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Sickle Cell Vaso-occlusive Crisis Is Associated with Abnormalities in the Ratio of Vasoconstrictor to Vasodilator Prostanoids

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ABSTRACT

Plasma levels of 6-keto-prostaglandin $F_{1\alpha}$ ($6kPGF_{1\alpha}$) and thromboxane (Tx) B_2 have been assessed in sickle cell disease (SCD) with discrepant results. Inasmuch as direct measurement of plasma prostanoids is fraught with the problem of interfering substances, we assessed plasma $6kPGF_{1\alpha}$ and TxB_2 levels in patients with SCD by RIA after extraction of eicosanoids and separation by HPLC. We demonstrate that the $6kPGF_{1\alpha}$ and TxB_2 levels in children with SCD in steady state as well as in vaso-occlusive crisis (VOC) are significantly lower when compared with those from age-matched controls. The VOC plasma $6kPGF_{1\alpha}$ and TxB_2 levels were, however, significantly elevated when compared with those from children in steady state. Changes similar to those noted with unpaired plasma samples were also observed when paired steady state and VOC plasmas from the same patients were assessed. The ratio of TxB_2 to $6kPGF_{1\alpha}$ was, however, significantly elevated in patients with SCD in crisis when compared with eicosanoid ratios obtained during steady state. In an attempt to understand whether the abnormality in $6kPGF_{1\alpha}$ was due to an impairment in endothelial cell prostacyclin-regenerating ability, we compared the ability of plasma from controls and children with SCD to activate arachidonic acid (AA) release and prostacyclin production by [^{14}C]AA-prelabeled bovine aortic endothelial cells. Our results suggest that the decreased $6kPGF_{1\alpha}$ levels in plasma from children with SCD was not due to an effect on substrate AA release but rather a modulatory effect of sickle plasma components on endothelial cell cyclooxygenase activity. Although a decreased production of prostacyclin has previously been suggested to play a role in the initiation and/or propagation of vaso-occlusion in SCD, our study demonstrates a relative increase during VOC of $6kPGF_{1\alpha}$ levels over those observed in steady state. However, the rise in $6kPGF_{1\alpha}$ is accompanied by an increase in levels of TxB_2 such that the ratio of TxB_2 to $6kPGF_{1\alpha}$ is significantly increased during VOC. The imbalance in the production of vasoactive eicosanoids, TxA_2 and prostacyclin, could potentially play a role in the potentiation of VOC in patients with SCD. (***Pediatr Res* 38: 95–102, 1995**)

Abbreviations

SCD, sickle cell disease
VOC, vaso-occlusive crisis
HBSS, Hanks' balanced salt solution
AA, arachidonic acid
PG, prostaglandin
$6kPGF_{1\alpha}$, 6-keto-prostaglandin $F_{1\alpha}$
Tx, thromboxane
HETE, hydroxyeicosatetraenoic acid
HEPES, *N*-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid

Intermittent episodes of vaso-occlusion are one of the major clinical problems in SCD. Vaso-occlusion is a complex process involving interactions of multiple components including sickle red blood cells, other circulating cellular elements of blood, plasmatic factors, and vascular endothelial cells (1, 2). Endothelial cells generate several vasoactive agents including prostacyclin, a potent vasodilator and anti-thrombotic eicosanoid, whereas activated platelets produce TxA_2, a potent vasoconstrictor and prothrombotic eicosanoid. Several previous studies have shown that the balance between the biosynthesis of these two vasoactive eicosanoids with contrasting biologic properties plays an important role in the maintenance of hemostasis (3, 4). Abnormalities in the production of these vasoactive agents have previously been observed in various pathologic conditions associated with vascular complications (5, 6). Circulatory levels of both $6kPGF_{1\alpha}$ and TxB_2, the stable nonenzymatic hydrolysis products of prostacyclin and TxA_2, respectively, in SCD have also been assessed by several investigators with discrepant results (7–12). These discrepancies are not surprising because the direct measurement of these eicosanoids in plasma or serum by RIA is fraught with the problem of interfering substances. In the present study, we have measured

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plasma levels of both $6kPGF_{1\alpha}$ and TxB_2 in patients with SCD by RIA after extraction of eicosanoids from plasma, and separation by reverse phase-HPLC. Our results demonstrate that the levels of both $6kPGF_{1\alpha}$ and TxB_2 in children with SCD in steady state are significantly lower when compared with those from age-matched controls. During VOC, although the levels rise when compared with steady state, they were still below normal control values. Additional *in vitro* studies suggest that the decreased $6kPGF_{1\alpha}$ level in plasma from patients with SCD in steady state was due to a modulatory effect of sickle plasma components on endothelial cell cyclooxygenase activity.

METHODS

Materials. Reference eicosanoid standards for chromatographic analysis were obtained from Biomol Research Laboratories (Plymouth Meeting, PA). AA, adenosine, theophylline, indomethacin, and nordihydroguaiaretic acid were purchased from Sigma Chemical Co. (St. Louis, MO). $[^3H]PGB_1$ (56 Ci/mmol), $[^3H]6kPGF_{1\alpha}$ (199 Ci/mmol), $[^3H]TxB_2$ (209 Ci/mmol), and $[1\text{-}^{14}C]AA$ (50–60 mCi/mmol) were obtained from Amersham Corp. (Arlington Heights, IL) or Dupont NEN (Boston, MA). Tissue culture supplies were purchased from Life Technologies, Inc. (Grand Island, NY).

