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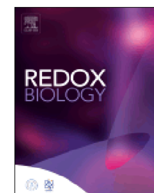

Research paper

Potential therapeutic action of nitrite in sickle cell disease

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ABSTRACT

Sickle cell disease is caused by a mutant form of hemoglobin that polymerizes under hypoxic conditions, increasing rigidity, fragility, calcium influx-mediated dehydration, and adhesivity of red blood cells. Increased red cell fragility results in hemolysis, which reduces nitric oxide (NO) bioavailability, and induces platelet activation and inflammation leading to adhesion of circulating blood cells. Nitric Oxide inhibits adhesion and platelet activation. Nitrite has emerged as an attractive therapeutic agent that targets delivery of NO activity to areas of hypoxia through bioactivation by deoxygenated red blood cell hemoglobin. In this study, we demonstrate anti-platelet activity of nitrite at doses achievable through dietary interventions with comparison to similar doses with other NO donating agents. Unlike other NO donating agents, nitrite activity is shown to be potentiated in the presence of red blood cells in hypoxic conditions. We also show that nitrite reduces calcium associated loss of phospholipid asymmetry that is associated with increased red cell adhesion, and that red cell deformability is also improved. We show that nitrite inhibits red cell adhesion in a microfluidic flow-channel assay after endothelial cell activation. In further investigations, we show that leukocyte and platelet adhesion is blunted in nitrite-fed wild type mice compared to control after either lipopolysaccharide- or hemolysis-induced inflammation. Moreover, we demonstrate that nitrite treatment results in a reduction in adhesion of circulating blood cells and reduced red blood cell hemolysis in humanized transgenic sickle cell mice subjected to local hypoxia. These data suggest that nitrite is an effective anti-platelet and anti-adhesion agent that is activated by red blood cells, with enhanced potency under physiological hypoxia and in venous blood that may be useful therapeutically.

1. Introduction

Sickle cell disease (SCD) is the most common genetic disease affecting about 2600 births a year in North America, while over 80% of patients are in Africa [1]. It is caused by a single mutation in the beta subunit of hemoglobin (glu → val) [2] that results in hemoglobin (Hb) polymerization under hypoxic conditions [3]. Polymerization distorts the shape of the red blood cell (RBC), a process known as sickling. It has

Abbreviations: Hb, hemoglobin; SCD, sickle cell disease; eNOS, endothelial nitric oxide synthase; L-NAME, L-NG-monomethyl arginine citrate; GSNO, S-nitrosoglutahthione; PRP, platelet rich plasma; RBC, red blood cell; DEANO, Diethylamine NONOate; HUVEC, human umbilical vein endothelial cells; LPS, lipopolysaccharide; WT, wild type; EPR, electron paramagnetic resonance; PBS, phosphate buffered saline; IVM, intravital microscopy; Hct, hematocrit; CFSE, Carboxyfluorescein succinimidyl ester; DI_{max}, maximum deformability index; Osm_{min}, minimum osmolality; Osm_{max}, maximum osmolality

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been shown that repeated cycles of sickling and unsickling lead to activation of the Gardos channel ($K_{Ca}3.1$), a calcium-activated potassium efflux channel, which is a major factor in RBC dehydration and reduced deformability and increased fragility [4,5]. Dehydration increases the intracellular concentration of Hb which enhances the rate of polymerization [6–8]. Red blood cell fragility results in hemolysis in SCD and other hemolytic diseases [9,10], diabetes, and transfusion of older stored blood; which in turn can lead directly to inflammation [11–13], and platelet activation [14–20]. There is abundant in vitro and animal data from multiple labs that support the notion that inflammation, cell adhesion, and platelet activation contribute to pathology in SCD [14,20–25], as well as other diseased conditions including atherosclerosis [26,27], diabetes [28], and sepsis [29–33]. Platelet activation can both initiate and accelerate inflammation as well as be induced by inflammation [26,27]. The role of platelet activation and its aggregation with neutrophils, in addition to adhesion of circulating blood cells to the endothelium, play major roles in pathology of SCD [25,34].

Since cell-free hemoglobin, released upon hemolysis, scavenges NO faster than that encapsulated in the RBC, low NO bioavailability contributes to pathology in sickle cell and other diseases [9,10]. Low NO bioavailability has been shown to promote platelet activation [35–37] and to increase cellular adhesion [38–40]. Inhibition of endothelial nitric oxide synthase (eNOS) with L-NG-monomethyl Arginine citrate (L-NAME) increased leukocyte rolling and adhesion [41]. In another study, blocking NO synthase led to an increase in platelet activation [42]. As a result, several different NO donating drugs have been explored as anti-platelet agents including NONOates, S-nitrosothiols, and organic nitrates [43,44]. Whereas organic nitrates have other vascular effects, S-nitrosoglutahthione (GSNO) has been shown to have some platelet specificity that has been demonstrated in clinical trials [45,46]. The potency of S-nitrosothiols and NO itself has been shown to be higher in platelet rich plasma (PRP) than when RBCs are present [20,47–49].

