Endothelial cell nitric oxide production in acute chest syndrome

SAMUEL I. HAMMERMAN,1 ELIZABETH S. KLINGS,1 KATHERINE P. HENDRA,1 GILBERT R. UPCURCH, J. R.,2 DAVID C. RISHIKOF,1 JOSEPH LOSCALZO,2 AND HARRISON W. FARBER1

1Pulmonary Center, 2Whitaker Cardiovascular Institute and Evans Department of Medicine, Boston University School of Medicine, Boston, Massachusetts 02118

Hammerton, Samuel I., Elizabeth S. Klings, Katherine P. Hendra, Gilbert R. Upchurch, Jr., David C. Rishikof, Joseph Loscalzo, and Harrison W. Farber. Endothelial cell nitric oxide production in acute chest syndrome. Am. J. Physiol. 277 (Heart Circ. Physiol. 46): H1579–H1592, 1999.—Acute chest syndrome (ACS) is the most common form of acute pulmonary disease associated with sickle cell disease. To investigate the possibility that alterations in endothelial cell (EC) production and metabolism of nitric oxide (NO) products might be contributory, we measured NO products from cultured pulmonary EC exposed to red blood cells and/or plasma from sickle cell patients during crisis. Exposure to plasma from patients with ACS caused a 5- to 10-fold increase in S-nitrosothiol (RSNO) and a 7- to 14-fold increase in total nitrogen oxide (NOx) production by both pulmonary arterial and microvascular EC. Increases occurred within 2 h of exposure to plasma in a concentration-dependent manner and were associated with increases in endothelial nitric oxide synthase (eNOS) protein and eNOS enzymatic activity, but not with changes in nitric oxide synthase (NOS) III or NOS II transcripts, inducible NOS (iNOS) protein nor iNOS enzymatic activity. RSNO and NOx, when measured, were not increased whether plasma was obtained from patients with ACS or other forms of vasoocclusive crisis. Furthermore, an oxidative state occurred and oxidative metabolites of NO, particularly peroxynitrite, were produced. These findings suggest that altered NO production and metabolism to damaging oxidative molecules contribute to the pathogenesis of ACS.

endothelium; endothelium-derived; sickle hemoglobinopathy; peroxynitrite; nitrogen oxide

Thus vasoocclusive processes are believed to account for much of the clinical picture. Several factors that may contribute to these vasoocclusive processes have been identified: 1) decreased red blood cell (RBC) deformability during sickling; 2) elevated blood viscosity secondary to poorly deformable and irreversibly sickled RBC; 3) bone marrow/myeloid emboli; and 4) increased adherence between sickled RBC and endothelial cells (EC) (12, 22, 27, 51, 64). Further definition and understanding of these and additional mechanisms involved in ACS are important because recurrent episodes not only develop in 20–80% of patients but also are the major risk for development of pulmonary hypertension, cor pulmonale and eventually death (5, 31, 49, 51).

In actuality, vasoocclusive crises (VOC) in SCD, such as ACS, are likely multifactorial and involve other factors not yet identified. Besides alterations in adherent properties of sickled erythrocytes and/or EC, alterations in EC metabolic functions could contribute to these vasoocclusive events or ACS. For example, plasma levels of endothelin-1 (ET-1), the most potent vasoconstrictor produced by the endothelium, are elevated in patients with SCD, especially during vasoocclusive events (19, 39). In addition, we have demonstrated that EC ET-1 mRNA and protein expression in vitro after exposure to plasma obtained serially from patients with SCD and plasma ET-1 levels in vivo correlate with stage of disease (19). Likewise, exposure of human umbilical vein EC to sickle RBC sickled in vitro induces ET-1 transcripts (48). Together, these studies suggest that increases in ET-1 levels contribute to the development of the clinical entities that complicate SCD.

Another important endothelium-derived molecule that might contribute to acute and chronic pulmonary complications of SCD is nitric oxide (NO). Aside from its role as a major EC product evoking smooth muscle relaxation and vasodilation, NO is also a potent inhibitor of platelet aggregation and adhesion and an inhibitor of smooth muscle and fibroblast migration and proliferation (15, 52). Several recent studies suggest that production and/or actions of NO may be altered in SCD (14, 23, 43, 53). To investigate EC metabolism of NO in SCD and the potential role of NO in the pulmonary complications of SCD, we measured NO products in supernatants of large vessel and microvascular pulmonary EC exposed to RBC and/or plasma of sickle cell patients in crisis. On the basis of the vasoocclusion associated with sickle cell crisis and increased ET-1 levels reported in several models of VOC, we expected NO production in this ex vivo model.
of ACS to be decreased. To our surprise, however, exposure to plasma from patients with ACS caused a marked increase in RSNO and total nitrogen oxide (NOx) production by both main pulmonary artery and pulmonary microvascular EC. Further examination of NOx products generated under these conditions and examination of the redox state of EC after exposure to plasma from patients with ACS suggested that altered NO production and its metabolism to potentially damaging oxidative molecules may contribute to the pathogenesis of vasoocclusive events, such as ACS, which are associated with SCD.

