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Research Article

Mechanisms of Enhanced Vascular Smooth Muscle Contraction Induced by Sickle Erythrocyte Constituents

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Summary: The mechanisms of the increased vascular tone associated with vaso-occlusive crisis of sickle cell disease have not been clearly defined. The goal of the present study was to examine the role of vascular smooth muscle membrane Na+-K+-ATPase enzyme activity as well as nitric oxide synthase inhibition on the contractile responses induced by sickle erythrocyte constituents. 2 mm ring segments of rabbit carotid arterial ring preparations were placed in 20 ml organ baths containing physiological salt solution (PSS) bubbled with 95% O2, 5% CO2, at 37oC and pH 7.4 and isometric contractions recorded, under an initial load of 2g. Arterial rings were exposed to 50 µl of each erythrocyte constituent at an adjusted haematocrit of 0.6. The magnitude of K+-induced relaxation of 10-7 M phenylephrine (PE)-precontracted rings exposed for 30 minutes to K+-free PSS (which inhibits Na+-K+ pump) was estimated in the absence (control) or presence of RBC constituents (ghosts, erythrocytes or haemoglobin solution) from Hb SS subjects. Secondly, the influence of 20-minute exposure of the rings to SS GHOSTS on acetylcholine-induced, endothelium-dependent relaxation of 10-7 M PE phenylephrine-precontraction (in the absence or presence of L-NAME) was evaluated. Our results show that K+-induced relaxation was significantly and differentially attenuated by erythrocyte constituents ($p<0.05$) in the order: SS GHOST > SS HBS > SS RBC. NO synthase inhibition with L-NAME further potentiated the enhanced PE contractions induced by SS GHOSTS and caused a greater attenuation of Ach-induced relaxation (compared with SS GHOSTS alone). The results suggest that SS erythrocyte GHOSTS induce enhancement of vascular smooth muscle tone via impairment of vascular Na+-K+ ATPase enzyme activity as well as attenuate endothelium-dependent relaxation. These functional changes in vascular smooth muscle and endothelial function may contribute to the pathophysiology of vaso-occlusive crisis of sickle cell disease.

Keywords*: Vaso-occlusive crisis, sickle cell disease, Na-K pump, erythrocytes, SS Ghost, nitric oxide, L-NAME.*

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INTRODUCTION

Vascular homeostasis is maintained by the endothelium through the release of endothelium derived relaxing factors (EDRF) including nitric oxide (NO), prostaglandins and endothelium derived hyperpolarizing factor (EDHF) (Reiter and Gladwin, 2003). The released NO could also open K+ channels (Feletou and Vanhoutte, 2006) contributing to the maintenance of adequate vascular function. In many vascular beds, intermediate and small conductance calcium-activated potassium channels play a prominent role in initiating hyperpolarization and modulating electrical conduction along the endothelium (Edwards *et al*., 2010). There is evidence that these channels play a role in the modulation of endothelial calcium signalling and nitric oxide release (Stankevicius *et al*., 2006). K+ channel opening hyperpolarizes smooth muscle, which, leads to vasodilatation by decreasing calcium entry through voltage-dependent Ca2+ channels (Nelson and Quayle, 1995). It was suggested that the relaxation response induced by high concentration of Ca2+ ions in rabbit aortic smooth muscle is endothelium-dependent and possibly mediated by the NO-guanylyl cyclase pathway (Azubuike-Osu and Ebeigbe, 2015). Tonic reduction of smooth muscle tone in vivo is the resultant effect of the vascular release of nitric oxide otherwise called endothelium derived relaxing factor (EDRF) (Furchgott and Zawadski, 1980; Ahmad *et al*., 2018). NO is a labile substance with a half-life of 4-50 seconds (Ebeigbe *et al*., 1990). Nitric oxide (NO) is synthesized from L-arginine by a nitric oxide synthase of the endothelial form in vascular endothelial cells (Tejero *et al*., 2019). Nitric oxide is involved in the regulation of many physiological and pathophysiological functions, including smooth muscle relaxation, platelet inhibition and immune regulation (Nussler and Billiar, 1993; Tykocki *et al*., 2017). NO induces vascular smooth muscle relaxation through the activation of guanylate cyclase leading to the accumulation of guanosine 3',5'-cyclic

monophosphate (cyclic GMP) (Moncada and Higgs, 1993).

