Acute hemolytic vascular inflammatory processes are prevented by nitric oxide replacement or a single dose of hydroxyurea

Camila Bononi Almeida¹,², Lucas Eduardo Botelho Souza³, Flavia Costa Leonardo¹, Fabio Trindade Maranhão Costa⁴, Claudio C. Werneck⁵, Dimas Tadeu Covas³, Fernando Ferreira Costa¹, Nicola Conran¹

¹Hematology Center - University of Campinas-UNICAMP, Campinas, Brazil; ² Instituto Israelita de Ensino e Pesquisa Albert Einstein, Hospital Israelita Albert Einstein, São Paulo, Brazil; ³ Faculty of Medicine of Ribeirão Preto - University of São Paulo, Ribeirão Preto, Brazil; ⁴ Department of Genetics, Evolution and Bioagents, Institute of Biology, University of Campinas-UNICAMP, Brazil; ⁵ Department of Biochemistry and Tissue Biology, Institute of Biology, University of Campinas-UNICAMP, Campinas, Brazil

Running Head: Hemolytic inflammation is prevented by hydroxyurea

Key Points

- Hemolytic processes induce rapid systemic and vascular inflammation in C57BL/6 mice that is abolished by a single dose of hydroxyurea (HU)
- HU exerts some NO-dependent effects and should be investigated as an acute treatment for SCD and for other hemolytic disorders

Corresponding author: Nicola Conran, Hemocentro, Rua Carlos Chagas 480, Cidade Universitária, Campinas SP, 13083-878, Brazil. E-mail, conran@unicamp.br; Tel. +55 19 3521 8533; Fax, +55 19 3289 1089

Word count: 3999
Abstract

Hemolysis, and consequent cell-free (CF) hemoglobin (Hb) release, impairs vascular nitric oxide (NO) bioavailability and causes oxidative and inflammatory processes. Hydroxyurea, commonly used as a therapy in sickle cell disease (SCD), induces fetal Hb production, and can act as a NO donor. We evaluated the acute inflammatory effects of intravenous water-induced hemolysis in C57BL/6 mice, and determined the abilities of a NO donor, diethylamine NONOate (DEANO), and a single dose of HU to modulate this inflammation. Intravenous water induced acute hemolysis in C57BL/6 mice, attaining plasma Hb levels comparable to those observed in chimeric SCD mice. This hemolysis resulted in significant and rapid systemic inflammation and vascular leukocyte recruitment within 15 min, accompanied by NO metabolite generation. The administration of another potent NO scavenger (2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide) to C57BL/6 mice induced similar alterations in leukocyte recruitment, while hemin-induced inflammation occurred over a longer timeframe. Importantly, the acute inflammatory effects of water-induced hemolysis were abolished by the simultaneous administration of DEANO or hydroxyurea, without altering CFHb, and in a NO-pathway-mediated manner. In vitro, hydroxyurea partially reversed the Hb-mediated induction of endothelial pro-inflammatory cytokine secretion and adhesion molecule expression. In summary, pathophysiological levels of hemolysis trigger an immediate inflammatory response, possibly mediated by vascular NO consumption. Importantly, hydroxyurea presents beneficial anti-inflammatory effects, inhibiting rapid-onset hemolytic inflammation via a NO-dependent mechanism, and independently of fetal-Hb elevation. Data provide novel insights into mechanisms of hemolytic inflammation and further support perspectives for the use of hydroxyurea as an acute treatment for SCD and for other hemolytic disorders.

Key words: Hemolysis, hydroxyurea, inflammation, nitric oxide, cell-free hemoglobin
Introduction

Red blood cell (RBC) destruction, or hemolysis, leads to the release of hemoglobin (Hb) into the plasma. Once in the plasma, cell-free hemoglobin (CFHb) is usually neutralized into less toxic metabolites by specialized scavenger proteins, such as haptoglobin and hemopexin.\textsuperscript{1,2} However, after procedures such as mismatched transfusions and hemodialysis, and in some diseases, including malaria, sepsis, and hemolytic anemias (sickle cell disease [SCD] and paroxysmal nocturnal hemoglobinuria, for example), levels of intravascular Hb can become excessive.\textsuperscript{3-5} Consequences of augmented CFHb include increased vascular nitric oxide (NO) consumption, oxidative stress, endothelial activation, and cytokine upregulation,\textsuperscript{3,6-9} all of which contribute significantly to the pathophysiology of these disorders. Endothelial activation leads to the expression of surface adhesion molecules, which can result in the recruitment of leukocytes and platelets, increasing inflammatory processes.\textsuperscript{10} In SCD, such leukocyte recruitment is significant as the adhesion of white and red cells to the vascular wall can trigger vaso-occlusive processes. Furthermore, once oxidized, hemoglobin releases hemin, a hydrophobic molecule recently demonstrated to induce neutrophil extracellular trap (NET) production,\textsuperscript{11} and cause toll-like receptor (TLR)-4-mediated stasis in murine SCD.\textsuperscript{12} As such, hemolysis constitutes a major disease mechanism, and failure to neutralize CFHb can cause vascular and organ dysfunction, leading to the adverse clinical effects that have been associated with hemolytic diseases, including leg ulcers, pulmonary hypertension and priapism.\textsuperscript{13}