METHODS

Cell cultures. Bovine pulmonary artery EC (BPAEC) were cultured in MEM containing 15% heat inactivated bovine calf serum (16, 62). Purity was verified by “cobblestone” appearance and labeling with fluorescent acetylated low-density lipoprotein. Human pulmonary microvascular EC (HMVEC-L) were obtained from Clonetics (San Diego, CA) and cultured according to the manufacturer’s directions. Purity was verified by presence of factor VIII-antigen and labeling with fluorescent acetylated low-density lipoprotein. For experiments, BPAEC or HMVEC-L from several different primary cell lines (passages 3–5) were subcultured enzymatically (trypsin/EDTA) onto 35-mm dishes (Costar, Boston, MA).

RBC and plasma. Peripheral blood samples were obtained under a protocol approved by the Boston City Hospital (now Boston Medical Center) Human Studies Committee from sickle cell patients during their hospitalization for either ACS (fever, chest pain, infiltrates on chest roentgenogram, hypoxemia) or nonulng VOC (nonpulmonary pain crisis) and/or at steady state during clinic visits. All patients admitted with ACS or VOC were eligible for the study and were interviewed by one of the authors; refusal to have blood drawn was the only criterion for exclusion from the study and occurred only once during the study period. A total of 20 different individuals provided samples for the current study (characteristics in Table 1). Samples from 15 consecutive patients with ACS were obtained before exchange transfusion within 6 h of admission to the Medical or Pediatric Intensive Care Unit and immediately after completion of the exchange; samples from five patients with VOC were obtained within 24 h of hospitalization; one individual with VOC refused inclusion in the study. Steady-state samples were obtained, in most cases, from the same individual recruited during hospitalization at clinic visits 3–6 mo after discharge. Samples were collected in heparinized syringes, placed on ice, and centrifuged at 2,000 g for 15 min. Platelet-poor plasma was prepared by centrifugation at 20,000 g for 20 min. Peripheral blood collected in a similar fashion from normal volunteers served as control (Table 1). Aliquots of plasma were preserved at −70°C until use.

Experimental protocol. BPAEC grown to confluence in 35-mm dishes were incubated with MEM, normal RBC, sickle RBC, or RBC ghosts at a final concentration equivalent to a hematocrit of 20% or with autologous plasma for up to 24 h. BPAEC were also exposed to plasma (with or without RBC) at a concentration of 10% in MEM for various periods up to 24 h or to plasma of varying concentrations for 20 h. HMVEC-L were incubated with either normal or sickle RBC suspended in EC growth medium (EGM) at a hematocrit of 20% or with autologous plasma diluted to 10% in EGM for a period of 24 h.

Preparation of ghosts. Hemolysate-free RBC ghosts were prepared by hypotonic lysis as previously described (7). One volume of normal or sickle RBC was added to thirty volumes of 3P buffer [3 mmol/l PBS, 1 mmol/l EDTA, 100 mmol/l phenylmethylsulfonyl fluoride (PMSF), pH 8.0]. After mixing was completed, the preparation was centrifuged at 22,000 g for 15 min at 4°C, the supernatant was removed, and the pellet was resuspended in 3P buffer. Mixing and centrifugation were repeated until absorption of supernatant at 385, 405, 560, 577, and 630 nm was < 0.01 optical density units.

Measurement of RSNO and free NO in media. Production of NOx (RSNO and free NO) was measured by photolysis-chromiluminescence with S-nitroso-glutathione as reference standard (57, 59, 65). Total NOx content (nitrite + RSNO) was determined by the Saville method (55).

Northern analysis. Total cellular RNA was extracted following the various experimental protocols by TriReagent (MRC, Cincinnati, OH) as described (29). Endothelial nitric oxide synthase III (NOS III) mRNA was detected using a cDNA insert from a bovine done provided by Drs. Thomas Michel and Santiago Lamas (accession no. M89952). NOS III mRNA was measured using a rat NOS II cDNA-containing plasmid prepared by polymerase chain reaction as previously reported (63). An insert of Kpn I-BamH I restriction fragment of the rat NOS II-containing plasmid (provided by Dr. Robert A. Star) was used as the cDNA probe for Northern analysis. Equal loading was assured using a mouse β-actin probe (gift of Dr. Alan Fine). Gels were exposed on Kodak X-OMAT film for 48 h at −70°C and densitometry performed using a computing densitometer (Molecular Dynamics, Sunnyvale, CA).

