Reduced sickle erythrocyte dehydration in vivo by endothelin-1 receptor antagonists

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Rivera A. Reduced sickle erythrocyte dehydration in vivo by endothelin-1 receptor antagonists. Am J Physiol Cell Physiol 293: C960–C966, 2007.—Elevated plasma levels of cytokines such as endothelin-1 (ET-1) have been shown to be associated with sickle cell disease (SCD). However, the role of ET-1 in the pathophysiology of SCD is not entirely clear. I now show that treatment of SAD mice, a transgenic mouse model of SCD, with BQ-788 (0.33 mg · kg⁻¹ · day⁻¹ intraperitoneally for 14 days), an ET-1 receptor B (ET_B) antagonist, induced a significant decrease in Gardos channel activity (1.7 ± 0.1 to 1.0 ± 0.4 mmol · 10¹³ cell⁻¹ · h⁻¹, n = 3, P = 0.019) and reduced the erythrocyte density profile by decreasing the mean density (D₅₀, n = 4, P = 0.012). These effects were not observed in mice treated with BQ-123, an ET-1 receptor A (ET_A) antagonist. A mixture of both antagonists induced a similar change in density profile as with BQ-788 alone that was associated with an increase in mean cellular volume and a decrease in corpuscular hemoglobin concentration mean. I also observed in vitro effects of ET-1 on human sickle erythrocyte dehydration that was blocked by BQ-788 and a mixture of ET_B/ET_A antagonists but not by ET_A antagonist alone. These results show that erythrocyte hydration status in vivo is mediated via activation of the ET_B receptor, leading to Gardos channel modulation in SCD.

Sickle erythrocytes have been shown to interact with vascular endothelial cells, stimulating the release of ET-1 and regulating the expression of the ET-1 gene in cultured endothelial cells (17). My coworkers and I have recently shown that the Gardos channel is coupled to ET-1, C-X-C (cytokines), and C-C (chemokines) receptors in both human (27) and mouse sickle erythrocytes (28). ET-1 acts via two major receptor isoforms, ET-1 receptor A (ET_A) and B (ET_B). The ET_A receptors are predominantly present in vascular smooth muscle cells mediating vasoconstriction, whereas ET_B receptors are mostly located in endothelial cells mediating vasodilatation. Both normal and sickle erythrocytes express ET_B receptors (27). In addition, my group has shown that ET-1 enhances the formation of dense cells in vitro. However, it is not known whether ET-1 can modulate Gardos channel activity and modulate erythrocyte cellular volume in vivo. It was hypothesized that blockade of ET-1 receptors in vivo would reduce formation of dehydrated sickle erythrocytes by decreasing Gardos channel activity in SCD.

In this study the in vivo role of ET-1 in sickle cell dehydration in a transgenic sickle mouse model was investigated using ET-1 receptor antagonists. It was observed that ET-1 receptor antagonists in vivo significantly decreased corpuscular hemoglobin concentration mean (CHCM) and the percentage of dense cells while increasing mean cellular volume (MCV) and decreasing Gardos channel activity. These results provide evidence that ET-1 receptor antagonists represent a novel target for the development of new therapeutic strategies to ameliorate the formation of sickle erythrocytes in SCD.

MATERIALS AND METHODS

Drugs and Chemicals

Charybdotoxin (ChTX), ET-1, calpastatin C, BQ-788 (selective ET_B receptor antagonist), and BQ-123 (selective ET_A receptor antagonist) were purchased from Sigma Chemical (St. Louis, MO). All peptides were prepared as indicated by the manufacturer and stored at −20°C for less than 2 mo. The A-23187 ionophore was purchased from Calbiochem (La Jolla, CA). ⁸⁶Rb was purchased from PerkinElmer Life Sciences (Boston, MA). All other reagents were purchased from Sigma Chemical.