Culture of endothelial cells. Fetal bovine aortic endothelial cells were isolated, identified, and cultured in minimal essential medium supplemented with 10% FCS as described by Glaser *et al.* (13). Cells from passages 8–16 were used in the experiments to be described, each passage representing two-cell doubling.

Blood collection and preparation of plasma. After informed consent, venous blood (5–10 mL) was obtained from healthy volunteers (n = 15, ages 2–21 y, mean age 9.6 y) and patients with SCD (n = 42, ages 2–22 y, mean age 11.9 y). The latter patient group included 35 patients homozygous for hemoglobin SS and seven patients with SC disease. The mean ages of both SS (ages 2–22 y, mean age 12.4 y) and SC (ages 2–21 y, mean age 9.6 y) patient groups were similar. Crisis blood samples (n = 19, ages 4–19 y, mean age 12.9 y) were obtained from patients in VOC within the first 24–48 h of the crisis event. Eight SS patients were studied under both steady state and crisis conditions. VOC was identified by the acute onset of bone pain in an afebrile patient who had no concomitant respiratory findings by clinical or radiologic examination and who had no clinical or laboratory evidence of infection. All patients in VOC were on analgesics (morphine, demerol, or codeine) and none was on nonsteroidal anti-inflammatory drugs. For evaluation of the effect of plasma on endothelial cell prostacyclin production, blood was collected in tubes containing either sodium heparin or acid-citrate-dextrose as an anticoagulant. For measurement of plasma $6kPGF_{1\alpha}$ and TxB_2 levels, blood was collected in acid-citrate-dextrose tubes containing nordihydroguaiaretic acid, indomethacin, adenosine, and theophylline added to a final concentrations of 25 μM, 30 μM, 100 mM, and 1 mM, respectively. The latter pharmacologic agents were added to suppress platelet activation and eicosanoid production. All blood samples were centrifuged at 3000 × g for 20 min at room temperature to remove platelets, white blood cells, and red blood cells. All plasma samples were stored frozen at −80°C until assayed.

Measurement of plasma $6kPGF_{1\alpha}$ and TxB_2 levels. Plasma (3–5 mL) was diluted with an equal volume of ice-cold methanol, 50,000 cpm of HPLC re-purified $[^3H]PGB_1$ was added to each sample to monitor for the recovery of prostanoids, and the mixture was then incubated overnight at −20°C to precipitate plasma proteins. The methanol content in the protein-free extract was adjusted to 10%, and eicosanoids in the methanolic extract were isolated using C-18 Maxi-Clean 600-mg cartridges (Alltech Associates, Deerfield, IL) as previously described (14). To the isolated eicosanoid fraction, 75 ng of unlabeled PGB_1 were added, and the samples were concentrated to dryness using a SpeedVac Concentrator. Lipids were reconstituted in the HPLC mobile phase and analyzed on a 4.6 × 125 mm Whatman's PartiSphere C-18 column (Whatman Chemical Separation, Clifton, NJ), and a Beckman liquid chromatograph (Beckman Instrument, Palo Alto, CA) using a stepwise gradient of acetonitrile and 0.1% acetic acid in water at a flow rate of 1 mL/min. Solvent A was acetonitrile-water-acetic acid (30:70:0.1%, by volume), and solvent B was acetonitrile (100%). The solvent program was as follows: 100% A for 13 min, a quick gradient from 0% to 24% B over 2 min, and the gradient was held at 24% B for an additional 10 min. The column was washed with 100% solvent B for 15 min between injections. The elution of reference prostanoids was monitored continuously at 210 and 280 nm with a Beckman 165 variable wavelength detector. Under these HPLC conditions, reference $6kPGF_{1\alpha}$, TXB_2, $PGF_{2\alpha}$, PGE_2, PGD_2, and PGB_1 eluted at 4.96 ± 0.08 (mean ± SD, n = 8), 9.01 ± 0.15, 12.84 ± 0.21, 15.37 ± 0.25, 17.23 ± 0.11, and 20.76 ± 0.08 min, respectively. Fractions (30 s) were collected for up to 25 min, and the material eluting in the regions of $6kPGF_{1\alpha}$, and TxB_2 were pooled separately. After evaporation of HPLC mobile phase, both $6kPGF_{1\alpha}$ and TxB_2 fractions were reconstituted in 500 μL of RIA buffer, and aliquots were assayed for $6kPGF_{1\alpha}$ and TxB_2 using commercially available RIA kits (Advanced Magnetics Inc., Boston, MA), respectively. The lower limits of these assays sensitivity were 7.4 and 22.2 fmol per 100 μL for $6kPGF_{1\alpha}$ and TxB_2, respectively. The elution of $[^3H]PGB_1$ was monitored with a Ramona-5-LS flow-through radioisotope detector (Raytest USA Inc., Pittsburgh, PA) equipped with a solid scintillator. The radioactivity eluting in the PGB_1 area was pooled, and activity determined in a LKB Mini-Beta liquid scintillation counter (Pharmacia Biotech Inc., Gaithersburg, MD) using Liquiscint (National Diagnostics, Atlanta, GA). The recovery of $[^3H]PGB_1$ through HPLC was 76 ± 1% (n = 18). In separate experiments, the recoveries of both $[^3H]6kPGF_{1\alpha}$ and $[^3H]TxB_2$ through HPLC were found to be similar to that of $[^3H]PGB_1$ when analyzed under identical experimental conditions. $6kPGF_{1\alpha}$ and TxB_2 levels corrected for $[^3H]PGB_1$ recovery were expressed as picomoles/mL plasma and ratios of TxB_2 to $6kPGF_{1\alpha}$ were determined for each individual evaluated.