Western blot analysis. EC, exposed to control media, RBC and/or plasma for 20 h, were harvested by scraping. After centrifugation, the cells were frozen at −70°C overnight. After thawing, the cell pellet was homogenized in buffer containing 0.32 mmol/l sucrose, 20 mmol/l HEPES, 0.5 mmol/l EDTA, 1 mmol/l dithiothreitol, 2.0 µmol/l leupeptin, 1.0 µmol/l pepstatin A and 1.0 µmol/l PMSF. Insoluble material was sedimented at 1000 g for 10 min at 2°C and the protein concentration of each supernatant was determined. After boiling for 2 min, samples (2.5 µg of protein) were loaded onto a 4% stacking/7.5% separating gel and electrophoresed at constant current overnight at 4°C. After transfer, blots were placed in 5% milk protein solution to block nonspecific binding. Blots were exposed to a human monoclonal antibody to endothelial NOS (eNOS) (1:1,500) or a human monoclonal antibody to inducible NOS (iNOS) (1:750) (Transduction Laboratories, Lexington, KY); eNOS from human EC (1.0 µg) or iNOS from human macrophages (1.0 µg) (Transduction Laboratories) served as control. The ECL Western blot analysis system (Amersham Life System, Amersham, England) was used to detect protein bands. Densitometry was performed using a computeraided densitometer (Molecular Dynamics, Sunnyvale, CA).

Table 1. Demographics of sickle cell patients and normal volunteers

<table>
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<tr>
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<th>ACS</th>
<th>VOC</th>
<th>Normal Volunteers</th>
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<tr>
<td>n</td>
<td>15</td>
<td>5</td>
<td>6</td>
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<tr>
<td>Age range, yr</td>
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<td>Sex (male:female)</td>
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<td>Folate</td>
<td>Folate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxygen</td>
<td>Oxygen</td>
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ACS, acute chest syndrome; VOC, vasoocclusive crisis. n, No. of patients. All ACS or VOC patients received analgesics (oxycodone, morphine, or meperidine), folate, and supplemental oxygen; all ACS patients received antibiotics (ceftriaxone ± macrolide).
UK) was used for detection. Immunoreactivity of NOS was determined by the conversion of L-[3H]arginine to L-[3H]citrulline in the presence of saturating cofactors as described (3). BPAEC were exposed to plasma at a 1:10 dilution for 20 h, washed twice, detached from the culture plate, centrifuged at 1,000 g for 10 min, and frozen overnight at −70°C. Cells were thawed and homogenized in the presence of dithiothreitol and protease inhibitors in buffer containing 0.32 mM sucrose, 0.5 mM EDTA and 20 mM HEPES, pH 7.0. The homogenate was centrifuged at 1,000 g for 20 min at 20°C and the soluble fraction added to a solution containing 2.0 mM NADPH, 0.05 mM L-arginine, 0.03 mM tetrahydrobiopterin, 1 mM CaCl2, 30 U/ml calmodulin, and 2.25 µCi/ml 2,3,4,5-L-[3H]arginine. The mixture was incubated for 60 min at 37°C and the reaction stopped using 1.5 ml of cold buffer (2 mM EDTA, 5 mM HEPES, pH 5.5). Samples were passed over a Na+ exchange column (Dowex AG50W-X8), and radioactivity of eluate determined using liquid scintillation spectrometry; eNOS activity was expressed as picomoles of L-citrulline per milligram cell protein per minute. For measurement of iNOS activity, calmodulin and CaCl2 were omitted from the same protocol.

Measurement of cellular thios. Total cellular thios were measured in EC exposed to 20% plasma from sickle cell patients or normal volunteers in MEM for 24 h using a modified Ellman assay (59). Briefly, cell lysates were suspended in PBS and 5,5-dithiobis(2-nitrobenzoic acid) (DTNB) was added to a final concentration of 0.1 mM. The increase in absorbance at 412 nm was followed until maximal absorbance was achieved. Free thiol concentration was calculated from the molar extinction coefficient of the nitrobenzoate ion (13,600/M/cm). All values were standardized for total protein content.

Measurement of cellular GSH. Cellular GSH was measured according to the method of Akerboom and Sies (1) modified for EC (6). After exposure to 20% plasma from sickle cell patients or normal volunteers, EC were sonicated in 0.05% perchloric acid and the supernatant stored at −20°C until evaluation. The lysates were thawed, the pH was adjusted to 7.0, 1.5 mg/ml DTNB was added, and the absorbance was measured at 412 nm. Known concentrations of GSH were used to generate a standard curve and results normalized to cell counts or total protein content.

Peroxynitrite production (nitrotyrosine residues). Peroxynitrite (ONOO−) formation was assessed by the presence of the stable end product of its interaction with cellular tyrosine residues, 3-nitrotyrosine, per the manufacturer’s protocol (Upstate Biotechnology). BPAEC were exposed to plasma from normal volunteers, patients with ACS preexchange transfusion or patients with ACS postexchange transfusion for 20 h and then fixed with ethanol-acetic acid (95:5). After a 30-min incubation with the blocking agent 8% BSA, the cells were incubated overnight at 4°C with a polyclonal rabbit anti-nitrotyrosine antibody (Upstate Biotechnology) at 10 µg/ml in 1% BSA followed by a goat anti-rabbit IgG antibody coupled to 3,3’-diaminobenzidine (DAB)-peroxidase (Vector Biotech). Presence of nitrotyrosine was detected by DAB staining and was compared with staining after incubation of BPAEC with normal plasma alone or normal plasma plus exogenous ONOO−. To determine if the presence of nitrotyrosine was dependent on NOS activity, BPAEC were incubated with appropriate plasma samples plus the NOS inhibitor Nω-monomethyl-L-arginine (L-NMMA; 0.27–2.70 mM). To determine further the specificity of this reaction, additional experiments used the same concentrations of the inactive isomer d-NMMA (Calbiochem).

