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Sodium nitrite potentiates renal oxidative stress and injury in hemoglobin exposed guinea pigs

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ABSTRACT

Methemoglobin-forming drugs, such as sodium nitrite ($NaNO_2$), may exacerbate oxidative toxicity under certain chronic or acute hemolytic settings. In this study, we evaluated markers of renal oxidative stress and injury in guinea pigs exposed to extracellular hemoglobin (Hb) followed by $NaNO_2$ at doses sufficient to simulate clinically relevant acute methemoglobinemia. $NaNO_2$ induced rapid and extensive oxidation of plasma Hb in this model. This was accompanied by increased renal expression of the oxidative response effectors nuclear factor erythroid 2-derived-factor 2 (Nrf-2) and heme oxygenase-1 (HO-1), elevated non-heme iron deposition, lipid peroxidation, interstitial inflammatory cell activation, increased expression of tubular injury markers kidney injury-1 marker (KIM-1) and liver-fatty acid binding protein (L-FABP), podocyte injury, and cell death. Importantly, these indicators of renal oxidative stress and injury were minimal or absent following infusion of Hb or $NaNO_2$ alone. Together, these results suggest that the exposure to $NaNO_2$ in settings associated with increased extracellular Hb may potentiate acute renal toxicity via processes that are independent of $NaNO_2$ induced erythrocyte methemoglobinemia.

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1. Introduction

Nitrite/nitrate salts are methemoglobin (metHb)-forming agents used to treat a number of medical conditions including cyanide poisoning, thermal injury, and sickle cell disease-associated leg ulcers (Baskin et al., 1992; Garner and Heppell, 2005; Minniti et al., 2014). Nitrites can oxidize Hb directly whereas nitrates are converted to nitrite in the gut. Low dose sodium nitrite ($NaNO_2$) administration is also being evaluated for treatment of pulmonary hypertension, post-hemorrhagic cerebral vasospasm, and myocardial infarction (Butler and Feelisch, 2008; Nossaman et al., 2010; Omar and Webb, 2014; Sinha et al., 2008). Therapeutic use of nitrite/nitrate salts can sometimes produce reversible cyanosis, typically associated with intra-erythrocyte metHb levels in a range of 30–50% (Attof et al., 2006; Baskin et al., 1992; Su et al., 2012; Umbreit, 2007; Wright et al., 1999). Under certain hemolytic conditions such as thermal injury, infection, sickle cell disease, and other hemoglobinopathies, free plasma Hb could be subjected to significant oxidation in cases of high nitrite exposure (Berkowitz, 1991; Francis et al., 2013; Hartmann et al., 1966; Lawrence and Atac, 1992; Rother et al., 2005). Individuals with glucose-6-phosphate dehydrogenase (G6PD) deficiency are also at a higher risk of developing hemolytic crisis when treated with metHb-forming drugs like $NaNO_2$ (Francis et al., 2013; Wright et al., 1999). Despite the extensive literature on the role of nitrites/nitrates in health and disease, the toxicological consequences of $NaNO_2$ exposure in subjects with intermittently elevated extracellular Hb are not well-defined (Butler and Feelisch, 2008; Nossaman et al., 2010; Omar and Webb, 2014; Sinha et al., 2008).

Inside erythrocytes, Hb is protected by antioxidant enzymes (e.g., superoxide dismutase, catalase), low molecular weight molecules (e.g., glutathione and ascorbate), and metHb reductase systems that preserve the oxygen-carrying reduced oxidation state (ferrous, Fe^{2+}) of Hb. In contrast, Hb released from erythrocytes is more susceptible to chemical oxidation to metHb (ferric, Fe^{3+}), ferryl Hb intermediate ($HbFe^{4+}$), hemichromes, and further breakdown to free heme or iron (Buehler et al., 2010; Schaer et al., 2013). These Hb redox reactions and breakdown products can

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trigger oxidative damage in the vasculature and tissues, although the mechanisms are not completely understood. Intravascular and tissue reductive systems as well as heme catabolic pathways can limit the progression of Hb-catalyzed oxidative damage, but these systems can be overwhelmed under certain settings of increased extracellular Hb exposure (Buehler et al., 2010; Schaer et al., 2013).

Previous non-clinical studies have reported the potential toxicity induced by $NaNO_2$ in the presence of free or modified Hbs in the central nervous system (CNS) and lungs of guinea pigs and swine, respectively (Buehler et al., 2011; Moon-Massat et al., 2012). In the present study, we hypothesized that acute $NaNO_2$ administration sufficient to induce a 45–60% rise in erythrocyte metHb levels in guinea pigs would cause extensive plasma metHb formation and subsequent end-organ injury in a model of extracellular Hb exposure. Here, we examined the kidney as a key site of toxicity given its sensitivity to oxidative stress and its role as a primary clearance organ for free Hb and $NaNO_2$. The guinea pig was selected as a relevant model in this setting because, like humans, this species lacks the rate limiting enzyme in the biosynthesis of ascorbic acid, which plays an essential role in regulating Hb oxidation in plasma (Buehler et al., 2007; Butt et al., 2010; Nandi et al., 1997). The present findings demonstrate that the exposure to $NaNO_2$ doses sufficient to simulate acute methemoglobinemia also produces renal oxidative stress and injury in guinea pigs co-exposed to extracellular Hb. Our data suggest that attention to signs and symptoms of acute kidney injury are warranted in circumstances when nitrite treatments lead to the oxidation of extracellular Hb.