Animals

SAD1 mice (8–10 mo old) were kindly provided by Dr. Seth Alper (Beth Israel Hospital and Harvard Medical School, Boston, MA). These studies were part of the research protocol A04-09-131R, which was submitted to and approved by the Children’s Hospital Animal
Treatment of Mice With ET-1 Antagonists

Transgenic SAD sickle cell mice have been widely used by various independent groups for ion transport and cellular dehydration studies (8, 10). Transgenic SAD sickle cell mice were placed on a regime for 14 days with ET-1 antagonist as follows: mice were divided into four groups of four animals per group and intraperitoneally injected for 14 consecutive days. ET-1 antagonists were diluted in sterile normal saline. SAD mice in the group A received sterile saline alone (0.1 ml). SAD mice in group B received 0.1 ml of a 0.1 mg/ml stock solution of the ET<sub>A</sub> antagonist BQ-123 (0.33 mg/kg). SAD mice in group C received the ET<sub>B</sub> antagonist BQ-788 (0.1 ml of 0.1 mg/ml stock). The mice in group D received 0.1 ml of mixed BQ-123 (0.2 mg/ml) and BQ-788 (0.2 mg/ml) dissolved into 1 ml of saline. At day 15, mice were killed, and whole blood was immediately collected into heparinized tubes for further experimentation.

ADVIA Hematological Parameters

Cell blood counts were determined using the ADVIA automated hematology analyzer (Bayer Diagnostics, Tarrytown, NY). Freshly isolated whole blood was collected in heparin-containing tubes, and an aliquot of 250 μL was used to perform the erythrocyte and reticulocyte counts and white blood differential for each sample by using a software program specific for mouse blood.

Measurement of Whole Blood Gardos Channel Activity

Freshly isolated blood collected from treated animals was used to determine the Gardos channel activity in whole blood as previously described in detail (28). Briefly, the whole blood sample was mixed with Tris-MOPS, pH 8.0, at 25°C (final concentration, 20 mM), 1 mM desoxynor-epinephrine, 0.15 MgCl<sub>2</sub>, and 2 CaCl<sub>2</sub> for 3 h (30% hematocrit) under a 10-20% oxygen-5% CO<sub>2</sub> balanced with N<sub>2</sub> gas. The gases were humidified by bubbling in a column that contained an isotonic saline solution at 37°C. The cell suspension was then transferred to an ice bath. Aliquots were obtained at different time points to evaluate the changes in gas levels during the 10-min cycles. Gases were measured using a Co-oximeter (model 845; Ciba-Corning, Medfield, MA).

Erythrocyte Fractionation

Freshly isolated erythrocytes were washed three times with normal saline and suspended at 10% in the same solution. Isotonic Stractan (arabinogalactan) solutions at different densities were prepared as described by Corash (6). Densities were prepared between 1.077 and 1.101 g/dl and carefully layered (1.0 ml of each density) in a 12-ml polypolyethylene tube. Cell suspensions (1 ml) were layered on top of the gradient and placed in a ultracentrifuge (Sorvall RC-28S) for 45 min at 72,000 g (8°C) in a swinging bucket rotor. Cells were divided into three fractions (top, middle, and bottom). The CHCM was measured using an aliquot of each cell fraction in an ADVIA 120 hematology analyzer.

Statistical Analysis

All values are means ± SE. When applicable, nonpaired t-test was used to calculate P values.

RESULTS

In Vivo Studies

Effects of ET-1 receptor antagonists on mouse hematological parameters. The in vivo effects of ET-1 receptor antagonists were studied in SAD transgenic mice. The hematological parameters after 14 days of administration of ET-1 receptor antagonists are shown in Table 1. MCV was significantly increased after treatment with a mixture of the ET-1 receptor antagonists BQ-123 and BQ-788. This change was accompanied by a decrease in CHCM and an increase in the percentage of hypochromic cells. In reticulocytes, similar effects were observed in MCV and CHCM values. These results suggest that ET receptor activation may play an important role in the regulation of cellular volume and hemoglobin concentration in sickle mice.