Webb and Bohr, (1978) assessed the degree of activity of Na+/K+-ATPase in vascular smooth muscles using potassium-induced relaxations. The endothelium-independent relaxations and hyperpolarizations obtained by re-admitting potassium ions after incubation in potassium-free solution suggest the presence of electrogenic sodium pumping in the smooth muscle of the rat mesenteric artery (Weston *et al*., 2002). There is evidence that Na+-K+ pump activation inhibits Ca2+ mobilization in endothelial cells and endothelium-dependent relaxation (Seol *et al*., 2004) while sodium calcium exchanger contributes to the endothelium-dependent control of vascular contractility (Schneider *et al*., 2002). Stimulation of enzymatic activity of Na+ -K+ ATPase by adenosine 3':5'-cyclic monophosphate (cyclic AMP) may lead to generation of the Na+ gradient necessary to exude Ca2+ via the Na+/Ca2+ exchanger or hyperpolarization of the membrane. An increase in Na+-K+ ATPase activity may induce smooth muscle relaxation by increasing Na+/Ca2+ exchange and reducing the Ca2+ influx through membrane potential-dependent calcium channels (Clausen and Nielsen, 1994). Bondarenko and Sagach, (2006) described interactions between the Na+- K+- pump and relaxations induced by acetylcholine. Ouabain inhibits the Na+-K+-ATPase (Therien and Blostein, 2000) and also induces an intracellular increase in Na+ and Ca2+ concentrations through the inhibition of the Na+/Ca2+-exchanger leading to an increment in vascular tone (Schoner, 2000).

Nitrosylated L-arginine derivatives are mostly used as inhibitors of nitric oxide synthase (Ea-Kim *et al*., 1992). The synthesis of nitric oxide is inhibited by guanidine-substituted L-arginine analogues e.g. L-NNA, L-NAME or L- NMMA. Arginine based nitric oxide synthase (NO) inhibitors constrict isolated, pressurized blood vessels having spontaneous myogenic tone in the absence of intraluminal flow (Undavia *et al*., 2003, Bai *et al*., 2004). The administration of L-arginine analogues in vitro results in a marked inhibition of endothelium dependent relaxations to various agonists including acetylcholine. In vivo, the systemic administration of either L-NMMA or L-NAME causes dose-dependent hypertension and regional vasoconstriction (Rees *et al*., 1990, Gardiner *et al*., 1990) and these effects are attributable to the inhibition of the basal release of nitric oxide from vascular endothelial cells. Feelisch *et al*., (1993) suggested the occurrence of the presynaptic effects of nitric oxide following the release of noradrenaline from sympathetic nerves.

Ghosts are fragmented red blood cell membranes. The red blood cell membrane is composed of: the glycocalyx which is rich in carbohydrates, the lipid bilayer which contains transmembrane proteins and the membrane skeleton which is a structural network of proteins located in the inner surface of the lipid bilayer. Increased wall shear stress, adhesion and the interaction between sickle red blood cells and endothelial cells including an increased viscosity and low oxygen tension are hall marks of endothelial dysfunction in sickle cell disease (Quyyumi *et al*., 1997). According to Stuart *et al*., (1999), the occurrence of vaso-occlusive crisis in sickle cell disease may be result from the increased interaction between sickle erythrocytes and the vascular endothelium. Following intravascular haemolysis is the impairment of nitric oxide bioavailability that results in a diminished blood flow, regional vasoconstriction and a remodelling of the blood vessel (Kato *et al*., 2017). Reduced deformability of red blood cells and accelerated de-oxygenation rates may reduce nitric oxide bioavailability, diminish vasodilatation and oxygen supply (Subashinghe and Spence, 2008). Very importantly, Mosseri *et al*., (1993) reported the attenuation of endothelial nitric oxide-dependent acetylcholine-induced relaxation by sickle erythrocytes. In a previous communication, we have reported a greater red blood cell- induced enhancement of histamine contractions when compared with phenylephrine (in AS and SS haemoglobin genotypes) which suggest a possible role for histamine in the increased vascular tone and vaso-occlusive crisis in sickle cell disease (Azubuike-Osu *et al*., 2017). However, the mechanisms of sodium potassium ATPase and nitric oxide synthase inhibition in sickle cell disease have not been examined. The goal of this study was to establish the mechanisms by which sodium potassium ATPase and nitric oxide synthase inhibition modulate contractile responses following exposure to sickle erythrocyte constituents.