Effect of plasma on endothelial cell prostacyclin regenerating ability. Endothelial cells from stock cultures were seeded into wells of six-well plates, grown to confluence, and then

prelabeled with [^{14}C]AA as previously described (15). In brief, confluent cell monolayers were incubated for 20 h with 2.5 μM [^{14}C]AA provided in 2 mL of minimal essential medium containing 10% FCS. Under this labeling condition, endothelial cells incorporated approximately 80% of the added [^{14}C]AA into the membrane phospholipids. The [^{14}C]AA-labeled washed endothelial cell monolayers were incubated for the indicated times in the presence of 1.5 mL of 1 to 1 diluted plasma in HBSS containing 1.3 mM $CaCl_2$ and 0.5 mM $MgCl_2$ buffered with 5 mM HEPES (HBSS-HEPES buffer) or HBSS-HEPES buffer alone. After incubation, metabolites from the pooled incubation medium and endothelial cells were extracted (16), evaporated, and reconstituted in 100 μL of chloroform. Aliquots were analyzed by thin layer chromatography on silica gel G plates (Analtech, Inc., Newark, DE) using the upper phase of ethyl acetate-isooctane-acetic acid-water (90:50:20:100, by volume) as a solvent system (17). Reference prostaglandins, HETE, and AA (5 μg/spot) were run on the same plates and visualized using a cupric acetate spray reagent (Applied Science Labs., Deerfield, IL). After radioautography, the eicosanoid bands were identified by comparing their retention factor values with those of authentic standards. Radioactive bands were scraped into scintillation vials and extracted with 500 μL of methanol, and radioactivity was determined using 4.5 mL of Liquiscint. The sum of radioactivity recovered in various prostanoids, HETE, and the free fatty acid fraction was used as a measure of endothelial cell AA release. Cyclooxygenase activity was assessed by measuring the radioactivity recovered in the various prostanoids including $6kPGF_{1\alpha}$ (the nonenzymatic hydrolysis product of prostacyclin), PGE_2, and $PGF_{2\alpha}$. Conversion of mobilized AA to prostacyclin or total prostanoids was expressed as the percent ratio of radioactivity recovered in products to total AA released.

Statistical analysis. Statistical evaluation was performed either by the unpaired or paired *t* test.

RESULTS

Plasma Prostanoid Levels in Children with Sickle Cell Disease

Comparison of plasma $6kPGF_{1\alpha}$ levels measured with direct assay of plasma versus assay after extraction and HPLC separation. Plasma $6kPGF_{1\alpha}$ levels were measured in blood samples obtained from 18 different donors either directly (using 25–100 μL plasma in the RIA) or following extraction of plasma eicosanoids and HPLC separation of $6kPGF_{1\alpha}$. When 100 μL plasma (the maximal volume that could be included in the RIA) was assayed directly, 10 out of 18 of these samples had measurable $6kPGF_{1\alpha}$ levels which ranged from 0.09 to 0.68 pmol/mL, whereas in the remaining eight samples the levels were either undetectable or below the lower limit of the assay's sensitivity. For calculation of mean $6kPGF_{1\alpha}$ levels, the plasma levels in these latter eight samples were assumed to be zero. The mean $6kPGF_{1\alpha}$ level measured in these plasma samples was 0.18 $\pm$ 0.06 (mean $\pm$ SE, n = 18) pmol/mL. Although all of these samples were assayed in triplicate volumes (25, 50, and 100 μL), only seven out of 18 of these samples had measurable eicosanoid levels with 50 μL plasma, whereas no $6kPGF_{1\alpha}$ was detected when 25 μL plasma were used in the assay. Although the variability among the duplicates generated using multiple aliquots of the same volume was within 5%, there was no linearity between the volume of plasma assayed *versus* $6kPGF_{1\alpha}$ levels measured. The $6kPGF_{1\alpha}$ levels measured per mL plasma using 50 and 100 μL of plasma in the RIA were significantly different (0.21 $\pm$ 0.04, and 0.47 $\pm$ 0.11 pmol/mL, respectively, n = 7, p <0.05). In contrast, when $6kPGF_{1\alpha}$ levels were measured in the same 18 plasma samples after extraction and HPLC separation, the values ranged from 0.15 to 9.93 pmol/mL with a mean ($\pm$ SE) value of 1.64 ($\pm$ 0.57) pmol/mL plasma. Further, there was a linear response between the volume of extract assayed *versus* $6kPGF_{1\alpha}$ levels measured. Finally, there was a 10-fold difference between the $6kPGF_{1\alpha}$ levels measured by direct assay of plasma (with 100 μL) and the levels measured after extraction and HPLC separation of eicosanoids. This difference was statistically significant at a p <0.025.