Hydroxyurea (HU) is a cytostatic drug, frequently used in the treatment of hematological diseases such as SCD, beta thalassemia, polycythemia vera, essential thrombocytemia and myelofibrosis, as well as non-hematological diseases, including HIV/AIDS.\textsuperscript{14-16} In SCD, HU modifies the disease process, improving hematological parameters and reducing hospitalization and mortality.\textsuperscript{17} One of the major mechanisms
of action of HU in SCD is believed to be its ability to induce the production of fetal Hb (HbF) in erythrocytes, reducing HbS polymerization and red cell sickling.\textsuperscript{18,19} However, it is becoming increasingly clear that HU also has immediate benefits that can be observed in SCD before elevations in HbF occur; such alterations include reductions in leukocyte counts and improved blood flow due to local vasodilation.\textsuperscript{20-22} A possible explanation for the acute effects of HU lies in its NO donor property, where previous reports have suggested that intravascular generation of NO occurs in SCD individuals following HU administration.\textsuperscript{23-25} NO, in turn, facilitates vasodilatation via activation of intracellular cyclic guanosine monophosphate (cGMP) signaling in smooth muscle cells and can reduce endothelial and leukocyte activation.\textsuperscript{20,26,27} In a recent study, we reported that HU has immediate and acute beneficial effects in a murine sickle cell model (BERK) of inflammatory vaso-occlusion, reducing leukocyte adhesion recruitment and secondary red cell interactions, particularly when administered together with a cGMP-amplifying agent.\textsuperscript{28}

The aim of the current study was to observe the acute inflammatory effects of hemolytic processes in C57BL/6 mice, and to compare these to inflammatory processes observed in chimeric SCD mice. Our data suggest that, in addition to its other well-documented effects,\textsuperscript{8} depletion of vascular NO by CFHb may contribute to the inflammatory effects of hemolysis. In addition, the abilities of a NO donor, diethylamine NONOate (DEANO), and a single-dose administration of HU to diminish hemolysis-induced inflammation were verified, as was the participation of NO/cGMP-dependent mechanisms in these effects.

**Methods**

**Animals**

C57BL/6 mice and chimeric SCD mice (generated from the transplantation of bone marrow from HbSS BERK SCD mice into lethally-irradiated male C57BL/6 mice) were
used in the study. For further information about animals, their diets, housing, transplantation and confirmation of human globin expression, please see Supplementary Information. All animal procedures were carried out in accordance with the ‘Principles of Laboratory Animal Care’ (http://grants.nih.gov/grants/guide/notice-files/not96-208.html), and in accordance with current Brazilian laws for the protection of animals; this study was approved by the Commission for Ethics in Animal Experimentation of the University of Campinas (CEUA/Unicamp, protocol 2360-1).

Hemolysis protocol and mouse treatments

Hemolysis was induced in mice by the intravenous injection of water (H₂O; 150 µl) at 15 to 60 minutes before analyses, unless otherwise indicated. An acute inflammatory reaction was induced in some mice by the injection of TNF-α (0.5 µg; i.p.) 180 minutes prior to analyses. Some mice were also treated with hydroxyurea (HU; 250 mg/kg; i.v.; dose determined based on body surface area normalization for mice and represents the equivalent of 20 mg/kg in humans), DEANO (0.4 mg/kg; i.v.), or vehicle (saline), concomitantly with TNF-α or H₂O administration (in separate boluses). In some experiments, mice were pre-treated, 75 minutes before H₂O administration, with a soluble guanylate cyclase (sGC) inhibitor, 1H-[1,2,4] oxadiazolo [4,3-a]quinoxalin-1-one (ODQ; 15 mg/kg; i.p.), or with 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO; 1 mg/kg; i.v.), a scavenger of NO that does not affect nitric oxide synthase (NOS) activity, or with vehicle (2% DMSO in saline, or saline). PTIO was also used alone, 15 min before analyses in some experiments. Hemin (40 µmols/kg; i.p. or i.v.), haptoglobin (7.14 mg/kg; i.v.; Molecular Innovations Inc., Novi, MI) or hemopexin (4.46 mg/kg; i.v.) were also administered, as indicated. All reagents and drugs were obtained from Sigma-Aldrich, unless stated (St Louis, MO).