Although once considered to be biologically inert in human physiology in terms of production of NO activity [50], nitrite is now recognized as a storage pool for NO activity that is harnessed under hypoxia [51]. Resting plasma nitrite is at least hundreds times more abundant than steady state plasma NO [52,53] and, preferentially under hypoxia, produces effects characteristic of those attributed to NO through conversion to other NO congeners or NO itself [54]. Unlike other NO donors, nitrite was thought to be ineffective in limiting cytokines associated with circulating blood cell adhesion [55]. It is now widely accepted that nitrite can be bioactivated by red blood cell (RBC) hemoglobin [49,56–58]. Contrary to the case of NONOates and GSNO, Srihirun, Schechter and coworkers showed that in the presence, but not absence, of RBCs nitrite inhibits platelet activation, and this activity is potentiated in hypoxia and inhibited by NO scavenging agents [49]. Antiplatelet effects of nitrite have been demonstrated in rodent [59] and human studies [60,61]. In addition, nitrite in the drinking water of mice reduced leukocyte adhesion that was due to a high cholesterol diet [62]. Nitrite therapeutics has gained increased attention through the nitrate-nitrite-NO cycle where dietary nitrate is partially reduced to nitrite by oral bacteria, nitrate and nitrite are released into blood, and then nitrate is taken back into the mouth by salivary glands [53].

In this paper, we have studied several potentially beneficial effects of nitrite as a therapeutic for SCD. We examined the potency of nitrite in terms of anti-platelet activity compared to other NO donors in vitro and then examined the ability of nitrite to reduce platelet and leukocyte adhesion in inflammation, hemolysis and sickle cell murine models in vivo. We directly compare the anti-platelet activity of 1 μM concentration of each, GSNO, Diethylamine NONOate (DEANO) and nitrite since plasma nitrite levels go up to about 1 μM on average after consumption of high nitrate-containing foods or beverages due to reduction of nitrate to nitrite by commensal oral bacteria [53,63]. Thus, our use of 1 μM concentration tests the effects of nitrite at levels achievable after dietary intervention but at concentrations of NO and GSNO that are about 1000 times greater than normal physiological levels [52,63,64]. Nitrite was also shown to improve RBC properties following calcium leak. We also explored the ability of nitrite to inhibit RBC adhesion in a microchannel assay. Finally, the potency of nitrite observed in vitro is recapitulated in our in vivo murine models where we used intravital microscopy to assess the effect of nitrite feeding on platelet and leukocyte adhesion.

2. Materials and methods

2.1. Materials

Blood was obtained from volunteers after obtaining written informed consent with all procedures having been approved by the Institutional Review Board of Wake Forest University Health Sciences. Pac-1 and CD61 monoclonal antibodies were purchased from Becton Dickinson Immunocytometry systems. FITC Annexin V Apoptosis Detection Kit l catalogue number 556547 was purchased from BD Pharmingen. DEANO was purchased from Cayman Chemicals and prepared in 0.01 N NaOH before dilution into PRP. GSNO was prepared by combination of acidified nitrite and glutathione followed by precipitation and drying under vacuum and stored at −80 °C until use. Concentrations of GSNO were determined spectrophotometrically. The amount of NO released from DEANO was determined by measuring the amount of methemoglobin formed from oxygenated hemoglobin using electron paramagnetic resonance (EPR) spectroscopy in a Bruker EMX X-band spectrophotometer at a temperature of 5 K. Lipopolysaccharide (E coli O111:B4 LPS), sodium nitrite and all other chemicals were purchased from Sigma/Aldrich (St. Louis, MO). Calcium ionophore A23187, acid free catalogue number 1234 was purchased from TOCRIS. Human umbilical endothelial cells (HUVEC) were purchased from LONZA. Micro- slide y-shaped flow chamber slides were purchased from ibidi (Germany). The wild type (WT: C57Bl/6; 6–8 week old) and Townes (B6;129-$Hba^{tm1(HBA)Tow}$ $Hbb^{tm2(HBG1,HBB*)Tow}$/$Hbb^{tm3(HBG1,HBB)Tow}$/J: 6–8 week old) mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). The animal studies were approved by the Institutional Animal Care and Use Committee of the Wake Forest School of Medicine and experiments were performed according to the NIH guidelines.

2.2. Methods

2.2.1. In vitro platelet assays

Platelet and RBC preparations to measure platelet activation were performed as described previously [57,58]. After discarding the first sodium citrate vacutainer of drawn blood into sodium citrate tubes (a common practice to avoid basal platelet activation), the blood was centrifuged at 120*g* for 10 min to collect PRP. RBCs were washed with phosphate buffered saline (PBS, pH 7.4) and deoxygenated for experiments in hypoxia by gentle rocking under a closed atmosphere with positive nitrogen pressure. The oxygenation state of the RBC hemoglobin (Hb) in all experiments was determined by measuring the near infra-red absorbance using a Cary 100 Varian spectrophotometer equipped with an integrating sphere to collect scattered light and fitting to basis spectra as described previously [65]. PRP was diluted into PBS 1:7; when present RBCs were then added to a final hematocrit of 15%, and the freshly prepared NO donors (all at 1 μM final concentration) were added immediately and incubated for five minutes at 37 °C, after which ADP (1 μM) was added. This mixture was incubated for another six minutes and 10 μl was taken and mixed with Pac-1 and CD61 antibodies for 15 min at room temperature in the dark and then fixed in 1% buffered formalin. Platelet activation was quantified using a BD Bioscience FACS Calibur flow cytometer.

2.2.2. Platelet aggregation assay

Whole venous blood was used to measure platelet aggregation,

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