Plasma $6kPGF_{1\alpha}$ levels in children with SCD. As seen in Fig. 1, *panel A*, plasma $6kPGF_{1\alpha}$ levels in control healthy children ranged from 1.08 to 9.93 pmol/mL (3.60 $\pm$ 0.88 pmol/mL, mean $\pm$ SE, n = 9). In contrast, the $6kPGF_{1\alpha}$ levels from children with SCD in steady state were significantly lower (0.46 $\pm$ 0.08 pmol/mL, n = 20, p <0.001). No differences in steady state $6kPGF_{1\alpha}$ levels were noted when the SS (0.46 $\pm$ 0.08 pmol/mL, n = 13) and SC (0.50 $\pm$ 0.15 pmol/mL, n = 7) groups were compared. The $6kPGF_{1\alpha}$ levels in plasma from patients with SCD in VOC were 1.41 $\pm$ 0.31 pmol/mL (n = 13). Although the levels of $6kPGF_{1\alpha}$ in this patient group were also significantly lower when compared with those from controls (p <0.025), they were significantly higher when compared with sickle cell patients in steady state (p <0.005). As depicted in Fig. 2, *panel A*, results similar to those found with unpaired plasma samples were also observed when paired steady state and VOC plasma from eight patients with SCD were compared. Although the mean $6kPGF_{1\alpha}$ level in steady state plasma was 0.31 $\pm$ 0.11 pmol/mL, during VOC the $6kPGF_{1\alpha}$ level in these patients was increased to 1.20 $\pm$ 0.36 pmol (n = 8, p <0.05).

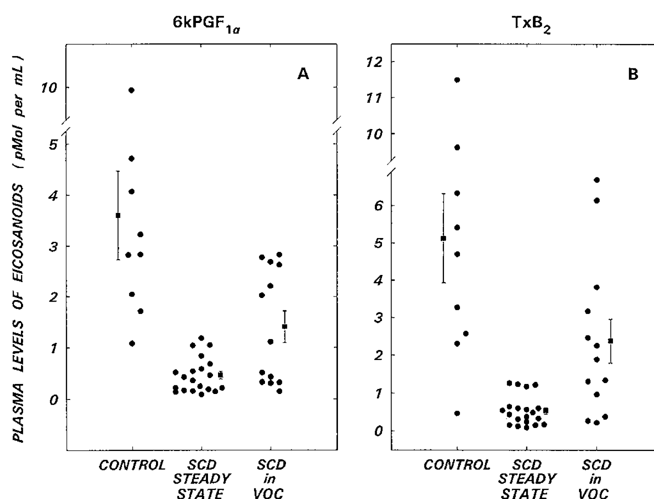

Figure 1. Plasma levels of $6kPGF_{1\alpha}$ (*panel A*), and TxB_2 (*panel B*) from control donors (n = 9) are compared with those from patients with SCD during steady state (n = 20) and in VOC (n = 13).

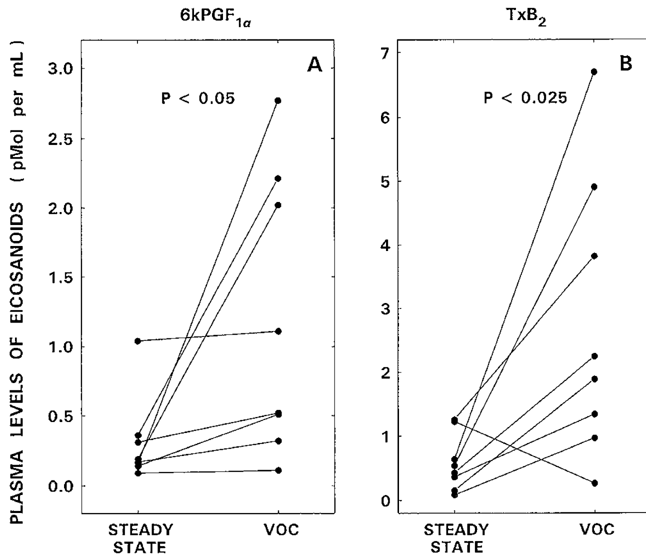

Figure 2. Plasma levels of $6kPGF_{1\alpha}$ (*panel A*), and TxB_2 (*panel B*) from eight patients with SCD in steady state are compared to plasmas obtained from the same patients during VOC.

Plasma TxB_2 levels in children with SCD. Changes similar to those seen with plasma $6kPGF_{1\alpha}$ were also observed when plasma TxB_2 levels were compared among these patient groups, *i.e.* TxB_2 levels were significantly lower in children with SCD in steady state (0.54 ± 0.09 pmol/mL plasma, mean ± SE, $n = 20$, $p < 0.001$) when compared with those from control healthy children (5.13 ± 1.19 pmol/mL, $n = 9$) (Fig. 1, *panel B*). There were no differences in steady state TxB_2 levels when patients with SS (0.51 ± 0.12 pmol/mL, $n = 13$) and SC (0.57 ± 0.12 pmol/mL, $n = 7$) disease were compared. Although the plasma TxB_2 levels in patients with SCD in VOC (2.38 ± 0.58 pmol/mL, $n = 13$) were also significantly decreased when compared with the values noted in controls ($p < 0.025$), the levels were significantly elevated when compared with those obtained during steady state ($p < 0.001$) (Fig. 1, *panel B*). Similar differences in TxB_2 levels in patients with SCD in steady state *versus* VOC were also observed when VOC plasma TxB_2 levels were compared with the patient's own steady state levels (2.77 ± 0.78 pmol/mL in VOC *versus* 0.59 ± 0.16 pmol/mL in steady state, $n = 8$, $p < 0.025$, Fig. 2, *panel B*). The ratios of TxB_2 to $6kPGF_{1\alpha}$ calculated for all patient groups included values of 1.99 ± 0.29 ($n = 9$), 1.41 ± 0.19 ($n = 20$), and 3.86 ± 1.23 ($n = 13$) for controls, and patients with SCD in steady state and crisis, respectively. Thus, a significant increase in the ratio of TxB_2 to $6kPGF_{1\alpha}$ was observed during VOC ($p < 0.025$) when compared with steady state. Such an increase was also noted when paired data comparing steady state and VOC ratios in the same patient were contrasted (1.61 ± 0.29 vs 3.62 ± 0.60, respectively, $n = 8$, $p < 0.025$).