In Vivo Imaging System (IVIS) protocol
A chemiluminescent probe (XenoLight Rediject Inflammation Probe; Perkin Elmer, MA) was used to evaluate in vivo myeloperoxidase (MPO) activity. Mice were pretreated with TNF-α, H2O, HU, DEANO, saline or vehicle and then anesthetized in a plastic chamber (2.5% isofluorane/oxygen). The probe (200 mg/kg) was then injected (i.v.) immediately before image capture (5 min exposure) and luminescent detection, using an IVIS Lumina System® (Caliper LifeSciences, MA). Isofluorane anesthesia (1.5%) was maintained during IVIS procedures. Signal intensity was quantified as the photon flux (photons per second) within the region of interest using IVIS Living Image 3.0 software (Caliper LifeSciences).

**Plasma hemoglobin and nitrate quantification**

Plasma was stored at -80°C for CFHb quantification using the Fairbanks All method, as described. Samples were centrifuged (1 200g, 10 min) before quantification and Hb concentrations are expressed as µM heme associated with Hb, assuming Hb to be tetrameric. Plasma nitrate was quantified using a nitrate/nitrite colorimetric assay (Cayman Chemical Company, Ann Arbor, MI).

**Intravital microscopy**

After treatment protocols, mice were anesthetized (2% α-chloralose/10% urethane in PBS) and tracheostomized. The cremaster muscle was surgically exteriorized and continuously superfused with bicarbonate-buffered saline (37°C, pH 7.4), equilibrated with a mixture of 95% N2 and 5% CO2. For all protocols, microvessels (8-15 for each mouse) were visualized after surgery using a custom-designed intravital microscope (Imager A2, Zeiss; 63X magnification) and images were recorded for 30-90 seconds using a video camera device (AxioCam HSM, Zeiss). Leukocyte (WBC) rolling, adhesion and extravasation were monitored and analyzed for 30-45 minutes after
surgery, unless otherwise stated. The definitions of leukocyte rolling, adhesion, and extravasation have been previously described.28,29

**Human umbilical vein endothelial cell (HUVEC) cultures and analyses**

For information regarding HUVEC cultures and analyses by flow cytometry and ELISA, please see Supplementary Information.

**Neutrophil isolation, incubation with RBC lysate and adhesion assays**

For information regarding *in vitro* neutrophil assays, please see Supplementary Information.

**Statistical Analyses**

Statistical analyses are described in Supplementary Information.

**Results**

**Intravenous administration of water induces rapid hemolysis in C57BL/6 mice**

Under basal conditions, or at 15-min post saline administration (*i.v.)*, C57BL/6 mice present low-level intravascular hemolysis, as evaluated by determining plasma CFHb (Figure 1A). In contrast, basal hemolysis was approximately 2.5-fold higher in chimeric SCD mice. Intravenous injection of sterile H$_2$O (150 µl) induces a marked increase in CFHb within just 15 min in C57BL/6 mice (Figure 1B), resulting in similar levels of CFHb to those of non-manipulated chimeric SCD mice (Figure 1A). In contrast, sterile H$_2$O injection does not induce further hemolysis in chimeric SCD mice within 60 min (Figure 1B). Intraperitoneal administration of neither the pro-inflammatory cytokine, TNF-α (0.5 µg; Figure 1C), nor hemin (40 µmols/kg; Figure 1D) was able to elevate plasma CFHb levels in either C57BL/6 mice or chimeric SCD mice, within 180 and 120 minutes, respectively.
Hemolysis induces extensive systemic inflammation in C57BL/6 mice, comparable to that of TNF-α cytokine administration

C57BL/6 mice received hemolytic or inflammatory stimuli, and systemic inflammation was quantified using a probe to detect MPO in activated neutrophils using in vivo imaging. Rapid hemolysis, induced by H2O administration, was accompanied by extensive inflammation that was comparable to that induced by the intraperitoneal administration of the inflammatory cytokine, TNF-α (Figure 2A-B). Inflammation, induced by both H2O and TNF-α, was observed in the entire body of the C57BL/6 mice, with a greater dissemination in the abdominal/peritoneal area. In contrast, the administrations of similar volumes of a vehicle solution or of saline were unable to produce inflammation. Interestingly, basal inflammation in chimeric SCD mice (Figure 2C-D) was found to be significantly higher than that of the basal inflammation of C57BL/6 mice, demonstrating levels that were similar to those of hemolysis-induced C57BL/6 mice. Intraperitoneal administration of TNF-α induced a further and significant systemic inflammatory response in these chimeric SCD mice (Figure 2C-D); however, the intravenous injection of H2O in chimeric SCD mice did not result in any further increase in inflammation, in keeping with the finding that this approach does not generate further hemolysis in chimeric SCD mice (Figure 2C-D).

