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Daily propranolol administration reduces persistent injury-associated anemia following severe trauma and chronic stress

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Abstract

Background—Following severe trauma, patients develop a norepinephrine-mediated persistent, injury-associated anemia. This anemia is associated with suppression of bone marrow erythroid colony growth, along with decreased iron levels, and elevated erythropoietin (EPO) levels, which are insufficient to promote effective erythropoiesis. The impact of norepinephrine on iron regulators such as ferroportin, transferrin and transferrin receptor-1 (TFR-1) are unknown. Using a clinically relevant rodent model of lung contusion (LC), hemorrhagic shock (HS), and chronic stress (CS), we hypothesize that daily propranolol (BB), a non-selective beta-blocker, restores bone marrow function and improves iron homeostasis.

Methods—Male Sprague-Dawley rats were subjected to LCHS±BB and LCHS/CS±BB. BB was achieved with propranolol (10mg/kg) daily until the day of sacrifice. Hemoglobin (Hgb), plasma EPO, plasma hepcidin, bone marrow cellularity and bone marrow erythroid colony growth were assessed. RNA was isolated to measure transferrin, TFR-1 and ferroportin expression. Data is presented as mean±SD; *p<0.05 vs. untreated counterpart by t-test.

Results—The addition of CS to LCHS leads to persistent anemia on post-trauma day 7, while the addition of BB improved Hgb levels (LCHS/CS: 10.6±0.8 vs. LCHS/CS+BB: 13.9±0.4* g/dL). Daily BB use following LCHS/CS improved BM cellularity, CFU-GEMM, BFU-E and CFU-E colony growth. LCHS/CS+BB significantly reduced plasma EPO levels and increased plasma hepcidin levels on day 7. The addition of CS to LCHS resulted in decreased liver ferroportin expression as well as decreased bone marrow transferrin and TFR-1 expression, thus, blocking

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Authors' contributions

I.G.A. and L.E.B. contributed to the formation of the experimental rodent model design, performed all rodent survival surgeries, assisted with tissue collection/processing, hematology and protein analysis, manuscript preparation, and statistical analysis of the manuscript. K.B.K. assisted with the formation of the experimental rodent model design, hematology, and protein analysis, performed tissue collection/processing, and protein isolation. H.R. performed sample preparations, tissues collecting/processing, protein and isolation. T.J.L. and P.A.E. provided assistance with the research question, interpretation of the data, statistical analysis, and writing of the manuscript. A.M.M. conceived the concept for the experimental rodent model design, research question, interpretation of data, and contributed significantly to the writing of the manuscript and final edits. All authors have read and approved the final manuscript.

iron supply to erythroid cells. However, daily BB after LCHS/CS improved expression of all iron regulators.

Conclusions—Daily propranolol administration following LCHS/CS restored bone marrow function and improved anemia after severe trauma. In addition, iron regulators are significantly reduced following LCHS/CS, which may contribute to iron restriction after injury. However, daily propranolol administration after LCHS/CS improved iron homeostasis.

Level of Evidence—Level II, therapeutic study

Keywords

erythropoiesis; hepcidin; transferrin receptor 1; ferroportin; transferrin; rat; trauma

Background

Immediately after trauma, severely injured patients experience a hypercatecholamine state as well as an inflammatory response to injury.^{1, 2} This initial sympathetic response is physiologic but when the hypercatecholamine state persists in critical illness it is damaging to vital organs.^{2–4} Both the bone marrow and the liver are highly innervated by the sympathetic nervous system.^{5, 6} Previous work with in vitro and vivo models demonstrated that norepinephrine (NE) rather than epinephrine was a key modulator of hematopoietic progenitor cell mobilization and an inhibitor of bone marrow erythroid cell growth.^{7–9} In the liver, NE can directly stimulate interleukin-6 (IL-6), a key pro-inflammatory cytokine.^{6, 10} IL-6 increases liver hepcidin, the main regulator of iron homeostasis that alters iron availability.^{10, 11} The true incidence of iron deficiency or functional iron deficiency in trauma patients is unknown.¹² While the etiology of anemia following trauma is multifactorial, the assessment of iron restriction is challenging to define in the context of persistent inflammation.¹² Yet, following severe trauma in humans and in rodent models of injury/shock/stress, the persistent elevation of NE is associated with bone marrow dysfunction and a persistent injury-associated anemia.^{13–15} In addition, in the context of tissue injury and hemorrhagic shock-induced bone marrow dysfunction, propranolol has been shown to reduce hematopoietic progenitor cell mobilization and reduce suppression of bone marrow erythroid colony growth.^{15–17}