Effect of Sickle Cell Plasma on Endothelial Cell Prostacyclin-Regenerating Ability

Time course of prostacyclin production and AA release. In our previous studies using bradykinin as a stimulus we have shown that the prostacyclin production by bovine aortic endothelial cells reached a maximum by 45 min (15). In preliminary experiments, endothelial cells incubated with buffer alone released 8354 to 9298 cpm [^{14}C]AA between 10 and 75 min. Endothelial cell monolayers incubated with both control plasma and plasma from patients with SCD released [^{14}C]AA in a time-dependent manner between 10 and 75 min with maximal stimulation observed by 45 min. The stimulatory responses noted at 60 and 75 min were not significantly different from that observed at 45 min. We have, therefore, selected a 45-min incubation time to compare the effects of plasmas from controls and patients with SCD on prostacyclin production and AA release.

Assay variabilities. In preliminary studies, we evaluated intra- and interassay variability on AA release and prostanoid production by endothelial cells using cultures from different passages. We have found that the intraassay variability was less than 5% among paired cultures from the same passage. However, there was considerable variation in AA release and prostanoid production among cultures from different passages with 20 to 300% interassay variability. Because of the latter, plasma prostacyclin-regenerating ability was expressed as percent of buffer control to facilitate comparisons among cultures from different passages. When the effect of steady state and VOC plasmas from the same patient with SCD was compared, paired endothelial cell monolayers from the same passage were employed.

Effect of plasma on prostacyclin generation. Using endothelial cell cultures, in preliminary studies we compared the effects of paired plasmas (prepared in tubes containing either citrate or sodium heparin as the anticoagulant) on endothelial cell prostacyclin production. We found no differences in prostacyclin production when the endothelial cells were incubated with plasma prepared using either anticoagulant (4060 ± 393 cpm $6kPGF_{1\alpha}$ per culture dish with citrated plasma *versus* 3995 ± 425 cpm with heparinized plasma, mean ± SE, $n = 5$, p = NS). Because citrated plasma in our preliminary experiments clotted when diluted with the calcium-supplemented buffer used in our prostacyclin-generating experiments, heparinized plasmas were used in our subsequent studies. Endothelial cells incubated with buffer alone produced 2521 ± 168 cpm ^{14}C-$6kPGF_{1\alpha}$ per culture dish ($n = 17$). As shown in Figure 3, plasma from control donors stimulated endothelial cell prostacyclin production by 77% over baseline (177 ± 17% of buffer control, $n = 15$). Plasma from patients with SCD in steady state had 29% stimulation over baseline (129 ± 8% of buffer control, $n = 15$), whereas VOC plasma stimulated the production of prostacyclin by 106% over baseline (206 ± 16% of buffer control, $n = 12$). Although the changes in prostacyclin-generating ability observed between control plasma and plasma from patients with SCD in VOC were not significantly different, the steady state plasma prostacyclin-regenerating activity was markedly decreased when compared with both control ($p < 0.001$) and VOC plasmas ($p < 0.025$). There were no differences when prostacyclin-regenerating ability of SS and SC plasma were compared. Differences similar to those observed with unpaired plasma samples were also found when paired steady state and VOC plasmas from the same patient

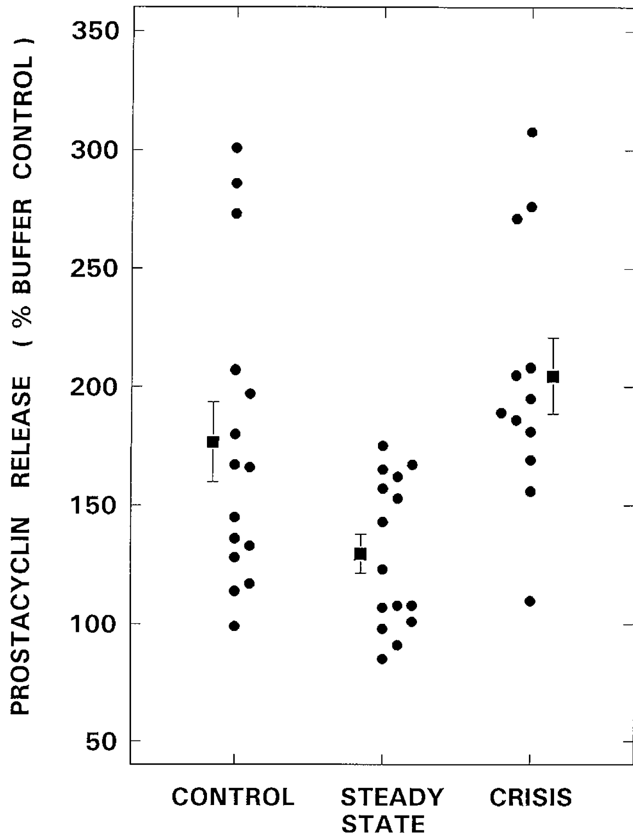

Figure 3. Comparative effects of plasmas from control donors (n = 15) and those from patients with SCD during steady state (n = 15) and in VOC (n = 12) on [^{14}C]prostacyclin production by [^{14}C]AA-labeled bovine aortic endothelial cells.

with SCD were compared (Fig. 4, *panel A*). Although steady state plasma produced 3907 ± 523 cpm 6kPGF$_{1\alpha}$ per culture dish (n = 7), VOC plasma released 5276 ± 353 cpm. These changes were significant at a p <0.025.