Rapid hemolysis induces vascular leukocyte recruitment in C57BL/6 mice, comparable to that of TNF-α administration

We then looked at the effects of H2O-induced rapid hemolysis on leukocyte dynamics in the microcirculation of C57BL/6 mice, using intravital microscopy. Previous studies32-34 have comprehensively shown the effects of inflammatory stimuli on the induction of leukocyte adhesion to the microvasculature. In BERK SCD mice, inflammatory stimuli lead to leukocyte recruitment, followed by secondary red cell capture and eventually
vaso-occlusive processes. In C57BL/6 mice, as expected, at 3-h post-TNF-α administration (0.5 µg; i.p.), leukocytes demonstrate decreased rolling and markedly increased adhesion to the microvasculature vessels walls, as well as augmented extravasation (Figure 3). Surprisingly, intravenous administration of H₂O in C57BL/6 mice, with ensuing hemolysis, caused a significant decrease in leukocyte rolling and increased leukocyte adhesion to the microvascular walls (Figure 3A-B), within 15 min. These effects were similar in magnitude to those of the TNF-α inflammatory stimulus.

**Scavenging of nitric oxide is associated with a rapid onset of vascular inflammation**

Consistent with reports that vascular hemolysis results in substantial NO consumption by CFHb, we found significant plasma nitrate generation within two minutes of i.v. H₂O administration in C57BL/6 mice (Figure 4A). Hypothesizing that NO depletion by CFHb may participate in the rapid onset of H₂O-induced hemolytic inflammation, we administered another potent NO scavenger in C57BL/6 mice, PTIO (1 mg/kg; i.v.), and observed leukocyte recruitment in the microcirculation after 15 min (Figure 4B-C). PTIO induced similar alterations in leukocyte rolling and adhesion to those provoked by i.v. H₂O, without changing plasma Hb (Figure 4D), indicating that NO consumption, *per se*, can induce vascular inflammation, in an Hb-independent manner.

Given the known effect that the Hb product, hemin, has on inflammatory processes, we administered hemin to C57BL/6 mice and evaluated leukocyte recruitment over time. Hemin, when given both via i.v. (Supplementary Figure 1A-C) and i.p. (data not shown), was indeed able to induce leukocyte recruitment, but only over a prolonged time period, with peak leukocyte recruitment occurring at approximately 2 hours post hemin administration. To further investigate the role of CFHb and hemin release in the rapid inflammation instigated by H₂O-induced
hemolysis, we induced hemolysis (H$_2$O \textit{i.v.}) and concomitantly administered either haptoglobin (extracellular Hb scavenger) or hemopexin (a hemin scavenger) and observed leukocyte recruitment after 15 min (Supplementary Figure 1D-F). While haptoglobin almost abolished the effects of hemolysis on leukocyte recruitment, hemopexin did not significantly modify the effects of H$_2$O-induced hemolysis on leukocyte adhesion and extravasation. Data are consistent with the hypothesis that the consumption of NO by CFHb may contribute to the acute and immediate inflammatory effects of H$_2$O-induced inflammation.

A nitric oxide donor reduces the inflammatory effects of hemolysis in C57BL/6 mice

DEANO, an NO donor, was intravenously injected concomitantly with H$_2$O in mice. As illustrated in Figure 4D, DEANO does not alter plasma CFHb levels and, therefore, does not prevent H$_2$O-induced hemolysis. However, DEANO significantly inhibited H$_2$O-induced hemolytic systemic inflammation in C57BL/6 mice (Figure 4E). Furthermore, while co-administration of DEANO with H$_2$O did not significantly alter hemolysis-induced WBC rolling (Figure 4F), significant reductions in WBC adhesion and extravasation (Figure 4G-H) were observed in C57BL/6 mice, compared to mice receiving H$_2$O and vehicle.

Hydroxyurea reduces the inflammatory effects of hemolysis in C57BL/6 mice

The effects of a single dose of HU (250 mg/kg, \textit{i.v.}) on acute H$_2$O-induced hemolytic inflammation were evaluated. Similarly to DEANO, administration of HU together with H$_2$O \textit{(i.v.)} did not abolish the H$_2$O-induced elevation in CFHb in C57BL/6 mice (Figure 5A). However, HU reversed the inflammatory effects of hemolysis, reducing systemic
inflammation (as measured by MPO detection/IVIS; Figure 5B) and significantly abrogating leukocyte recruitment in the microvasculature (reversing the effects of intravenous H₂O on leukocyte rolling, adhesion and extravasation, Figure 5C-E). At the lower dose of 100 mg/kg, HU also significantly reduced hemolysis-induced leukocyte adhesion and extravasation in the microcirculation, but did not inhibit alterations in leukocyte rolling mechanisms (See Supplementary Figure 2).

**Hydroxyurea reduces hemolysis-induced inflammation via a nitric oxide-dependent pathway**

Hypothesizing that the acute beneficial effects of HU upon hemolytic inflammatory processes are due to NO release *in vivo* and/or stimulation of cGMP-dependent signaling, we administered H₂O (*i.v.*) in C57BL/6 mice together with HU and the NO scavenger PTIO or ODQ (a sGC inhibitor). Figure 6 demonstrates that both PTIO and ODQ abrogate the beneficial effects of HU on WBC rolling, adhesion and extravasation.