2. Materials and methods

2.1. Materials

Human stroma free Hb was prepared as a 6% solution in 0.9% sodium chloride with an endotoxin content of less than 1 EU/mL (Elmer et al., 2009). The Hb solution contained >95% ferrous Hb ($HbFe^{2+}$). $NaNO_2$ was prepared daily as a 20 mg/mL solution in 0.9% sodium chloride solution and kept on ice in a light resistant container over the course of each experiment. All solutions were filtered using a 0.22 μm syringe filter prior to administration.

2.2. Antibodies

Rabbit polyclonal anti-heme oxygenase-1 (HO-1) was purchased from Assay Designs (Ann Arbor, MI). Rabbit polyclonal antibodies to nuclear factor erythroid 2-related factor 2 (Nrf-2) and β-actin were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA). Rabbit polyclonal antibody to histone H3 was obtained from Cell Signaling Technologies (Beverly, MA). Goat polyclonal antibodies to ionized calcium binding adaptor molecule 1 (Iba-1), kidney injury-1 marker (KIM-1), liver-fatty acid binding protein (L-FABP), rabbit polyclonal antibody to Wilms Tumor protein (WT-1), rabbit monoclonal antibody to podocin, and mouse monoclonal antibody to Hexanoyl-Lys adducts (HEL) were obtained from Abcam (Cambridge, MA). Mouse monoclonal antibody to 4-Hydroxy-2-Nonenal adducts (4-HNE clone J-2) was purchased from Genox (Baltimore, MD).

2.3. Animal experiments

Male Hartley guinea pigs (Charles Rivers Laboratories, Wilmington, MA) were acclimated for one week upon arrival to the FDA/Center for Biologics Evaluation and Research (CBER) animal care facility. All animals weighed 400–450 g before surgery. Animal protocols were approved by the FDA/CBER Institutional Animal Care and Use Committee with all experimental procedures performed in adherence to the National Institutes of Health guidelines on the use of experimental animals. Surgical preparation was performed as previously described (Buehler et al., 2007). Hb (300 mg, 5 mL) was administered as a 20% blood volume top-load infusion via a jugular catheter. After 30 min $NaNO_2$ was administered via the jugular catheter at a rate of 0.1 mg/min over a period of 2 h (12.5 mg cumulative dose). Additional groups included surgically-instrumented sham control animals that did not receive any treatment, and animals infused with either Hb alone or the identical $NaNO_2$ dosing scheme alone. At 4 and 24 h after Hb infusion, animals were humanely euthanized with Euthasol and perfused via the arterial catheter with 60 mL cold saline to remove blood. Kidneys were excised and fixed in 10% formalin or frozen at −80 °C.

2.4. Measurement of Hb concentrations

Total, ferrous (Fe^{2+}), and ferric (Fe^{3+}) Hb concentrations were determined using a photodiode array spectrophotometer (Model 8453 Hewlett Packard, Palo Alto, CA) and multi-component analysis based on the extinction coefficients for each Hb species (Buehler et al., 2007). Baseline plasma samples for each animal were used to correct for background interference and turbidity. For erythrocytes, packed cells (0.5 mL) were lysed with 0.5 mL cold sterile water for injection. Samples were allowed to sit on ice for one hour and centrifuged at 6000 rpm for 20 min. Hb was separated from membrane and measured as described above. To account for water lysis volume (0.5 mL) on actual concentration, a dilution factor of 2 was applied.

2.5. Western blotting

Kidney whole cell extracts were prepared in ice cold RIPA lysis buffer (Millipore, Temecula, CA) containing protease inhibitors (Roche, Indianapolis, IN). Nuclear lysates were prepared with the NE-PER kit as per the recommended protocol (Thermo Scientific, Rockford, IL). Lysates were separated on 4–12% Tris-glycine gels (Invitrogen, Inc., Carlsbad, CA) and transferred to polyvinyl difluoride (PVDF) membranes (Bio-Rad Labs, Hercules, CA). The membranes were blocked in Tris-buffered saline with 0.1% Tween 20 (TBST) containing 5% nonfat dry milk and incubated with primary antibodies against Nrf-2, HO-1, histone H3, and β-actin. After multiple TBST washes, the membranes were incubated with appropriate HRP-conjugated secondary antibodies and developed using the ECL Plus chemiluminescence kit (Amersham, Arlington Heights, IL). Densitometry analysis was performed using the ImageJ software (NIH, Bethesda, MD). Equal protein loading was verified by stripping and reprobing membranes for histone H3 or β-actin.

2.6. Non-heme iron histochemistry with DAB intensification

Non-heme ferric iron deposition in formalin-fixed paraffin-embedded kidney sections was detected using Perls method with diaminobenzidine (DAB) intensification as previously described (Butt et al., 2010).

2.7. Tissue iron measurements

Kidney samples (100 mg) were homogenized in double deionized H_2O at 1:10 w/v. Homogenates were mixed with 500 μL of an acid mixture containing 1 mM HCl and 10% trichloroacetic acid, and incubated at 50 °C for 1 h with intermittent shaking (Carter, 1971). The samples were then centrifuged at 15,000 × *g* for 15 min at room temperature. The clear supernatant (90 μL) was mixed with 30 μL of 20 mg/mL ascorbic acid followed