To confirm the oxidative state of the plasma, BPAEC were exposed to plasma from normal volunteers or plasma from patients with ACS with or without addition of diethylamine NONOate (1 µM), a known NO donor for 1 min, 10 min, or 60 min and ONOO− production assessed as above.

Physicochemical characterization of the plasma factors. Organic extraction of plasma was achieved by combining 4.5 ml of plasma with 5.5 ml of chloroform:methanol (2:1) (9, 10). The organic (lipid) and aqueous (protein) phases were separated; each fraction was dried under a nitrogen stream and reconstituted in 1 ml MEM. Two milliliters of experimental media (1% BCS in MEM) were added to each 1-ml sample; the resultant solution was incubated with confluent BPAEC for 24 h. At that time, supernatants were collected and assayed for total NOx production. Heat stability was determined by heating the aqueous or organic fractions to 56°C for 30 min or to 100°C for 3 min. Samples were then incubated with confluent BPAEC for 24 h; at that point supernatants were collected and assayed for total NOx production.

Data. Each experiment was performed 3–12 times, and data were expressed as means ± SE. In some cases, representative examples of at least three experiments are presented; the results were qualitatively similar and reproducible in all individuals examined and independent of passage number or primary cell line of EC used. In most cases Student’s t-test or one-way ANOVA, followed by the Student-Newman-Keuls multiple comparison test, were used to compare means. In the thiol and GSH experiments, means were computed for each group of values in each experiment and results compared by a Wilcoxon matched pairs signed-ranks test. Differences were considered significant at P < 0.05.

RESULTS

RSNO and total NOx production in cultured EC. Compared with plasma from normal volunteers, plasma from patients with ACS or VOC caused a striking increase in RSNO production from either BPAEC (Fig. 1) or HMVEC-L (Fig. 2). Likewise, total NOx production increased markedly in both BPAEC (Fig. 1) and HMVEC-L (Fig. 2) during exposure to plasma from patients with ACS or VOC. This increase in RSNO and total NOx occurred only after exposure to plasma from patients in crisis; exposure to plasma from patients who were clinically stable (outpatient clinic) caused no such increase in HMVEC-L (Fig. 2). Increases in RSNO and total NOx from BPAEC appeared at all time points examined (2–24 h; Fig. 3) and occurred in a plasma concentration-dependent manner (Fig. 4); similar results were found when HMVEC-L were the target cell in the assay (data not shown). Likewise, similar increases in RSNO and total NOx production were observed whether plasma was obtained from patients with ACS or VOC (data not shown). When RBC were included, either alone or with plasma, there was no increase in RSNO or total NOx production. In comparison to RBC from normal volunteers, however, a greater amount of RSNO or total NOx was measured in every experiment after exposure to sickle RBC whether they were obtained from patients in steady-state or from patients with ACS or VOC (Figs. 1 and 2; statistical significance was not reached in Fig. 1A). When RBC
ghosts were substituted for RBC in these experiments, there was no increase in BPAEC RSNO or total NOx production under any of these conditions; however, the difference in RSNO or total NOx between RBC from normal volunteers and sickle patients noted above was abolished (data not shown).

NOS III and NOS II transcripts in cultured EC. To investigate the mechanism of the increase in RSNO or total NOx production under any of these conditions; however, the difference in RSNO or total NOx between RBC from normal volunteers and sickle patients noted above was abolished (data not shown).

NOS III and NOS II transcripts in cultured EC. To investigate the mechanism of the increase in RSNO or total NOx, we evaluated NOS III expression after exposure to RBC and/or plasma from normal volunteers and sickle cell patients during crisis (Fig. 5). Plasma from normal volunteers did not alter NOS III transcripts. Despite marked increases in RSNO production, no change in steady-state NOS III mRNA was found in BPAEC exposed to any concentration of plasma from patients with ACS or VOC at any time point examined (Fig. 5, A and B). In contrast to experiments with plasma, exposure to RBC from normal volunteers upregulated NOS III mRNA (Fig. 5C). Exposure to RBC from crisis patients also increased NOS III expression; however, in each experiment this increase was less than that observed with RBC from normal volunteers. Under the same experimental conditions, NOS II transcripts in the same or different blots were undetectable (data not shown).

eNOS and iNOS protein expression in cultured EC. Compared with normal volunteers, eNOS protein was twofold higher in BPAEC exposed to plasma from patients with ACS (Fig. 6). In contrast, under the same experimental conditions, iNOS protein was undetectable (data not shown).

eNOS and iNOS enzymatic activity in cultured EC. Compared with normal volunteers, eNOS enzymatic activity was almost 50% higher in BPAEC exposed to plasma from patients with ACS (Fig. 7). In contrast, under the same experimental conditions, iNOS enzymatic activity was undetectable (data not shown).