**Effects of hemoglobin on endothelial cells can be partially inhibited by hydroxyurea**

Given that CFHb may exert some of its inflammatory effects via endothelial activation, we looked at the effect of the inhibition of endothelial adhesion molecules on H₂O-induced hemolytic inflammation, *in vivo*. The co-administration of antibodies that neutralize the activities of the adhesion molecules, E-selectin and ICAM-1, together with the intravenous H₂O stimulus, each abolished the immediate effects of hemolysis on leukocyte recruitment in the microcirculation (Supplementary Figure 3). As such, we investigated whether HU can protect endothelial cells (HUVECs) from some of the
effects of Hb, *in vitro*. HUVECs were incubated with a hemoglobin (10 mg/ml; 4h) suspension, concomitantly with HU (100 µM). Hb slightly increased VCAM-1 expression (P>0.05) and significantly augmented ICAM-1 and E-selectin expressions on the HUVEC surface (Figure 7A-C). The presence of HU in cultures partially inhibited the effects of Hb on endothelial VCAM-1 and E-selectin expression. Cytokines released by HUVECs after treatments were also evaluated; Hb significantly intensified endothelial release of MCP-1, IL-6 and IL-8 (Figure 7D-F). Furthermore, the presence of HU slightly, but significantly, inhibited the release of MCP-1 and IL-8 by HUVECs.

**Induction of neutrophil adhesion by RBC lysates is partially prevented by hydroxyurea**

Neutrophils from healthy individuals were isolated and incubated with autologous RBC lysate (1.5 mg/ml Hb) in the presence or absence of HU (100 µM), and their capacity to adhere to ICAM-1 was evaluated using two assays; a static adhesion assay (5 min incubation with RBC lysate; Figure 7G) and a microfluidic adhesion assay (4 h incubation with RBC lysate; Figure 7H). After 4 h of incubation with RBC lysate, the adhesive properties of human neutrophils were significantly increased, while at 5 min this increase was not significant. The presence of HU in cultures significantly diminished these RBC lysate-induced neutrophil adhesive properties.

**Discussion**

Under normal conditions, Hb is compartmentalized inside erythrocytes, where its primary function is to transport oxygen throughout the organism; however, under certain circumstances, and in a number of diseases, such as SCD, the erythrocyte
membrane is disrupted and Hb released into the plasma. If not neutralized immediately, CFHb reduces endothelial cell-derived NO bioavailability and is oxidized into methemoglobin (metHb), before releasing hemin,\textsuperscript{2,36} with significant pathophysiological consequences. As hemolysis occurs exponentially over time during blood storage, CFHb also has potentially important implications for transfusion procedures, in addition to the known hazards of hemolytic transfusion reactions.\textsuperscript{9,37}

New insights into the potent inflammatory effects of hemolytic products have emerged following reports that heme activates inflammasome formation in macrophages,\textsuperscript{38} and induces NET release in neutrophils of BERK SCD mice, as well as TLR-4-mediated signaling in endothelial cells.\textsuperscript{11,12} In support of these data, we induced intravascular hemolysis in C57BL/6 mice, elevating CFHb to levels equivalent to those of a chimeric SCD mouse model (approximately 2.5-fold higher); \textit{in vivo} imaging demonstrated that this hemolysis was associated with a rapid generation of systemic inflammation that was similar to the inflammation observed in TNF-α-stimulated mice and in non-manipulated chimeric SCD mice. Furthermore, intravital microscopy showed that intravenous H\textsubscript{2}O-induced hemolysis in C57BL/6 mice was associated with augmented leukocyte recruitment in venules, similar to that observed in TNF-α-stimulated C57BL/6 mice, and previously reported to trigger vaso-occlusive events in BERK SCD mice.\textsuperscript{29} Consistent with the observation that \textit{i.v.} water does not augment hemolysis in chimeric SCD mice, we did not see further increases in neutrophil recruitment or systemic inflammation in chimeric SCD mice following water administration (data not shown). These findings probably reflect the decreased osmotic fragility of sickle RBC\textsuperscript{39} and the fact that BERK SCD mice already demonstrate high levels of intravascular hemolysis.\textsuperscript{40}

While others have successfully demonstrated the direct inflammatory effects of hemin administration \textit{in vivo},\textsuperscript{11,12,38} we opted to employ intravenous H\textsubscript{2}O administration to achieve red cell rupture, with ensuing and measurable Hb release, as previously
described, rather than to administer the product of Hb processing, hemin, as the oxidation of Hb to metHb may constitute an important step in in vivo hemolysis. This approach effectively elevated CFHb and induced inflammation in C57BL/6 mice within 15 minutes of H2O administration. Although known to destabilize the erythrocyte membrane, under the conditions employed, hemin did not induce rapid hemolysis in either the C57BL/6 or the chimeric SCD mice.