ET-1 receptor antagonist affects erythrocyte dehydration in vivo in sickle erythrocytes. Treatment with the ET<sub>A</sub> receptor antagonist BQ-123 did not significantly affect the density of the sickle erythrocytes (data not shown). However, BQ-788, an ET<sub>B</sub> receptor antagonist, induced a significant change in the density profile of the entire red cell population (27).

Cyclic Deoxygenation-Oxygenation Experiments In Vitro

Blood was collected into tubes containing heparin and prepared as described in detail previously (27). Briefly, freshly isolated human erythrocytes were incubated in a plasmalike buffer containing (mM) 145 NaCl, 2 KCl, 25 NaHCO<sub>3</sub>, 10 glucose, 0.06 adenosine, 0.04 inosine, 0.15 MgCl<sub>2</sub>, and 2 CaCl<sub>2</sub> for 3 h (30% hematocrit) under a 10-20% oxygen-deoxygenation cycle. Each cycle provided 3 min of 15% O<sub>2</sub>-5% CO<sub>2</sub> balanced with N<sub>2</sub> and 7 min of 5% CO<sub>2</sub> balanced with N<sub>2</sub> gas. The gases were humidified by bubbling in a column that contained an isotonic saline solution at 37°C. The cell suspension was then transferred to an ice bath. Aliquots were obtained at different time points to evaluate the changes in gas levels during the 10-min cycles. Gases were measured using a Co-oximeter (model 845; Ciba-Corning, Medfield, MA).
Table 1. *In vivo* effects of 14 days of treatment with ET-1 receptor antagonists on hematological parameters in SAD mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>BQ-123</th>
<th>BQ-788</th>
<th>Mixture</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Erythrocytes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCV, fL</td>
<td>44.7±0.6</td>
<td>45.3±1.0</td>
<td>43.3±1.5</td>
<td>47.5±0.2*</td>
</tr>
<tr>
<td>CHCM, g/dl</td>
<td>29.4±0.1</td>
<td>29.1±0.1</td>
<td>28.9±0.5</td>
<td>28.5±0.2*</td>
</tr>
<tr>
<td>CH, pg</td>
<td>13.1±0.1</td>
<td>13.1±0.3</td>
<td>12.5±0.6</td>
<td>13.5±0.1</td>
</tr>
<tr>
<td>RDW, %</td>
<td>16.8±0.6</td>
<td>17.8±0.5</td>
<td>17.4±0.8</td>
<td>17.4±0.8</td>
</tr>
<tr>
<td>HDW, g/dl</td>
<td>2.07±0.1</td>
<td>2.42±0.1</td>
<td>2.33±0.1</td>
<td>2.2±0.1</td>
</tr>
<tr>
<td>Hypochromic, %</td>
<td>0.2±0.0</td>
<td>0.43±0.1</td>
<td>0.3±0.1</td>
<td>0.4±0.1*</td>
</tr>
</tbody>
</table>

| **Reticulocytes** | | | | |
| Reticulocytes, % | 3.2±0.8 | 4.2±1.7 | 2.8±0.5 | 3.5±1.1 |
| MCV, fL | 49.8±0.8 | 48.5±1.1 | 48.3±0.3 | 52.1±0.1* |
| CHCM, g/dl | 28.9±0.1 | 28.6±0.2 | 28.5±0.3 | 27.9±0.4* |
| CH, pg | 14.3±0.2 | 13.7±0.3 | 13.5±0.2 | 14.1±0.5 |
| HDW, g/dl | 2.7±0.2 | 3.3±0.4 | 3.2±0.3 | 3.0±0.3 |
| RDW, % | 17.2±1.2 | 20.9±0.9* | 20.5±1.3 | 24.5±5.3 |
| Hypochromic, % | 1.6±0.5 | 2.18±0.5 | 2.38±0.7 | 4.45±2.5 |
| L retic | 74.8±3.0 | 79.2±0.9 | 78.8±2.0 | 79.5±5.0 |
| M retic | 24.0±3.0 | 19.1±0.8 | 20.2±2.3 | 18.9±5.5 |
| H retic | 1.18±0.3 | 1.68±0.25 | 1.0±0.5 | 1.7±0.6 |