Total cellular thiols in cultured EC. Total cellular thiols were measured as a reflection of the redox state of EC and thus the oxidant state of the plasma to which they were exposed under the various experimental conditions (Table 2). There was no difference in thiols in BPAEC exposed to plasma from normal volunteers or from sickle patients at baseline. In contrast, exposure
to plasma from patients with ACS or VOC caused a significant decrease in BPAEC thiols (43%); however, there was no significant difference whether the plasma was obtained from patients with ACS or VOC.

GSH in cultured EC. As a further reflection of the redox state of EC and the oxidant state of the plasma to which they were exposed, EC GSH was measured under the various experimental conditions (Table 2). Compared with plasma from normal volunteers, exposure to plasma from sickle patients at baseline caused a decrease in BPAEC GSH (61%). There was an even more dramatic decrease in BPAEC GSH exposed to plasma from patients with ACS or VOC (425%); again, there was no significant difference whether plasma was obtained from patients with ACS or VOC.

ONOO⁻ production (nitrotyrosine residues). Through detection of nitrotyrosine residues, we found evidence of substantial ONOO⁻ production after exposure of BPAEC to plasma from five different patients with ACS (Fig. 8); plasma from patients with VOC was not tested in these experiments. BPAEC exposed to plasma from normal volunteers did not contain significant nitrotyrosine residues, whereas normal plasma plus exogenous ONOO⁻ caused marked DAB staining. Plasma from patients with ACS caused DAB staining of similar intensity as normal plasma plus exogenous ONOO⁻. Of interest, there was no difference in the intensity of DAB staining whether the plasma used in the experiments was obtained pre- or postexchange transfusion. ONOO⁻ production (nitrotyrosine residues) was completely blocked by incubation of BPAEC with all concentrations of L-NMMA tested (0.27–2.70 mM); however, it was unaffected by incubation with the same concentrations of the inactive isomer D-NMMA (Fig. 8).

Incubation of BPAEC with plasma from patients with ACS plus 1 µM diethylamine NONOate for 60 min
resulted in significant production of ONOO⁻ (nitrotyrosine residues), whereas incubation of BPAEC with plasma from normal volunteers plus NONOate for 60 min caused no such production (Fig. 9). Likewise, incubation of BPAEC with ACS plasma alone (without NONOate) for 60 min caused no increase in nitrotyrosine residues confirming experiments demonstrating that the increase in NOx products required a 2-h incubation with ACS plasma. Shorter incubations of BPAEC (1 min or 10 min) with ACS plasma plus NONOate did not significantly increase production of ONOO⁻ (data not shown).

Physicochemical characterization of the plasma factor(s). Compared with the organic (lipid) and aqueous (protein) phases of plasma from normal volunteers, both the aqueous and organic phases of plasma from patients with ACS markedly increased NOx production by BPAEC; the degree of stimulation of NOx production by each fraction was approximately equal (Table 3). The active factors in both the organic and aqueous phases of plasma from patients with ACS were heat stable whether exposed to 56°C for 30 min or to 100°C for 3 min (Table 3).

**DISCUSSION**

In the current study, we investigated whether NO and/or its metabolites might play a role in ACS by measuring NO products in supernatants of BPAEC or HMVEC-L exposed to the RBC and/or plasma from sickle cell patients in crisis. Exposure to crisis plasma caused marked increases in RSNO and total NOx production from both BPAEC and HMVEC-L. Moreover, similar increases in RSNO and NOx were observed whether plasma used in this in vitro system was obtained from patients with ACS or VOC. Additional studies demonstrated that increases in RSNO and total NOx appeared as soon as 2 h after exposure to crisis plasma and occurred in a plasma concentration-dependent manner, were not associated with alterations in NOS III or NOS II transcripts, were not secondary to changes in iNOS protein or enzymatic activity, but were associated with increases in eNOS protein and eNOS enzymatic activity. Furthermore, we found that crisis plasma was profoundly oxidative in nature and that under these conditions oxidative metabolites of NO, such as ONOO⁻, could be formed.
Despite the frequency of ACS and the morbidity associated with it, few data concerning mechanisms leading to its development exist. Similar to other forms of VOC in SCD, ACS is characterized by vasoocclusive events in numerous vessels, ranging from the microvasculature to the muscular arteries (12, 27). The processes contributing to VOC that characterize SCD are likely complex, multiple and interrelated. Although deoxygenation for a critical period is certainly an important factor resulting in sickling of erythrocytes containing HbS, there is increasing evidence that extra-erythrocytic factors, such as EC activation, may influence the pathophysiology of the vasoocclusive state. Moreover, it is not unreasonable to believe that events that lead to development of ACS are similar to phenomena observed or suggested during VOC within other organ systems. Many studies of these organ systems suggest direct involvement of endothelium in these processes. Early histological and many subsequent in vivo and in vitro studies have demonstrated increased adherence between sickled erythrocytes and EC, particularly during VOC (26, 27, 32). In addition to alterations in adherent properties of sickled erythrocytes and/or EC, alterations in EC metabolic functions, including production of vasoactive mediators, could contribute to these vasoocclusive events (40, 58).