Consumption of NO by CFHb to form metHb may constitute one of the major effects of intravascular hemolysis. We found that, in C57BL/6 mice, H2O-induced hemolysis was accompanied by rapid NO metabolite generation, indicative of NO consumption, while the administration of another potent (non-heme) NO scavenger was able to induce similar inflammation to that of H2O-induced hemolysis. Furthermore, the immediate inflammatory effects of H2O-induced hemolysis were abolished by haptoglobin, a scavenger of extracellular Hb, but not by hemopexin, a hemin scavenger, while the effects of hemin on leukocyte recruitment occurred over a longer time period, appearing to peak at about 2 h-post-hemin administration. Of course, these findings do not exclude a participation for heme in the immediate inflammatory effects of acute hemolysis, but they suggest that other more instantaneous effects, such as NO consumption and consequent reactive oxygen species production, may be more at play during the acute inflammation seen in the model that we have used.

Thus, we hypothesized that restoration of NO could inhibit or reverse the rapid and potent immediate inflammatory effects of intravascular hemolysis. Accordingly, when a NO donor (DEANO) was administered together with H2O in C57BL/6 mice, we observed a significant inhibition of ensuing systemic inflammation and leukocyte recruitment and extravasation that was not mediated by changes in CFHb levels. Data indicate that the rapid release of NO, afforded by DEANO within minutes of administration, is able to spare the inflammatory effects of CFHb.
HU is commonly used for the treatment of SCD, amongst other hematological
and non-hematological diseases, in a chronic regime, due to its ability to increase
levels of HbF, and reduce platelet and leukocyte counts, with consequent clinical
benefits for patients. However, HU may have important effects that are independent
of HbF elevation, as this molecule can donate NO. Additionally, reports suggest that
HU stimulates NOS activity and may provoke cGMP-dependent signaling, in vivo, by
direct nitrosylation of the sGC enzyme. As NO bioavailability is apparently reduced
in SCD patients, it is reasonable to assume that HU exerts some of its beneficial
effects by improving NO bioavailability. In support of this hypothesis, we recently
found that the administration of HU to BERK SCD mice, together with a cGMP-
amplifying agent, abolished TNF-α-induced vaso-occlusive mechanisms and increased
animal survival in a NO-dependent manner. In the present study, administration of
HU to C57BL/6 mice, at the time of hemolytic induction, inhibited ensuing systemic
inflammatory processes and leukocyte recruitment, without altering plasma CFHb
levels. HU was also administered, in H2O-induced hemolytic C57BL/6 mice, at the
same time as NO-signaling modulating agents. Accordingly, the effects of HU
administration on hemolytic vascular inflammation in C57BL/6 mice were abrogated in
the presence of a NO scavenger and a sGC inhibitor, indicating that, in this model, HU
exerts these anti-inflammatory effects via a NO/cGMP-dependent pathway, possibly via
the generation of intravascular NO. While the anti-inflammatory effects of HU, observed
herein, are consistent with the idea that the acute hemolytic inflammation generated in
this model may occur via rapid NO consumption, the possibility that stimulation of
NO/cGMP-dependent signaling by HU exerts a protective effect in this setting, rather
than an abrogating effect, should not be overlooked.

Hemolysis-induced leukocyte recruitment, in vivo, was found to be dependent
on endothelial E-selectin and ICAM-1 activity. To clarify whether HU exerts effects via
modulation of cellular mechanisms, in vitro assays were employed to determine the
direct effects of Hb on endothelial cell activation and leukocyte adhesive properties. In *vitro*, significant effects of Hb were observed after a longer time frame of 4 h, compared to the induction of inflammation by hemolysis, *in vivo*, which occurred within just 15 min. As *in vitro* Hb preparations are rapidly oxidized in air, the *in vitro* effects observed were likely mediated by metHb/hemin. Hb increased the expression of adhesion molecules involved in leukocyte recruitment on the surface of HUVECs and significantly induced their secretion of pro-inflammatory cytokines, indicating an activation of cells to a pro-inflammatory phenotype. In the presence of HU, these alterations were slightly, but significantly, abrogated. Additionally, Hb, acquired from autologous RBC lysis, induced neutrophil adhesion to ICAM-1 and the presence of HU effectively reduced this induction of neutrophil adhesion. While *in vivo* data indicate that the ability of HU to abrogate hemolytic inflammation may be due primarily to its ability to counteract the immediate depletion of NO by Hb, HU may also diminish the subsequent effects that hemoglobin has on leukocyte and endothelial activation, potentially reducing the ability of the endothelium to capture and tether leukocytes in response to hemoglobin, or its products, and reducing the subsequent ability of neutrophils to firmly adhere to endothelial ICAM-1. Given that on-going clinical trials suggest prospectives for the use of pan-selectin inhibitors for ameliorating leukocyte adhesive interactions in SCD, the use of HU in combination with this approach should be explored.