MATERIALS AND METHODS

Blood Samples: Blood samples were obtained from sickle cell subjects attending the University of Benin Teaching Hospital. Erythrocytes (RBCs) were prepared according to the method of Caughley and Watkins, (1985) and as modified by Ajayi and Ebeigbe, (2014) by washing with normal saline, to obtain a clear supernatant. The cells were re-suspended to make up 6-8% packed cell volume. Pre-washed erythrocytes were mixed with distilled water and centrifuged. The supernatant obtained is the haemoglobin solution. To prepare erythrocyte ghosts, 1 ml of blood sample was mixed with 1 ml of distilled water and spun slowly in a centrifuge (1000 rev/min) for 5 minutes. The supernatant was decanted and the resulting sediments washed with normal saline (Ajayi and Ebeigbe, 2014).

Preparation of Arterial Rings: Segments of the carotid arteries were obtained from freshly sacrificed, New Zealand rabbits, cleaned free of adhering connective tissues and cut into 2 mm ring segments. The rings were placed between L-shaped wire loops and suspended in 20 ml organ baths containing physiological salt solution (PSS). The lower loop was attached to the base of the organ bath while the upper end was attached to a Grass model FT03 force transducer connected to a Grass model 7P polygraph (Grass Instrument Co, Quincy, MA, USA). The composition of the normal PSS was (mM): 119 NaCl, 4.7 KCl, 1.6 CaCl2, 1.2 MgSO4, 1.2 KH2PO4, 24.9 NaHCO3 and 11.5 glucose and the composition of K+ free PSS was (mM): 123.7 NaCl, 0 KCl, 1.2 NaH2PO4, 1.2 MgSO4, 1.6 CaCl2, 24.9 NaHCO3 and 11.5 glucose. The PSS was bubbled with 95% O2-5% CO2 gas mixture. The rings were given an initial load of 2g, at 37oC and pH 7.4 and were allowed to equilibrate for 90 minutes.

Experimental protocol:
Two protocols were examined in this study:

K^+-induced relaxation: The rabbit carotid rings were exposed to K+-free PSS in the absence (control) or presence of RBC constituents (ghosts, erythrocytes or haemoglobin solution) from Hb SS subjects for 30 minutes and contracted with 10-7 M PE. Thereafter, re-introduction of K+ (5 mM) to the bath caused relaxation due to increased Na-K pump activity and hyperpolarization of the membrane. The influence of erythrocyte constituents from Hb genotype SS subjects on the sodium-potassium pump activity was examined by estimating the magnitude of such K+-induced relaxation in control rings as well as in rings exposed (separately), to erythrocyte ghosts, red blood cells and haemoglobin solution.

Nitric oxide synthase inhibition: The rabbit carotid rings were exposed to normal PSS in the absence (control) or presence of SS Ghosts for 30 minutes, with or without 10-5 M L-NAME (10-minute exposure). Thereafter, rings were precontracted with EC70 (M) Phenylephrine. Dose-response tests to phenylephrine and acetylcholine were carried out in control rings and following exposure to SS Ghosts. Contractile responses to PE were obtained by cumulative additions of the drug to the organ bath; the next higher concentration was added when response to the previous concentration had stabilized.
Endothelium-dependent acetylcholine-induced relaxation responses were obtained by addition of 10-5M Ach to rings pre-contracted with EC70 (M) Phenylephrine in control rings as well as in rings exposed to SS Ghosts, with or without L-NAME.

Data analysis
Results are presented as means ± SEM. Comparison of the means was done using student's t-test and the MicroCal Origin 5.0 software. A p value < 0.05 was considered statistically significant. EC70 and IC50 (M) values represent the concentrations which produced 70% contraction and 50% inhibition, respectively.

Chemicals: The chemicals used were phenylephrine hydrochloride, acetylcholine, N G-nitro-L-arginine methyl ester (L-NAME), salts, all purchased from Sigma Aldrich.

