidative stress per se induces expression of endothelin-1 (18) and the synthesis of thromboxanes (19). In our study we considered another possibility, namely, isoprostanes. These vasoconstrictive oxidant-generated prostanooids are increased in states of oxidative stress attended by altered vascular reactivity (32). Plasma concentrations of free 8-isoprostane are increased in sickle mice, thus raising the possibility that these species may counterbalance the effects of increased amounts of cGMP.

Studies of cardiac output and the systemic hemodynamic profile are of particular interest in the transgenic sickle mouse examined in our study. Patients with sickle cell disease exhibit increased cardiac output and decreased vascular resistances (3). An elevation in cardiac output in this transgenic sickle mouse, in the presence of unchanged mean arterial pressure, would reflect decreased total peripheral resistances. Such reduction in vascular resistances in this sickle mouse would underscore the dominant vasorelaxant effects of NO-dependent mechanisms, peroxides, and other vasodilatory systems in the basal state.

In sickle populations, systemic blood pressures are commonly reduced, and renal plasma flow rates are often increased, before the development of progressive renal disease (29, 31, 37). These hemodynamic findings may also reflect the vasorelaxant effects of NO-dependent mechanisms, peroxides, and other vasodilatory systems. Variation in blood pressures, however, occurs within sickle populations, some patients exhibiting normal rather than low blood pressures; it is possible that the transgenic sickle mouse that we studied may be more representative of this latter subset of patients.

Several mechanisms may account for oxidative stress in sickle cell disease. Sickle erythrocytes may release sickle hemoglobin (14), the latter representing an unstable heme protein that may foster heme-dependent oxidative stress (14). Sickle erythrocytes may directly generate reactive oxygen species (14, 24) and/or stimulate such generation from endothelial cells (41); additionally, increased hemodynamic stress imposed upon the endothelium, because of the abnormal rheology in sickle cell disease (5), may stimulate endothelial production of oxidants (17). Oxidants may also originate in leukocytes, because stimulated leukocytes from sickle patients release more superoxide anion than similarly treated cells from healthy individuals (4); additionally, sickle erythrocytes adhere to neutrophils and activate the respiratory burst (16).

The increased oxidative stress we observed in the vasculature is relevant to an emerging view that the endothelium is activated in sickle cell disease (39, 40). Sickle patients, irrespective of clinical status, exhibit increased numbers of activated circulating endothelial cells (40); activation of these cells is indicated by increased expression of ICAM-1, VCAM-1, E-selectin, and P-selectin (40). Endothelial activation, in conjunction with increased expression of tissue factor (39), may promote occlusive and nonocclusive vascular disease. Interestingly, the expression of ICAM-1, VCAM-1, E-selectin, P-selectin, and tissue factor can all be upregulated by oxidative stress. Redox alterations in the sickle vasculature may contribute to the activated and procoagulant phenotype of the endothelium, a phenotype implicated in the pathogenesis of crisis syndromes and chronic vasculopathy observed in this disorder.

**Perspectives**

On the basis of studies in a transgenic sickle mouse, we provide evidence of altered vascular responses and the presence of oxidative stress in the sickle vasculature. We suggest that the concomitant upregulation of opposing vasoactive systems imparts an inherent vascular instability in sickle cell disease. For example, in microcirculatory beds in which red cell transit may be delayed, this increased availability of hemoglobin in congegated red cells may draw nitric oxide away from the vasculature, thereby leaving vasoconstictors relatively unopposed; additionally, precipitants of sickle crisis (for example, hypoxia and sepsis) and/or sickling of erythrocytes may further upregulate such vasoconstictors as endothelin, thromboxanes, and isoprostanes through oxidant and nonoxidant pathways, again tilting the vasoactive balance decidedly toward vasoconstriction. That increased amounts of vasoconstictors lurk constantly in the sickle vasculature and are only held in check by commensurate upregulation in vasodilators raises the possibility that, in relevant clinical settings, this tenuous vasoactive balance may be destabilized, thereby contributing to the initiation and/or maintenance of vasooclusive disease. Finally, we suggest that redox alterations in the sickle vasculature may contribute to the activated and procoagu-
lant phenotype of the endothelium, a phenotype recently recognized in this disorder and implicated in the pathogenesis of sickle crisis syndromes and chronic vasculopathy.

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