In summary, induction of hemolysis in C57BL/6 mice, to levels similar to those seen at baseline in chimeric SCD mice, triggers a rapid systemic and vascular inflammatory response, possibly mediated by vascular NO consumption. Such an acute inflammatory response is likely to play an important role in the pathophysiology of hemolytic reactions and diseases, including SCD, with significant clinical consequences. Acute administration of HU, at the time of the hemolytic assault, abolished the effects of CFHb on systemic and vascular inflammation via activation of a
NO-cGMP-signaling pathway. HU may exert its immediate beneficial effects by restoring vascular Hb-scavenged NO, in addition to diminishing endothelial activation and leukocyte adhesive interactions induced by Hb products. In addition to providing evidence that HU may hold potential for countering hemolytic inflammation in diverse disorders, this study further highlights perspectives for the use of hydroxyurea as an acute treatment for SCD, an approach that could have major clinical implications.

Acknowledgments

This work was supported by FAPESP, Brazil (grants 2008/50582-3; 11/50959-7 [CBA]) and CNPq (grants 565036/2010 and 307784/2013-4 [CCW]). The authors are grateful to Dr. John Belcher, University of Minnesota, for assistance with plasma hemoglobin determination methodology. We also thank CEMIB, the Instituto de Pesquisas Energéticas Nucleares (IPEN), São Paulo and Dr. Flávia R. Pallis and Dr. Carla F. Penteado for support with animal procedures.

Authorship

C.B.A. and N.C. conceived and designed the study, interpreted data and wrote the manuscript; C.B.A also performed experiments; L.E.B.S performed and analyzed IVIS experiments, F.C.L. performed in vitro experiments; F.T.M.C., C.C.W., D.T.C., F.F.C. participated in study design, revised and approved the final manuscript.

Conflict-of-interest disclosure

The authors declare no competing financial interests relevant to this study.
References


Figures Legends

Figure 1. H₂O administration, but not TNF-α or hemin, increases plasma hemoglobin (Hb) levels in C57BL/6 mice

Plasma Hb concentration in C57BL/6 mice or chimeric SCD mice (SCD) (A) at baseline or 15 min after saline administration (150 μl, i.v.; n=5-9); (B) at 15 min after saline administration or 15 and 60 min after H₂O administration (150 μl i.v.; n=4-9 mice); (C) at 180 min post-TNF-α or vehicle administration (0.5 μg i.p.; n=4-6 mice); (D) at 120 min post-hemin or vehicle administration (40 μmols/kg i.p.; n=3-6 mice). Plasma Hb is expressed as μM heme associated with Hb, assuming Hb to be tetramic. **p<0.01, compared to basal C57BL/6; #, p<0.05, ## p<0.01, ###p<0.001 compared to C57BL/6 plus saline; •• p<0.01 compared to vehicle (C57BL/6).

Figure 2. Bioluminescence imaging of myeloperoxidase activity in C57BL/6 and chimeric SCD mice after TNF-α and H₂O administration

Quantification of systemic inflammation in (A) C57BL/6 and (C) chimeric SCD mice (SCD) after treatment with TNF-α (0.5 μg; i.p.; 180 min; n=3-5 mice) or vehicle, and H₂O (150 μl; i.v.; 15 or 60 min; n=5-12 mice) or saline. *p<0.05, ***p<0.001, compared to vehicle and ###p<0.001, compared to saline. Illustrative images from each treatment in (B) C57BL/6 and (D) chimeric SCD mice (SCD). Mice were injected with a chemiluminescent probe that reacts with myeloperoxidase produced by activated phagocytes/neutrophils. Signal intensity was quantified as the photon flux (photons per second) within the region of interest.

Figure 3. Rapid hemolysis caused by the intravenous administration of H₂O is associated with increased leukocyte (WBC) recruitment in C57BL/6 mice
WBC rolling (A); adhesion (B); extravasation (C) in the cremaster microcirculation, determined at 180 min post administration of TNF-α (0.5 µg, i.p.) or vehicle or 15 min post H2O (150 µl, i.v.) or saline. ***p<0.001 compared to vehicle; ##p<0.01, ###p<0.001 compared to saline; n= 28-53 venules from 3-6 mice. (D) Representative images of C57BL/6 mice venules after treatments. White stars represent adherent WBC; white arrow indicates the direction of blood flow. Scale bar: 15 µm.