RESULTS

Dose response to Phenylephrine and Acetylcholine
Cumulative increases in phenylephrine concentrations resulted in concentration-dependent contractions which were significantly enhanced in rings exposed to SS Ghosts (Fig. 1) when compared with control rings that were not exposed to the ghosts. On the other hand, the endothelium-dependent relaxation responses induced by acetylcholine were significantly attenuated following exposure of the rings to SS Ghosts.

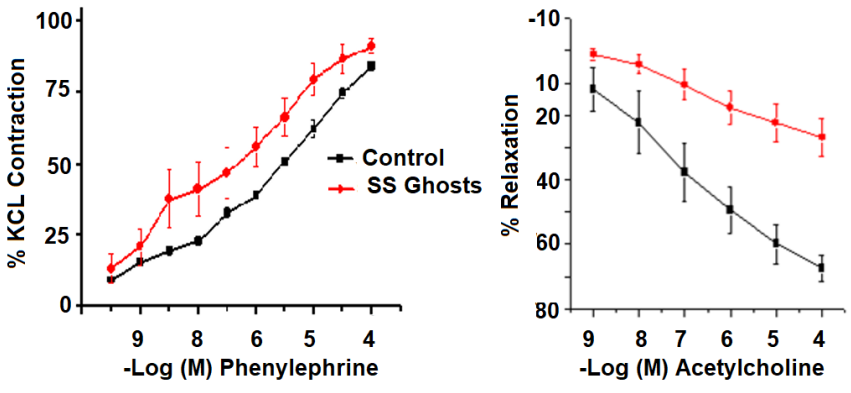

Figure 1:
Concentration-response curves for phenylephrine (PE) contraction (left) and acetylcholine-induced relaxation (right) of rabbit carotid arterial rings in the absence of ghosts (control, n=8; n=7) and following exposure to erythrocyte ghosts from Hb SS subjects (n= 6). Results are expressed as means ± SEM. PE contractions were significantly enhanced by ghosts from Hb SS subjects particularly, at higher doses while SS ghosts attenuated acetylcholine relaxation significantly when compared to the control, $p < 0.05$.

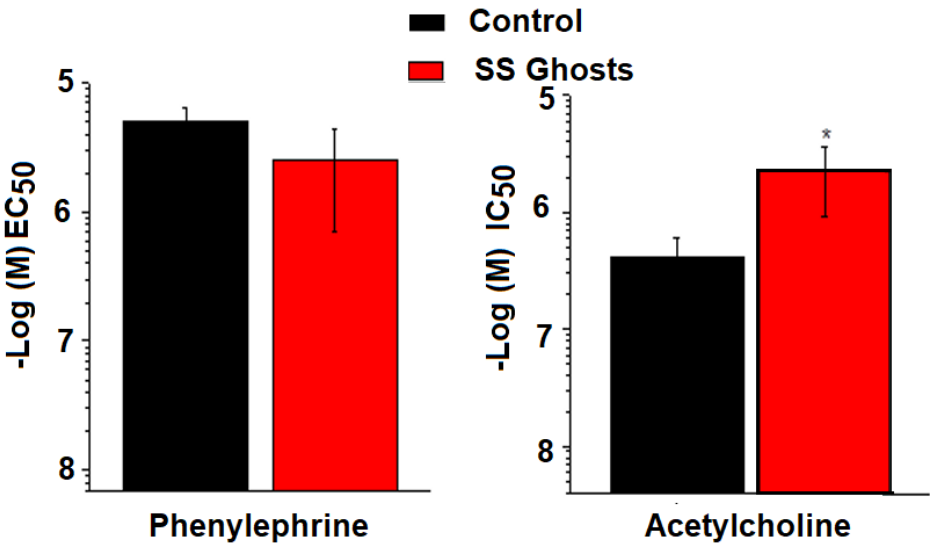

Figure 2:
Comparison of the mean EC_{50} (M) values of phenylephrine contraction and IC_{50} of acetylcholine-induced relaxation in the absence of SS Ghosts (control, n=7; n=8) and following exposure to SS Ghosts (n=6; n=7). While exposure to SS Ghosts enhanced phenylephrine contraction (lower EC_{50}); SS Ghosts significantly attenuated relaxation responses to acetylcholine. Asterisks* denote significant differences, $p < 0.05$.