With regard to endothelium-derived vasoconstrictor molecules, plasma levels of ET-1 are elevated in patients with SCD, especially during vasoocclusive events (19, 39). We have found that exposure of BPAEC to plasma from sickle cell patients with ACS or VOC causes upregulation of ET-1 transcripts (19). In contrast, plasma obtained months later from the same individuals as stable outpatients caused no such upregulation. As in the current study, we found no difference in ET-1 upregulation whether plasma samples were obtained from patients with ACS or VOC. Moreover, plasma levels of ET-1 were two- to threefold higher in patients hospitalized with any form of crisis and decreased during outpatient follow-up. Interestingly, plasma ET-1 levels measured in one patient during a stable clinic visit 6 mo after hospitalization, but 4 days before hospitalization for VOC, were again elevated; this plasma also upregulated ET-1 transcripts in cultured BPAEC. Similarly, in another recent study, exposure of human umbilical vein EC to sickle RBC sickled in vitro to simulate VOC also induced ET-1 (48). Together, these studies suggest that ET-1 is an addi-
tional factor contributing to development of the clinical entities that complicate SCD and that increases in plasma levels may precede the clinical onset of ACS or VOC.

Whether alterations in production of the endothelium-derived vasodilator NO also occur in SCD is not clear; however, several recent studies suggest that this indeed may be the case. Mosseri et al. (43) demonstrated that, in the presence of sickle RBC, vasorelaxation of rabbit aortic strips stimulated with ACh was decreased suggesting that NO formation might be impaired in the presence of sickle RBC. Rees et al. (53) found that, compared with controls, plasma nitrite levels were elevated in sickle cell patients in crisis; however, these levels were not significantly different from plasma nitrite levels in sickle cell patients at baseline. Head et al. (23) demonstrated an increase in oxygen affinity of sickle RBC after short-term NO treatment (up to 80 ppm for <60 min) both in vitro and in vivo; this was accomplished without significant increase in methemoglobin levels. With the use of the rat stroke model, French et al. (14) demonstrated by direct visualization or laser-Doppler flowmetry a marked decrease in cerebral blood flow during infusion of sickle RBC and the

Fig. 5. A: NOS III mRNA expression in BPAEC exposed to plasma obtained during ACS. CL, media alone; SS-1%, 1% plasma from patient with ACS; SS-5%, 5% plasma from patient with ACS; SS-10%, 10% plasma from patient with ACS; SS-20%, 20% plasma from patient with ACS; SS-40%, 40% plasma from patient with ACS; SS-60%, 60% plasma from patient with ACS. B: NOS III mRNA expression in BPAEC exposed for various periods of time to plasma obtained during ACS. SS(4), plasma from patient with ACS (4 h exposure); SS(6), plasma from patient with ACS (6 h exposure); SS(8), plasma from patient with ACS (8 h exposure); SS(24), plasma from patient with ACS (24 h exposure). C: NOS III mRNA expression in BPAEC exposed to RBC obtained during ACS. STSS, RBC from patient during stable clinic visit; SS, RBC from patient with ACS. In all these experiments, β-actin was used to demonstrate equal loading on Northern analysis.

Fig. 6. A: endothelial NOS (eNOS) protein in BPAEC exposed to plasma obtained during ACS. Values are means ± SE; n = 3–6 patients for all conditions. *P < 0.05 compared with normal volunteer. B: Western blot analysis of eNOS protein in BPAEC exposed to plasma from six different individuals during ACS (lanes 1–6).

Fig. 7. eNOS enzymatic activity in BPAEC exposed to plasma obtained during ACS. Values are means ± SE; n = 3–6 for all conditions. *P < 0.05 compared with control.
NO in Sickle Cell Crisis

Table 2. Endothelial cell antioxidants during exposure of BPAEC to plasma from patients with sickle cell disease

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<th>Group</th>
<th>Total Thiol Content, ng/µg protein</th>
<th>GSH, nmol/10^6 cells</th>
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<tr>
<td>Normal volunteers</td>
<td>0.160 ± 0.13</td>
<td>2.10 ± 0.36</td>
</tr>
<tr>
<td>Sickle cell – baseline</td>
<td>0.150 ± 0.16</td>
<td>1.30 ± 0.41*</td>
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<tr>
<td>ACS/VOC</td>
<td>0.112 ± 0.07∗†</td>
<td>0.40 ± 0.15†</td>
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Values are means ± SE; n = 4–12 for thiol experiments; n = 3–9 for GSH experiments. BPAEC, bovine pulmonary artery endothelial cells. *P < 0.05 compared with normal volunteers; †P < 0.05 compared with sickle cell – baseline.