The effect of severe trauma and chronic stress on iron metabolism has not been clearly defined. The liver plays an essential role, as the primary site for production of iron-regulating proteins and as a storage site for excess iron, which may be mobilized to the circulation to meet metabolic requirements.¹⁸ Ferroportin, the sole exporter of iron, releases iron from hepatocytes into the circulation and hepcidin regulates iron by degrading ferroportin to block iron release.^{18, 19} Transferrin, a plasma glycoprotein, has a high affinity for iron and transports it to other tissues.^{11, 20} Following blood loss, the maturation of erythroid cells are necessary for production of red blood cells and developing erythrocytes have high iron demand.^{21, 22} The uptake of iron in erythroid cells is mediated by transferrin and its receptor, transferrin receptor-1 (TFR-1).²³ Approximately 80% of TFR-1 is located in bone marrow erythroid cells.²³ Erythropoietin (EPO) is essential in the early stages of erythroid cell growth, including the survival of colony-forming unit erythroid cells (CFU-

Es).²⁴ Hecidin also plays an important role in effective erythropoiesis. Under normal homeostasis, there is a balance of the EPO-hecicin axis. Following severe injury, plasma EPO levels are high relative to the degree of anemia.²⁵ Previous work in severe trauma and chronic stress has demonstrated that EPO suppresses plasma hecicin and iron levels remain low and anemia persists.^{26–27}

In this study, we use a rodent model of lung contusion (LC), hemorrhagic shock (HS) and chronic stress (CS) to evaluate iron regulation and bone marrow erythroid function. We hypothesize that daily administration of propranolol (BB) following LCHS/CS restores bone marrow erythroid function, improves iron homeostasis, and abrogates persistent injury-associated anemia.

Methods

Animals

Male Sprague-Dawley rats (Charles River, Wilmington, MA), weighing 280 to 315g were housed in pairs with free access to food and water during daily night/day cycles of 12 hours each. Female animals were excluded due to estrous cycle variability and its impact after hemorrhagic shock. The animal protocol had been approved by the University of Florida Institutional Animal Care and Use Committee.

Experimental Design

Rodents were randomly assigned into one of the five groups (n=8/group): 1) naïve control; 2) lung contusion followed by hemorrhagic shock (LCHS); 3) LCHS+propranolol hydrochloride (LCHS+BB) (Sigma-Aldrich, St Louis, MO); 4) LCHS followed by daily chronic stress (LCHS/CS); 5) LCHS/CS+BB. Propranolol (10mg/kg) was administered by the intraperitoneal route ten minutes following LCHS and daily for six days. In groups subject to LCHS/CS, BB was given daily immediately after CS. Naïve rodents underwent daily handling. All groups were sacrificed on day 7. Liver, bone marrow and peripheral blood samples were collected and stored in a –80°C freezer.

Lung Contusion and Hemorrhagic Shock

As previously described, after intraperitoneal pentobarbital, a unilateral lung contusion (LC) was made by a percussive nail gun (Sears Brand, Chicago, IL) applied directly to a 12mm metal plate that was placed in the right axilla of the rodent.¹⁵ Ten minutes after LC, the right femoral artery and right internal jugular vein were cannulated using heparinized saline (10units/ml). The femoral artery tubing was connected to a continuous BP-2 Digital Blood Pressure Monitor device (Columbus Instruments, Columbus, Ohio) for measurement of mean arterial pressure and heart rate. Blood was then withdrawn from the internal jugular to maintain a mean arterial pressure of 30–35 mmHg for 45 minutes. Shed blood is then reinfused.

Chronic Restraint Stress

Chronic stress (CS) consisted of two hours of restraint in a nose cone rodent cylinder (Kent Scientific Corporation, Torrington, CT, USA) daily for six days.¹⁴ Rodents were

repositioned in the cylinder every 30 minutes to prevent habituation and during which alarms sounded for two minutes. Those rodents in LCHS models were restricted from food and water during CS periods.