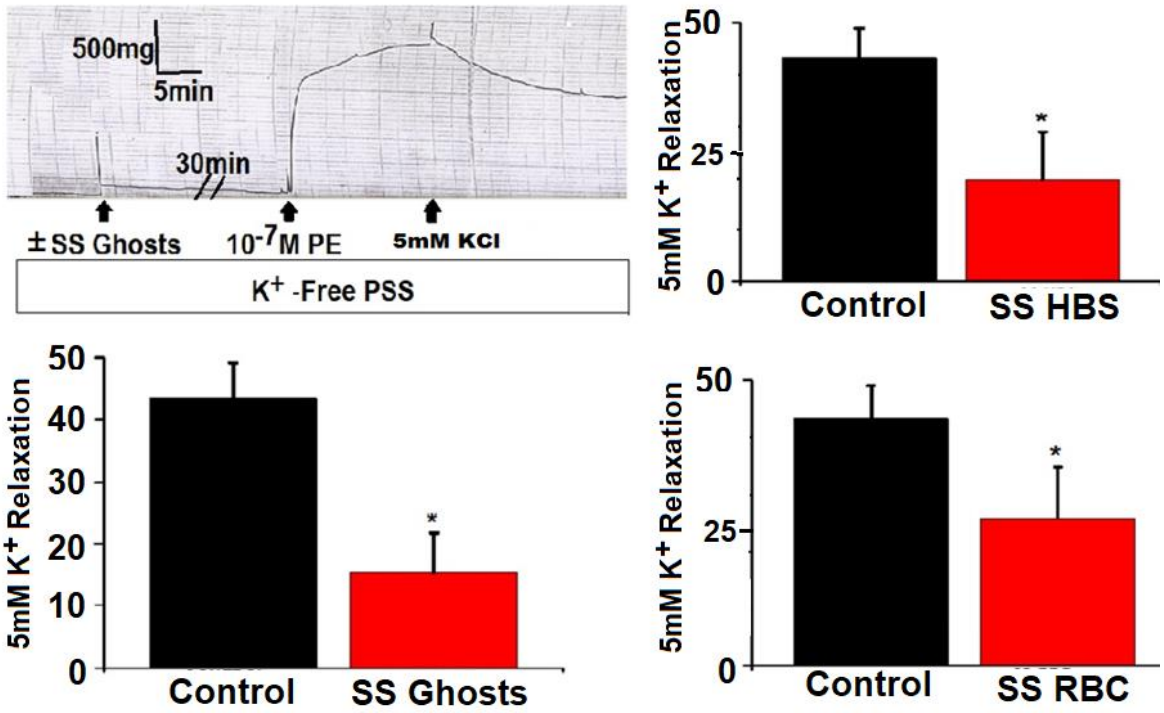

Figure 3:
The tracing (top left) explains the experimental protocol using SS Ghost as an example. The histograms show the magnitudes of 5 mM K^+-induced-relaxation responses of 10^{-7} M PE pre-contracted rabbit carotid arterial rings in the absence of erythrocyte constituents (control, n=7) and following exposure to SS Ghosts, SS HBS and SS RBC from HbSS subjects in K^+ free PSS; n=13, 8, 6 respectively. All data were expressed as means ± SEM. *denotes significant difference, $p < 0.05$.

The respective EC_{50} and IC_{50} values for phenylephrine contraction and acetylcholine relaxation (in the absence or presence of SS Ghosts) are shown in Fig. 2.

Potassium-induced Relaxation: The protocol for studying potassium-induced relaxation is illustrated in the tracing on Figure 3 which shows a typical experimental recording. Re-introduction of 5mM K+ to rings precontracted with PE in K+-free medium results in rapid relaxations. The influence of erythrocyte constituents (SS Hb solution, SS RBC and SS Ghosts) on the magnitude of relaxation responses induced by re-introduction of 5mM K+ (during K+-free exposure) is summarized in the histograms below. K+-induced relaxation responses were attenuated in the order: SS Ghosts > SS HbS > SS RBC.