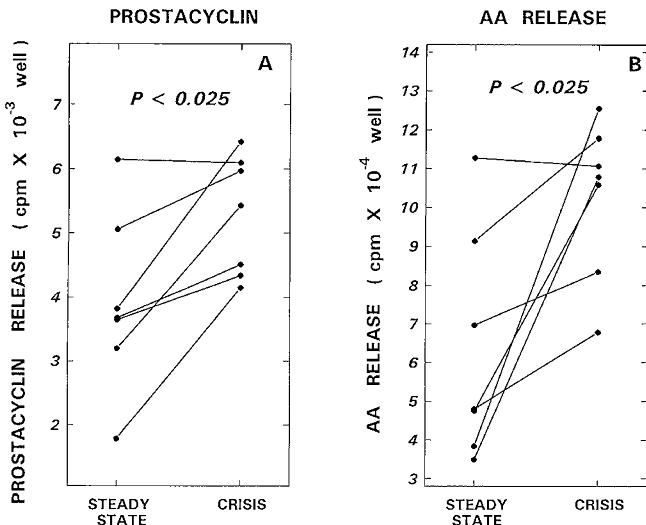

Figure 4. Comparative effects of plasmas obtained from the same patients with SCD (n = 7) during steady state and in VOC on prostacyclin production (*panel A*) and AA release (*panel B*) by [^{14}C]AA-labeled bovine aortic endothelial cells.

Localization of Defects in SCD Plasma on Endothelial Cell Prostacyclin-Regenerating Ability

Effect of plasma on AA release. Endothelial cells incubated with buffer alone released 13058 ± 1063 cpm (mean ± SE, n = 17) [^{14}C]AA per culture dish. As depicted in Fig. 5, both control and steady state plasma stimulated AA release to the same extent. While control plasma stimulated AA release by 432% over baseline (532 ± 64% of buffer control, n = 15), SCD steady state plasma stimulated AA mobilization by 331% over baseline (431 ± 56% of buffer control, n = 15). An even greater stimulatory response on AA release was seen with plasma from SCD patients in VOC with 885% stimulation over baseline (985 ± 156% of buffer control, n = 12). The AA mobilizing effect of VOC plasma was statistically different from both control plasma- and steady state plasma-induced changes at p <0.01, and p <0.005, respectively. As seen in Fig. 4, *panel B*, results similar to those obtained with unpaired treatments were also noted when paired plasma samples obtained from the same individual under both steady state and VOC conditions were concomitantly evaluated. Although steady state plasma released 63,237 ± 11,117 cpm AA per well (n = 7), VOC plasma mobilized 102,706 ± 7623 cpm. This response was statistically significant at a p <0.025.

Effect of plasma on conversion of released AA to prostacyclin. Endothelial cells treated with control plasma converted 8.89 ± 0.11% (mean ± SE, n = 15) of the released AA into prostacyclin. Conversion of the mobilized AA to prostacyclin

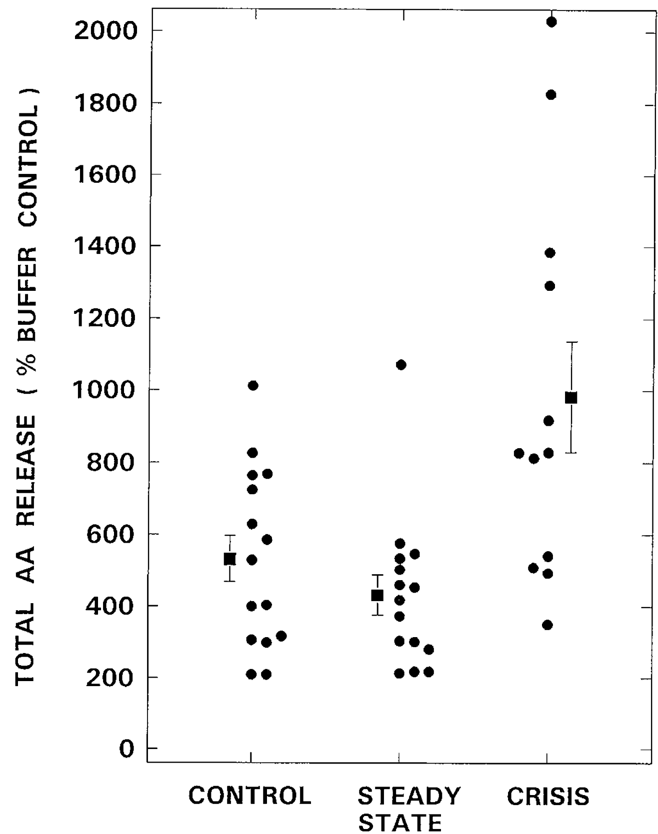

Figure 5. Comparative effects of plasmas from control donors (n = 15) and those from patients with SCD during steady state (n = 15) and in VOC (n = 12) on [^{14}C]AA release by [^{14}C]AA-labeled bovine aortic endothelial cells.

was significantly reduced in cells incubated with plasma from patients with SCD in steady state (6.21 ± 0.66%, $n = 15$, $p < 0.01$). Such a reduction in AA metabolism was also observed in endothelial cells incubated with plasma from SCD patients in VOC (6.86 ± 0.72%, $n = 12$, $p < 0.05$). Thus, although steady state and VOC plasma mobilized differing amounts of AA from endothelial cells (Fig. 5), the conversion of mobilized AA to prostacyclin was similar under both steady state and VOC conditions. Similar results were also obtained when AA conversion to prostacyclin was analyzed in the presence of the paired steady state and VOC plasma samples from the same individual patient with SCD (6.48 ± 0.68% *versus* 6.43 ± 0.43%, $n = 7$). When the effect of plasma on endothelial cell cyclooxygenase activity was assessed, although control plasma and VOC plasma enhanced prostanoid formation by 40% (140 ± 12% of buffer control, $n = 15$) and 57% over baseline (157 ± 12% of buffer control, $n = 12$), the prostanoid-regenerating ability of steady state plasma was significantly decreased (108 ± 6% of buffer control, $n = 15$). These latter changes were statistically significant at $p < 0.025$ and $p < 0.05$, respectively.