**Figure 4.** Nitric oxide scavenging induces leukocyte recruitment and nitric oxide donation reverses the systemic inflammation and leukocyte recruitment induced by H2O in C57BL/6 mice

(A) Plasma nitrate (NO3-) concentration in C57BL/6 mice at 2-30 minutes post H2O (150µl; i.v.) or saline administration; n=3 mice. (B) WBC rolling and (C) adhesion determined 15 min post administration of saline, H2O (150 µl; i.v.) or PTIO (1.0 mg/kg, i.v.); n= 34-48 venules from 4-6 mice). (D) Plasma Hb levels in C57BL/6 mice 15 min after receiving saline (150 µl; i.v.), H2O (150 µl; i.v.), PTIO (1.0 mg/kg, i.v.), or DEANO (0.4 mg/kg, i.v.), co-administered with either saline or H2O (150 µl; i.v.); n=3-11 mice; (E) inflammatory status was analyzed by IVIS at 15 min post H2O or saline administration (150 µl; i.v.) in the presence/absence of DEANO (0.4 mg/kg, i.v.; n=3-6 mice); (F) WBC rolling; (G) adhesion; and (H) extravasation determined 15 min post co-administration of DEANO or vehicle (0.4 mg/kg, i.v.) plus saline or H2O (150 µl; i.v.) (n= 24-28 venules from 3 mice). *p<0.05, **p<0.01, ***p<0.001 compared to saline; ""p<0.01 compared to saline + DEANO; **p<0.01 compared to H2O; "p<0.05, ###p<0.001 compared to H2O + vehicle.
Figure 5. A single dose of hydroxyurea abolishes the induction of systemic inflammation and leukocyte recruitment by H₂O-mediated hemolysis in C57BL/6 mice.

(A) Plasma Hb levels in C57BL/6 mice at 15 min after receiving H₂O or saline (150 µl; i.v.), in the presence of HU or vehicle (250 mg/kg, i.v.), n=4-11 mice; (B) inflammatory status was analyzed by IVIS at 15 min post H₂O or saline (150 µl; i.v.), in the presence of HU (250 mg/kg, i.v.) or vehicle, n=4-9 mice; (C) WBC rolling; (D) adhesion; and (E) extravasation determined 15 min post co-administration of saline or H₂O (150 µl; i.v.) plus HU or vehicle (250 mg/kg, i.v.), n=28-33 venules in 3-4 mice. *p<0.05, **p<0.01, ***p<0.001 compared to saline; "p<0.05 compared to saline + HU; *p<0.05, **p<0.01, ***p<0.001, compared to H₂O; ##p<0.01 and ###p<0.001, compared to H₂O + vehicle.

Figure 6. Hydroxyurea exerts its effects via a NO-cGMP-dependent pathway

WBC rolling (A), adhesion (B) and extravasation (C) in H₂O-treated C57BL/6 mice after administration of HU (250 mg/kg, i.v.) or vehicle, in the presence of ODQ (15 mg/kg, i.p.) or PTIO (1 mg/kg, i.v.) or vehicle. *p<0.05, ***p<0.001 compared to vehicle + H₂O + vehicle. n=22-33 venules from 3-4 mice.

Figure 7. Hydroxyurea reverses effects of hemoglobin on endothelial cell activation and neutrophil adhesion, in vitro

The expression of adhesion molecules on the HUVEC surface was determined by flow cytometry following incubation with 10 mg/ml hemoglobin (Hb) in the presence/absence of HU (100 µM) or vehicle for 4 hours, (A) VCAM-1 (anti-CD106-FITC); (B) ICAM-1 (anti-CD54-PE); (C) E-selectin (anti-CD62E-APC); n=9. Cytokines in HUVEC-conditioned culture medium were quantified by ELISA, (D) MCP-1; (E) IL-6; (F) IL-8;
n=4-14. Adhesion of human neutrophils to ICAM-1 (10 µg/ml) ligand, evaluated after incubation with autologous RBC lysate (Hb; 1.5 mg/ml) in the presence/absence of HU (100 µM) (G) by static adhesion, after 5 min of treatment, neutrophil adhesion was quantified using a colorimetric assay, n=8; (H) using a microfluidic platform, after 4 h of treatment. For the microfluidic assay, neutrophil adhesion was quantified in a 400-µm-width channel with an applied shear stress of 0.5 dynes/cm²; data were analyzed using DucoCell analysis program, recording the mean number of neutrophils adhered to an area of 0.08mm² (n=13-17). TNF-α (10 ng/ml) was used as a positive control. *p<0.05, **p<0.01, ***p<0.001 compared to basal without HU; #p<0.05, ##p<0.01 compared to Hb. or RBC lysate without HU.
Figure 2
Figure 3

A: Rolling WBC (min⁻¹)

B: Adherent WBC (100 μm⁻¹)

C: Extravasated WBC (per 100x50 μm²)

D: Images of different groups: vehicle, TNF-α, saline, H₂O
Figure 4
Figure 5
Figure 6
Figure 7
Acute hemolytic vascular inflammatory processes are prevented by nitric oxide replacement or a single dose of hydroxyurea

Camila Bononi Almeida, Lucas Eduardo Botelho Souza, Flavia Costa Leonardo, Fabio Trindade Maranhão Costa, Claudio C. Werneck, Dimas Tadeu Covas, Fernando Ferreira Costa and Nicola Conran