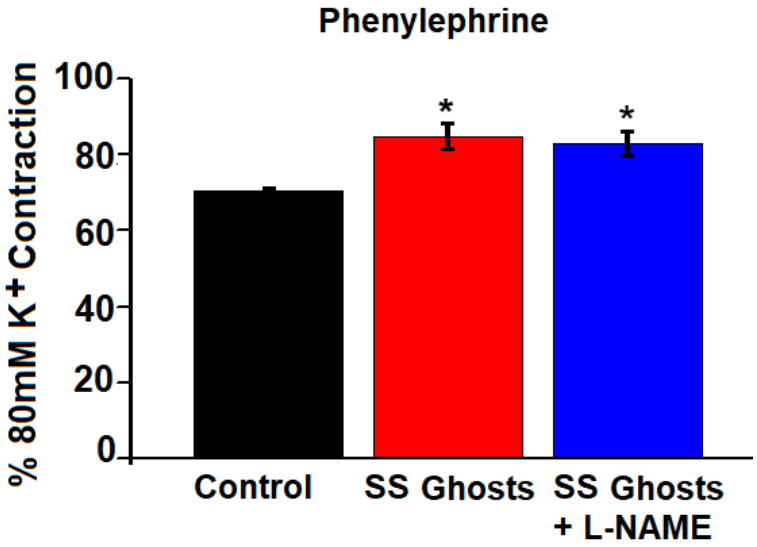

Fig. 4: Contractile responses to 10^{-7} M phenylephrine (PE) in the absence of SS Ghosts (control, n=8), following exposure to ghosts from Hb SS subjects (SS Ghost, n=7) and following exposure to both SS Ghosts and 10^{-5} M L-NAME (SS Ghosts + L-NAME, n=6). *denotes significant difference, $p < 0.05$. Both SS Ghosts and SS Ghosts + L-NAME significantly enhanced PE contractions. Values are means ± SEM.

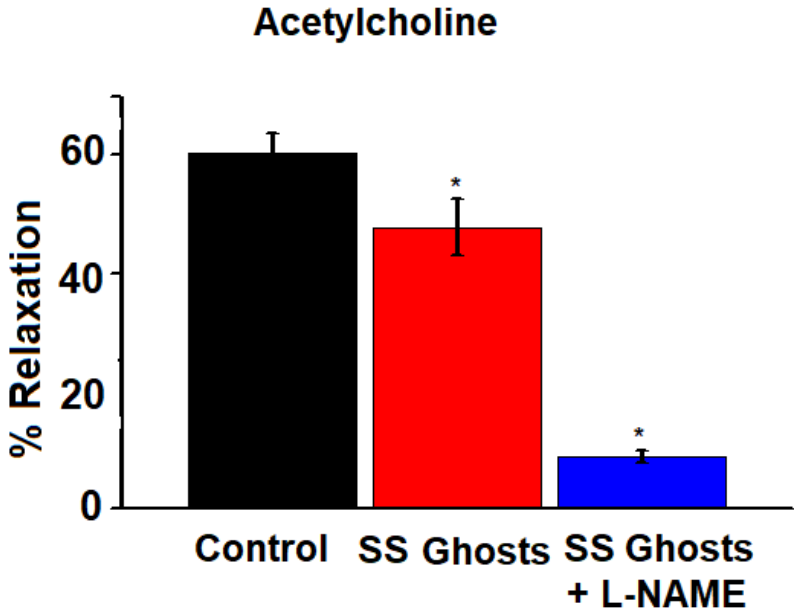

Fig. 5: Relaxation responses to 10^{-5} M acetylcholine in carotid arterial rings precontracted with 10^{-7} M phenylephrine, in the absence of blood constituents (control, n=8), in the presence of SS Ghosts, n=7 and following exposure to both SS Ghosts and L-NAME, n=6. The relaxation responses were significantly attenuated by SS Ghosts + L-NAME. *denotes significant difference, $p < 0.05$. Values are as means ± SEM.

NO Synthase Inhibition: Since SS Ghosts elicited the greatest attenuation of K^+-induced relaxation as compared with SS RBC and SS HbS, we further examined the effects of SS Ghosts and NO synthase inhibition with L-NAME, on phenylephrine contraction as well as endothelium-dependent acetylcholine-induced relaxation responses. L-NAME enhanced the increased contraction induced by SS Ghosts (Fig. 4) but further attenuated the decreased acetylcholine-induced relaxation responses induced by SS Ghosts (Fig. 5).