NOS inhibitor N-4-nitro-L-arginine methyl ester. The studies by Mosseri et al. (43) and French et al. (14) are compatible with our findings that sickle RBC may not bind NO as well as normal RBC. In contrast with Rees et al. (53), who found no difference in plasma nitrate levels between sickle patients in crisis or at baseline, we found marked differences in the effect of plasma on cultured EC, depending on the clinical status of the patient. Although it is difficult to compare all of these studies because of marked differences in study design, our observations together with these studies do suggest that alterations in the metabolism of NO may be an additional component contributing to the pathogenesis of vasoocclusive events in SCD and that similar mechanisms contribute to VOC in both microvessels and large vessels.

Initially, we considered the possibility that the increase in NO production might be a compensatory response to an increase in vasoconstrictor molecules, such as ET-1, because the amount of NO, necessary to “neutralize” any induced vasoconstrictor molecule is unknown. However, our observations of marked decreases in endothelial antioxidant defense mechanisms (thiols and GSH), which were presumably secondary to the oxidant state of the plasma to which they were exposed, combined with previous reports of antioxidant abnormalities in SCD suggested the intriguing possibility that NO metabolic pathways are altered during ACS and that toxic metabolites, such as ONOO−, are produced. For example, the observed decrease in superoxide dismutase in SCD (56) could increase the quantity of superoxide available to react with NO, thereby increasing production of ONOO−. In addition, decreases in catalase observed in SCD could increase the quantity of hydrogen peroxide available to react with NO thereby producing nitrite and nitrate (25). The increase in nitrotyrosine residues we observed in cultured EC after exposure to plasma from patients with ACS and VOC implies substantial ONOO− was generated during this interaction and that this is a tenable hypothesis.

Recent literature further supports this provocative hypothesis. Numerous studies now demonstrate that NO can be metabolized to ONOO− under many different conditions and that ONOO− is an extremely damaging oxidant with multiple noxious effects at many different cellular sites (13). Lewis et al. (36) have elegantly demonstrated that the fate of NO and metabolites generated depends on the concentration of oxidants in the milieu. In addition, Miles et al. (42) have observed that under specific conditions (i.e., absence of reduced iron) equimolar fluxes of NO and superoxide can interact to yield potent oxidants; in addition, nitrosation and oxidation of thiol by NO and superoxide are determined by their relative oxidants (66). Recent studies (28, 66) have also implicated GSH in the transport and catabolism of NO. Together, these studies suggest that oxidants and oxidant scavengers within the milieu (i.e., crisis plasma in our experiments) are essential determinants of the NO metabolites produced in a given situation and modulate, in part, the physiological or pathological effects of NO production.

NOx products generated during sickle cell crisis may also be dependent on the amount of NO that is protected from environmental factors noted above; an emerging model suggests that Hb may have evolved both electronic and conformational switching mechanisms to achieve NO homeostasis through the formation of S-nitroso-hemoglobin (30, 60). NO in this form may possess characteristics that distinguish it from NO itself, may elicit responses of which NO is incapable, may not be subject to the same diffusional constraints imposed by the high concentration of Hb in blood and may be protected from usual NO metabolic pathways. However, currently available studies have only evaluated the S-nitrosation of HbA; whether similar interactions or whether reactions of similar degrees occur with other hemoglobin, in particular HbS, have not been determined. In fact, French et al. (14) questioned whether sickle hemoglobin may adversely affect synthesis, delivery or regulation of NO. This is important because there is increasing evidence that NO directly regulates eNOS transcripts and activity (34, 37). For example, altered redox chemistry at the heme iron site of HbS (i.e., a greater propensity to oxidize NO by HbSF e[111]) could lead to increased nitrite production. Alternatively, an altered globin thiol site in HbS could react more readily with NO to form HbS-SNO; this nitrosated species engages in thiol-S-nitrosotiol exchange reactions more readily than heme Fe-N0 and could increase low-molecular-weight RSNO pools (38, 59). The greater amount of RSNO or total NOx found after exposure to sickle RBC, as well as the elimination of these differences after exposure to RBC ghosts, are the initial observations to suggest that HbS may not be as efficient as HbA at interacting with NO. Although further studies are necessary to verify these findings, if this were the case, NO produced during SCD may not be as well protected from a hostile environment and could then be metabolized to toxic products; likewise, production of beneficial NO metabolites would be diminished. Together, these studies suggest that oxidants and oxidant scavengers within the milieu and the ability of Hb to bind and protect NO are essential determinants of the NO metabolites produced in a given situation, and modulate, in part, the physiological or pathological effects of NO production.

Our findings of substantial ONOO− production imply that the oxidant status of the milieu (crisis plasma) and
local cells is extremely important in the fate of the increased NOx we observed and support our contention that the milieu into which EC NOx is released during ACS is substantially altered and oxidative in nature. With the use of detection of nitrotyrosine residues as evidence of ONOO−, we found substantial ONOO− production during exposure of PAEC to plasma from five patients with ACS; ONOO− production was completely blocked by incubation of PAEC with various concentrations of L-NMMA but unaffected by incubation with the same concentrations of the inactive isomer D-NMMA. These findings suggest that the ONOO− was derived from the interaction of oxidants in plasma from patients during ACS with increased NOx.