DISCUSSION

We measured plasma levels of $6kPGF_{1\alpha}$ and TxB_2—the stable nonenzymatic hydrolysis products of prostacyclin and TxA_2, respectively, in patients with SCD after extraction and HPLC separation of these eicosanoids. Our results demonstrate that the levels of $6kPGF_{1\alpha}$ in patients with SCD in steady state are significantly lower when compared with those from age-matched controls. During VOC, although the levels tend to rise above steady state, they were still below the normal range. Similar changes in serum $6kPGF_{1\alpha}$ levels between control donors and patients with SCD in steady state have previously been reported by Longenecker and co-workers (Table 1). These investigators also found that the crisis $6kPGF_{1\alpha}$ levels were marginally higher than the steady state levels (Table 1). In contrast to our present results and those of Longenecker, other investigators have found increased $6kPGF_{1\alpha}$ levels in plasma from patients with SCD (Table 1). In concomitant studies, we also measured plasma TxB_2 levels in these patients and demonstrated changes in this eicosanoid similar to those observed with $6kPGF_{1\alpha}$, *i.e.* the steady state TxB_2 levels were significantly lower when compared with those from age-matched healthy controls. Although crisis TxB_2 levels were significantly elevated compared with those in steady state levels, they were still reduced when compared with the levels assayed in controls. Such changes in basal TxB_2 levels in patients with SCD in steady state and crisis have previously been documented in one study (11), whereas others have shown elevated levels of this eicosanoid in steady state patients with the levels either remaining unchanged or increasing further during crisis (9, 12).

The discrepancies in published results suggest differences in patient groups and methodologic differences in blood sampling and assays for $6kPGF_{1\alpha}$ and TxB_2. All of the blood levels hitherto reported were obtained from direct assay of either plasma or serum samples employing different pharmacologic agents to suppress eicosanoid production by the cellular elements of blood. In addition, as shown in this study as well as by other investigators (18, 19), direct RIA of prostanoids in biologic fluids is subjected to either positive or negative interferences. Finally, the eicosanoid levels in several plasma samples reported here and in many of the published reports were either below the detection level or very close to the lower limit of the assay's sensitivity when plasma was assayed directly. To obviate these previously identified methodologic problems, in this investigation the eicosanoids were extracted from larger sample volumes of plasma (3–5 mL), separated by reverse phase-HPLC, and only then assayed by immunoassay procedures.

Vascular endothelium is the major source of plasma prostacyclin. Previous studies have shown that plasma stimulates prostacyclin production by endothelial cells (20–22). Using Remuzzi *et al.*'s (23) exhausted human umbilical arterial seg-

Table 1. *Plasma $6kPGF_{1\alpha}$ levels in children with SCD*

Reference	Methodology	Observation
Mehta and Albiol (7)	Plasma prepared in the presence of 1 mM aspirin was assayed directly. Most of the control levels were below detectable levels	$6kPGF_{1\alpha}$ levels in children with steady state SCD were significantly higher compared to age- and sex-matched controls
Longenecker and Mankad (8)	Serum prepared in the presence of indomethacin was assayed directly	$6kPGF_{1\alpha}$ levels in children with steady state SCD were significantly lower compared to age-matched controls. Crisis levels were higher compared with steady state levels
Buchanan and Holtkamp (9)	Plasma prepared in the presence of 25 μg/mL indomethacin was assayed directly. Pediatric patients were compared with controls comprising at least 75% adult population	Steady state $6kPGF_{1\alpha}$ levels were higher than the control levels. Crisis levels were similar to those obtained with steady state
Koren (Kurlat) and Halevi (10)	Plasma was assayed directly	$6kPGF_{1\alpha}$ levels in children with steady state SCD were slightly higher than in age-matched control. Crisis levels were significantly lower compared with steady state levels
Longenecker *et al.* (11)	Serum prepared in the presence of 13.9 μM indomethacin was assayed directly	$6kPGF_{1\alpha}$ levels in children with steady state SCD were significantly lower compared to age- and race-matched controls. Early crisis levels were lower than the steady state levels