DISCUSSION

The results of the present study provide support for the notion that enhanced vascular smooth muscle tone as well as impaired endothelium-dependent relaxation following exposure of carotid arterial smooth muscle to erythrocyte constituents, may contribute to the pathophysiology of vaso-occlusive crisis in sickle cell disease.

Vasoactive agents that modulate vascular smooth muscle tone have been well reported to act via the vasoconstrictor or vasorelaxation pathway (Webb, 2003). In this study, we have employed two (2)

protocols: contractile response to phenylephrine as well as relaxation response to acetylcholine and K^+, to examine possible mechanisms by which erythrocyte constituents may alter the reactivity of rabbit carotid arterial smooth muscle.

The enhancement of phenylephrine contractions (Figs. 1 and 4) by exposure of the rings to SS Ghosts and the further potentiation by inhibition of NO synthase with (ω)-nitro-L-arginine methyl ester (L-NAME) suggests that SS Ghosts and reduced NO levels/availability work synergistically to elicit increased vascular tone. L-NAME is known to inhibit the synthesis of nitric oxide by inhibiting nitric oxide synthase (Rees *et al*., 1990). As reported by Ea-Kim *et al*., (1992), inhibition of NO can induce an endothelium-dependent and enantiomerically specific contraction of the vascular smooth muscle, confirming that there is a continuous use for L-arginine for the basal release of NO. Endothelial dysfunction that occurs in sickle cell disease (SCD) may prevent the arteries of patients with SCD from adapting to chronic or acute shear stress elevations (Belhassen *et al*., 2000) hence sickle red blood cell membranes are less deformable. The deformability of red blood cell membranes depends on cellular properties like surface to volume ratio, intracellular calcium concentration, activation of calcium ATPase, sodium-potassium ATPase activation, pH or messenger like prostaglandins and importantly, nitric oxide (Bruckdorfer, 2005).

As shown in Fig. 3 (tracing), K^+-free exposure blocks the Na-K pump, resulting in increased intracellular Na^+ and depolarization, increased calcium influx and contraction. Potassium-induced relaxation following K^+-free exposure results from electrogenic Na^+ pumping and hyperpolarization (Webb and Bohr, 1978) and is an indirect indicator of the Na^+-K^+ ATPase enzyme activity. The Na-K pump is blocked by the cardiac glycoside, ouabain as well as by exposure to K^+-free medium. Studies by various workers have established a relationship between Na^+-K^+ATPase activity, endothelium-dependent relaxation and intracellular calcium concentration. McCaron and Halpern, (1990) reported that Na^+-K^+ pump activation relaxes vascular smooth muscle by hyperpolarizing the membrane. Na^+-K^+ pump inhibition contracts vascular smooth muscle through activating the reverse mode of the Na^+/Ca^{2+} exchanger by Na^+ accumulation in the myoplasm (Fernandez-Alfonso *et al*., 1992). Woolfson and Poston, (1991) reported that Na^+-K^+ pump inhibition affects the synthesis or release of endothelium-derived relaxing factors. Observations from the present study interestingly show that impairment of Na^+-K^+ pump was greatest in rabbit carotid arterial rings exposed to SS Ghosts in comparison with SS HbS and SS RBC. The results also suggest that SS erythrocyte Ghosts mediate the impairment of vascular Na^+-K^+ATPase enzyme activity as well as endothelial dysfunction in sickle cell disease.

The greater attenuation by a combination of SS Ghosts and L-NAME of acetylcholine-induced relaxation is in line with a possible effect of SS Ghosts in mediating endothelial dysfunction in sickle cell disease. It is therefore reasonable to suggest that SS Ghosts inactivate NO function by impairing nitric oxide synthase, thus, providing a role for nitric oxide in modulating vascular reactivity changes induced by exposure to sickle cell ghosts.

In conclusion, our study suggests that sickle erythrocyte ghosts might increase vasoconstriction and vasospasm that characterize vaso-occlusive crisis in sickle cell disease by not only impairing vascular Na^+-K^+ATPase enzyme activity but also by impairment of vascular endothelial function.

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