Values are means ± SE of 4 animals in each group. Control values represent parameters measured after 14 days of sterile saline injections; experimental values were obtained after 14 days of treatment with the ET-1 receptor antagonists for subtype A (BQ-123) and B (BQ-788) or a mixture of both. Hematological parameters were measured in heparinized whole blood collected immediately after death, using the hematology analyzer ADVIA 120. MCV, mean cellular volume; CHCM, corpuscular hemoglobin concentration mean; CH, cellular hemoglobin; HDW, hemoglobin density width; RDW, red cell density width; hypochromic, percentage of red cells that are hyperchromic. L, M, and H retic are indicators of the reticulocyte maturation profile. *P < 0.035.

The channel activity was measured in ex vivo conditions (Table 1). Together these results are in agreement with the MCV and CHCM changes observed under similar conditions (Table 1).

Gardos channel activity reduced by ET-1 receptor antagonists in vivo. The channel activity was measured in ex vivo heparinized whole blood to preserve the conditions in circulation of SAD mouse erythrocytes. Figure 2 shows that in SAD mice, treatment with BQ-123 antagonist did not significantly change the channel activity, which is in agreement with the lack of an effect on cellular dehydration. However, BQ-788 antagonist treatment significantly reduced the activity of the channel (from 1.7 ± 0.1 to 1.0 ± 0.4 mmol·10¹³ cells⁻¹·min⁻¹, n = 4, P = 0.035). Treatment with a mixture of both antagonists produced an even greater inhibition on the channel activity, which was consistent with the changes observed in the density profile (Fig. 1). Together these results strongly support the hypothesis that the ET-1 receptor antagonists are changing cellular volume via Gardos channel inhibition in vivo.

In Vitro Studies

Cellular dehydration via ET-1 receptor activation. To investigate the pathways by which activation of the ET-1 receptors induces cellular dehydration, freshly isolated sickle erythroid cells were injected intraperitoneally with saline (○), BQ-788 (□), or a mixture of BQ-123/BQ-788 receptor antagonists (□) for 14 days. Mice were killed at day 15, and an erythrocyte density profile was determined using the phthalate method in heparinized whole blood. Data are means ± SE of 4 experimental determinations (n = 4 mice).
erythrocytes were exposed to deoxygenation-oxygenation cycles for 3 h. This procedure has been well described by my group (27) and others (17a, 32a) to induce significant sickle erythrocyte dehydration. Aliquots were removed and gases were determined. Figure 3 shows that during the 10-min cycle, the pH and PCO2 gas levels did not significantly change. Variation of PO2 was observed as expected when the system was switched from O2 to N2. A concomitant increase in the PO2 and O2 saturation (SO2) was observed during those 3 min of oxygenation. After 3 min, PO2 and SO2 started to decrease as the O2 gas was switched off, demonstrating the cycling pattern during the deoxygenation-oxygenation cycle protocol. Under these experimental conditions, erythrocytes from SCD patients were incubated with 500 nM ET-1 in the presence or absence of 1 μM of either ETB (BQ-788) or ETA antagonist (BQ-123) during deoxygenation-oxygenation cycles. The presence of 1 μM BQ-788 induced a leftward shift of the red cell density profile in the presence of 500 nM ET-1 (D50 from 1.1027 to 1.098; data not shown). Figure 4 shows that ET-1-induced cellular dehydration was blocked by BQ-788 and a mixture of ETB and ETA antagonists but not by ETA antagonist alone. These data support the contention that ETB receptors are involved in cellular dehydration in sickle erythrocytes and are consistent with the in vivo findings in mice.