Table 3. Physicochemical characterization of plasma factor(s)

<table>
<thead>
<tr>
<th>Condition</th>
<th>ACS 1</th>
<th>ACS 2</th>
<th>Normal Volunteer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unfractionated plasma</td>
<td>19.8 ± 2.6*</td>
<td>11.6 ± 2.0*</td>
<td>3.3 ± 0.4</td>
</tr>
<tr>
<td>Lipid fraction</td>
<td>12.8 ± 1.4*</td>
<td>11.4 ± 1.2*</td>
<td>4.1 ± 0.5</td>
</tr>
<tr>
<td>Lipid fraction (56°C)</td>
<td>13.2 ± 2.1*</td>
<td>6.1 ± 0.9*</td>
<td>1.8 ± 0.2</td>
</tr>
<tr>
<td>Protein fraction</td>
<td>11.6 ± 1.6*</td>
<td>11.0 ± 1.8*</td>
<td>4.6 ± 0.6</td>
</tr>
<tr>
<td>Protein fraction (56°C)</td>
<td>13.3 ± 1.8*</td>
<td>8.1 ± 1.3*</td>
<td>4.4 ± 0.8</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 3; *P < 0.05 compared with normal volunteers. ACS, acute chest syndrome. Either unfractionated plasma or the designated plasma fraction was incubated with BPAEC for 24 h; supernatants were collected and assayed for total nitrogen oxide production (see METHODS).
generated by the increase in NOS protein and/or activity under these conditions. Although the role of ONOO - in physiological or pathophysiological processes is controversial (44), it has been implicated in the initiation and propagation of several types of lung injury (18, 29, 35, 45) and in other forms of tissue injury.

Although the environment into which NO is released might predispose to the production of toxic metabolites, another question arises. Is our hypothesis compatible with NO formation by the constitutive rather than the inducible enzyme? It is generally thought that eNOS produces small, physiological amounts of NO whereas iNOS is the isoform that responds to cytokines and other stimuli to produce the large quantities of NO that can result in tissue damage or death. However, recent studies (41, 46, 47) of tissue ischemia/reperfusion and stroke have demonstrated that constitutive NOS can produce cytotoxic quantities of NO. Moreover, Rosenkranz-Weiss et al. (54) have demonstrated that, in human umbilical vein EC, inflammatory cytokines increase endothelial production of NO by augmenting the specific activity of eNOS. Thus our findings that increased NO production during exposure to plasma from sickle cell patients with ACS is associated with increases in the specific activity of eNOS represent another example of what will likely be a growing number of instances in which constitutive NOS is responsible for the NO that can produce tissue damage. As in the studies of Rosenkranz-Weiss et al. (54), we could not detect iNOS activity nor demonstrate an increase in NOS III transcripts despite an increase in eNOS activity.

An additional question arising from these studies is the identity of the substance(s) that induces the increase in NOS activity in our model of ACS. Numerous proinflammatory cytokines and EC modulators are likely released during VOC and any of them could induce the NO production we have observed (8, 11). Our initial studies to identify the plasma factor(s) responsible for the stimulation of EC NO production imply that several factors, both lipid and protein in nature, exist. Further studies are underway to define and clarify this aspect of our findings.

In summary, we have demonstrated dramatic increases in RSNO and total NO production from both BPAEC and HMVEC-L exposed to plasma from sickle patients during ACS or VOC. These increases appeared within 2 h of exposure to crisis plasma and occurred in a plasma concentration-dependent manner and were not associated with alterations in NOS III or NOS II transcripts, but they were associated with increases in eNOS protein and eNOS enzymatic activity. Although it seems counterintuitive that a vasodilatory molecule, such as NO, could be detrimental during a vasoocclusive state, our observations of a decrease in EC antioxidant defense molecules reflecting an oxidative state of crisis plasma, substantial increases in nitrotyrosine residues implying production of ONOO - , other reports of diminished antioxidant mechanisms in SCD and the reliance of NO metabolic pathways on the oxidant state of the environment suggest to us that an overabundance of toxic NO metabolites might be produced during ACS and that these metabolites could contribute to the subsequent cellular and tissue damage. Although these initial findings are provocative, they are limited because of the nature of the system used. Whether similar findings occur in vivo during ACS and, if so, whether this scenario is limited to ACS or occurs with other inflammatory lung processes is not yet clear. Further studies will be necessary to investigate our hypothesis and to increase understanding of the pathogenesis of ACS. Such an understanding is important because recurrent episodes of ACS are the major risk for development of pulmonary hypertension, cor pulmonale and eventual death in SCD (15, 17, 18).

S. I. Hammerman, E. S. Klings, and K. P. Hendra contributed equally to this work.

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Address for reprint requests: H. W. Farber, Pulmonary Center, Boston Univ. School of Medicine, 715 Albany St., R-304, Boston, MA 02118 (E-mail: hfarber@lung.bumc.edu).

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