ments, we have previously demonstrated that the *in vitro* prostacyclin-regenerating ability of steady state SCD plasma is markedly decreased when compared with control plasma (24). These studies, therefore, suggest that the abnormal circulatory $6kPGF_{1\alpha}$ levels in SCD may be due to modulation of endothelial prostacyclin production by sickle plasma components. Biochemical changes that occur in cells after activation include mobilization of AA from membrane phospholipids and conversion of the mobilized AA via the cyclooxygenase and prostacyclin synthase pathway to prostacyclin. Modulation of one or more of these biochemical events by sickle plasma may result in a decrease in circulatory $6kPGF_{1\alpha}$ levels. The decreased ability of steady state SCD plasma to stimulate prostacyclin production *in vitro* appears to be due to a modulatory effect of SCD plasma components on processes distal to AA mobilization, because both control and steady state SCD plasma stimulated the release of AA almost to the same extent (Fig. 5). Our results also indicate that the activity of cyclooxygenase is affected by SCD plasma, because both total prostanoid production (a measure of cyclooxygenase activity) and prostacyclin formation decreased in parallel. Plasma components could interfere with the cyclooxygenase reaction either by reducing the availability of free AA for enzymatic reaction or by inhibiting the enzyme directly. These plasma effects have not been further characterized. Another possible mechanism by which steady state SCD plasma may induce changes in prostacyclin production includes depletion of the substrate as a result of continuous or repeated endothelial activation. This possibility is seemingly excluded from our *in vitro* studies, because the cells subjected to treatment with different plasmas had similar amounts of substrate arachidonic acid in their membrane phospholipids. However, this could be an additional factor in modulating prostacyclin production *in vivo* in patients with SCD.

During VOC there appear to be higher circulating levels of $6kPGF_{1\alpha}$ than in steady state. This finding goes hand in hand with our further observation that prostacyclin-regenerating activity is also relatively increased. The increased prostacyclin-regenerating activity that we have observed in the presence of crisis plasma was not due to an activation of the enzymes cyclooxygenase or prostacyclin synthetase by crisis plasma components, but rather due to an increased release of the substrate, arachidonic acid, from the storage pools for enzymatic conversion. This conclusion was supported by the observations that the crisis plasma mobilized 2–4 times more AA, compared with steady state plasma, and that the ratio of prostanoids produced to the amount of AA mobilized remained the same with both steady state and crisis plasma preparations.

Vaso-occlusion in SCD is a complex and a multifactorial process (1, 2). Increased adherence of sickle red cells to endothelium is suggested to be one of the important events in the initiation and propagation of vaso-occlusion. Although altered erythrocyte membrane surface properties of sickle red cells and plasmatic factors have been reported to play important roles in the adhesiveness of red cells, the adherence process is further potentiated by microvascular hemodynamics and cellular factors including platelet-platelet, platelet-endothelial, and leucocyte-endothelial interactions (1, 2). Prostacyclin (a potent vasodilator and anti-thrombotic eicosanoid) and TxA_2 (a potent vasoconstrictor and prothrombotic eicosanoid) may modulate the pathogenesis of vaso-occlusion by affecting one or more of these processes. The role of cyclooxygenase metabolites including prostacyclin on red cell-endothelial cell adherence has previously been evaluated with conflicting results. Wautier and co-workers found that although exogenously provided prostacyclin stimulated adherence, inhibition of endogenous prostacyclin production by flurbiprofen (a cyclooxygenase inhibitor), had no effect on the adherence process (25, 26). In contrast, other investigators have demonstrated that iloprost (a stable analog of prostacyclin) inhibited the adherence process (27). In a recent study using human retinal microvascular endothelial cells, we have demonstrated that carbacyclin (a stable synthetic prostacyclin analog with biologic properties similar to prostacyclin) had no effect on the adherence process when evaluated at concentrations between 10 pM and 1 μM (28). The conflicting results reported in the literature on the role of prostacyclin on red cell-endothelial cell adherence could be due to differences in the cell systems employed and also due to methodologic differences associated with these studies. Although the levels of prostacyclin are, therefore, seemingly not relevant to the adherence process *per se*, prostacyclin can alter microcirculatory perfusion either through a direct vasodilatory effect or by counteracting the vasoconstrictor effect of TxA_2, and thus by these latter mechanisms potentially affect the vaso-occlusive phenomenon. Prostacyclin may also play an important role in the pathogenesis of vaso-occlusion in SCD by modulating the interaction of platelets and leukocytes with the endothelium, because this eicosanoid inhibits a variety of platelet and leukocyte functions either directly or through its counter effect on the potentiating effects induced by TxA_2 (29). Thus, an abnormal balance between the synthesis of these two vasoactive eicosanoids favoring vasoconstriction may play an important role in the initiation and propagation of VOC in patients with SCD. Although a decreased release of prostacyclin by endothelium has been previously suggested to be one of the factors precipitating VOC, our study demonstrates a relative increase during VOC of $6kPGF_{1\alpha}$ levels over those observed in steady state. However, when the ratio of TxB_2 to $6kPGF_{1\alpha}$ was calculated, a significant increase in this ratio, *i.e.* a preponderance of the vasoconstrictor eicosanoid was noted during VOC compared with steady state. Because activated platelets release TxA_2, the relative increase in plasma TxB_2 observed in this study in patients with SCD during VOC could be a consequence of platelet activation, a pathophysiologic process that has been shown to occur *in vivo* in sickle cell patients during crisis when compared with steady state (30–32).

In summary, we have demonstrated that the $6kPGF_{1\alpha}$ and TxB_2 levels in children with SCD in steady state are significantly lower when compared with those from age-matched controls. Decreased circulatory $6kPGF_{1\alpha}$ levels in patients with SCD in steady state may be due to sickle plasma-induced impairment in endothelial cell prostacyclin production. Although the plasma level of $6kPGF_{1\alpha}$ increases during VOC, the rise is accompanied by an increase in levels of TxB_2 such that the ratio of TxB_2 to $6kPGF_{1\alpha}$ is significantly increased in crisis when compared with the steady state. This imbalance in the

ratio of constrictor to dilator eicosanoids could potentially play a role in the potentiation of VOC in patients with SCD.

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