To study the effect of deoxygenation-oxygenation-stimulated ET-1 cellular dehydration on Gardos channel activity, sickle erythrocytes were incubated with ET-1 in the presence or absence of 10 nM ChTX or 10 μM clotrimazole (CLT) during 3 h of deoxygenation-oxygenation cycles (Fig. 5). Both ChTX and CLT are well-described inhibitors of the Gardos channel. ET-1-induced cellular dehydration was inhibited in the presence of either ChTX or CLT, indicating that ET-1-induced cellular dehydration is mediated by the activation of the Gardos channel under deoxygenation-oxygenation conditions. These results support the hypothesis that in vivo, the Gardos channel plays an important role in cellular dehydration.

Protein kinase C (PKC) has been reported by my group (28) and others (14) to activate the Gardos channel in erythrocytes.

Furthermore, activation of PKC activity is seen upon activation of ET-1 receptors. To test whether PKC plays a role in ET-1-induced cellular dehydration, red blood cells were incubated with ET-1 in the presence or absence of 1 μM calphostin C (CC) under deoxygenation-oxygenation conditions for 3 h (Fig. 6). Similar experiments were performed with 1 μM chelerythrine, a structurally distinct PKC inhibitor. Inhibition of PKC activity completely blocked the ET-1-induced cell dehydration in sickle erythrocytes, suggesting that PKC activity is involved in cellular dehydration and dense cell formation in deoxygenation-oxygenation conditions.

**ET-1 receptor activation is hyperresponsive in discocytes from sickle cell anemia subjects.** It has been described that a subpopulation of erythrocytes, discocytes, have the tendency to dehydrate at a higher rate than other subpopulations, leading to...
activation of the Gardos channel through ETB receptor stimulation. In addition, these in vitro studies now show that the channel activity in vitro in the presence of exogenous ET-1 results support and extend previous studies showing increased sickle cell mice treated with ET-1 receptor antagonists. These cellular dehydration and dense cell formation was shown in receptor for ET-1 in promoting cellular dehydration was evaluated in a transgenic model of SCD. Reduction of cellular dehydration and dense cell formation was shown in sickle cell mice treated with ET-1 receptor antagonists. These results support and extend previous studies showing increased channel activity in vitro in the presence of exogenous ET-1 (27). In addition, these in vitro studies now show that the mechanism for this reduction in cellular dehydration is via activation of the Gardos channel through ETB receptor stimulation and a PKC-dependent mechanism. Together, these results suggest an in vivo coupling of sickle cell formation and ETB receptor activation.

A role for ET-1 levels in SCD pathophysiology has been previously suggested. It is possible that suppression of ET-1 expression may lead to changes in erythrocyte volume and, hence, hemoglobin S polymerization in vivo. Consistent with this hypothesis, ET-1 levels, erythrocyte dehydration, and Gardos channel activity are decreased when sickle transgenic animals are fed a diet supplemented with arginine (29). These data suggest that arginine diet supplementation interferes with ET-1 expression and/or release in sickle cell transgenic mice and support the hypothesis that modulation of ET-1 levels is associated with cellular hydration status.

ET-1-induced Gardos channel activity was significantly inhibited when cells were incubated in the presence of the ETB receptor antagonist but not the ETA antagonist (27). However, a mixture of both ETA and ETB antagonist in vivo showed a further decrease in the activity of the Gardos channel from that observed with ETB alone, suggesting that both receptors are involved in the regulation of dense erythrocyte formation. It is possible that the presence of ETA antagonist may enhance the specific effects of the ETB antagonist. This proposal is supported by previous observations showing that a PCR product corresponding to ETB and not ETA was identified in human erythroid precursor cDNA with the use of primers for ETB (27). Alternatively, the presence of ETA receptor antagonists may induce a decrease in the vessel tone that reduces shear stress of the sickle erythrocytes, leading to reduced local production of endogenous ET-1 or/and activation of the endothelium. This effect may lead to the reduction in the overall levels of ET-1 and/or other cytokines in the circulation.

The role of hypoxia in SCD has been well established, but the contribution of cytokines to hypoxia-mediated cellular dehydration is not clear. Hypoxia has been shown to increase ET-1 levels in vivo (23). ET-1 induces an increase in cellular dehydration that leads to enhanced dense cell formation under hypoxic conditions in vitro. It is possible that in sickle transgenic mice, hypoxia-induced dense cell formation in vivo may
be blocked by treatment with these antagonists. This is strongly supported by the effects of these antagonists on hypoxia-induced cellular dehydration in sickle erythrocytes (Fig. 4). Reduction of hypoxia-induced sickle erythrocyte dehydration in vivo by clotrimazole or Mg²⁺ supplementation, both blockers of K⁺ transport, has been shown in a mouse model of SCD (9). However, the effect of these blockers on cytokine and chemokine release has not been investigated.

ET-1 receptor antagonists have been used as a potential therapeutic approach to pulmonary hypertension and asthma. Both conditions have been shown to be associated with increased plasma levels of ET-1 (12, 15, 22) as observed in SCD. In patients with pulmonary hypertension, a pilot study has shown therapeutic efficacy of selective antagonists to ET-1 receptors (1). In addition, in a mouse model of pulmonary hypertension, this therapy showed benefits in both prevention and reversal of hypoxia-induced pulmonary vascular remodeling. Furthermore, treatment with bosentan, a nonsel ective blocker of ET-1 receptors, has been reported to reduce the levels of proinflammatory molecules that are associated with the development of asthma (15). A clinical trial is currently underway to assess the effect of bosentan on pulmonary vascular resistance and exercise capacity in SCD patients (http://clinicaltrials.gov/ct/show/NCT00313196) (5).

Briehl and Christoph (3) found that a 15% change in hemoglobin concentration increased hemoglobin polymerization rate fivefold. Inhibition of the Gardos channel by clotrimazole proved to be effective in the reduction of cellular dehydration in only a subset of sickle cell patients (4, 10). In the present study, only 14 days of ET-1 receptor antagonist treatment changed the CHCM by 3% in sickle cell mice (Table 1). These changes are smaller, albeit significant, compared with what has been observed with clotrimazole, which specifically targets Gardos channel activity, for 14 days in human (4%) or sickle mice (12%) (4, 10). However, it is possible that increasing either the exposure or dose of the ET receptor antagonists will lead to similar effects on CHCM as observed with clotrimazole. Nonetheless, these observations suggest that suppression of specific endothelial cytokine signaling not only mediates reduction of hemoglobin S polymerization but also may interfere with ET-1 effects on the endothelium in vivo. Consistent with my original observations (26), a preliminary report now suggests that treatment with bosentan reduces hypoxic-induced kidney injury in SAD mice (32). Although the mechanism(s) for this effect were not described, they provide further evidence of the role of ET-1 in the pathophysiology of SCD that supports the observations presented in the present study.

A search for novel therapies based on regulation of signaling pathways that affect both K⁺ loss and volume regulation continues to provide valuable insight into the progression of SCD. Consistent with this approach, the new Gardos channel blocker ICA-17043, a clotrimazole analog (34) that is currently in phase III clinical trials, and arginine diet supplementation (30), which is in phase II clinical trials, have been shown to modulate red cell volume in vivo in sickle cell animal models via reduction of Gardos channel activity. The present results suggest an important interplay between the endothelium via endothelium-derived cytokine/chemokines and erythrocyte volume regulation in vivo. Most importantly, these studies demonstrate a role for ET-1 in the pathophysiology of SCD. Thus these results add to the arsenal of potential therapies that are designed to reduce dense cell formation in SCD and suggest that a combination of therapies such as ICA-17043 together with arginine diet supplementation and ET-1 receptor antagonist may have synergistic effects on erythrocyte volume regulation and as such warrant further investigation.

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GRANTS

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REFERENCES