Hemoglobin Analysis

Heparinized blood samples were used for the analysis of hemoglobin using a VetScan HM5 Hematology Analyzer (Abaxis, CA, USA).

Bone Marrow Cellularity and Erythroid Progenitor Colonies

Bone marrow was harvested from the left femur using a 19-gauge needle and flushing 1ml of Iscove's Modified Dulbecco's Medium (IMDM) (Lonza, Walkersville, MD, USA) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Flowery Branch, GA, USA). Bone marrow cell viability was determined by 0.4% Trypan blue staining and cellularity was assessed on a hemocytometer.

As previously described, bone marrow erythroid progenitor colony growth assays, including colony-forming units-granulocyte-, erythrocyte-, monocyte- megakaryocyte (CFU-GEMM), burst-forming unit-erythroid (BFU-E) and CFU-E were performed.^{16, 17} Briefly, bone marrow mononuclear cells at 1×10^6 cells/mL in IMDM were plated in duplicate with Methocult media (Stemcell, Vancouver, BC, Canada). Plates were supplemented with 1.3 U/mL rhEpo and 6 U/mL rhIL-3 for BFU-E and CFU-E or 3 U/mL rhGM-CSF for CFU-GEMM. Plates were then incubated at 37°C in 5% CO₂ incubator. A blinded reader assessed colony growth of CFU-E on day 7 BFU-E colonies on day 14, and CFU-GEMM on day 17.

Erythropoietin and Hepcidin Measurements

Plasma EPO was evaluated using the standard sandwich ELISA (R&D Systems, Minneapolis, MN, USA) and plasma hepcidin was measured using the standard competitive ELISA kit (MyBioSource.com, San Diego, CA, USA). All samples were run in duplicate following the manufacturer's protocol.

Ferroportin, Transferrin, and TFR-1 Expression

Liver and bone marrow RNA were isolated used RNeasy Mini Kit (Qiagen, Germantown, MD, USA). Complementary DNA (cDNA) was then prepared from the RNA using a cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). Liver ferroportin (35 cycles, annealing temperature 56°C), bone marrow transferrin (35 cycles, annealing temperature 54°C) and TFR-1 (32 cycles, annealing temperature 56°C) polymerase chain reaction (PCR) was performed using the following primers: ferroportin: 5'-CCCTGCTCTGGCTGTAAAAG-3, 5'-TAGGAGACCCATCCATCTCG-3, transferrin: 5'-TCAAGAGCTGCTGCAGAAAA-3, 5'-CCTGTTCCACACTGGACTT-3, TFR-1: 5'-TCTGACCCTCACTGGGATTC-3, 5'-GCCAAAAACGACAGAGCTTC-3. The following β -actin primers (annealing temperature 60°C) were used: 5'-AGCCATGTACGTAGCCATCC-3, 5'-CTCTCAGCTGTGGTGGTGAA-3. Amplified bands were visualized with 1.5% agarose gels and densitometry was measured using Image Processing and Analysis (ImageJ-NIH, Bethesda, MD).

Statistical Analysis

Graph Pad Prism Version 6 (San Diego, CA, USA) was used to perform all statistical analysis. All data was presented as mean $\pm$ standard deviation and assessed for normality using the D'Agostino/Pearson omnibus test. Normally distributed variables were compared using t-tests while Mann-Whitney U-test was used to compare all other variables. Significance was defined as * $p < 0.05$ vs. naïve controls and ** $p < 0.05$ vs. untreated counterpart.

Results

Propranolol Restored Erythroid Function

Figure 1A demonstrates that seven days of LCHS and LCHS/CS resulted in a significant decrease in BM cellularity by 24–27% compared to naïve. However, daily BB administration to LCHS and LCHS/CS rodents significantly improved cellularity compared to untreated counterparts (Figure 1A). The use of BB following LCHS and LCHS/CS restored bone marrow cellularity to that of naïve animals.

On day seven, LCHS and LCHS/CS resulted in suppression of bone marrow CFU-GEMM, BFU-E, and CFU-E erythroid progenitor colonies (Figure 1B–D). The addition of CS to LCHS resulted in an additional 10–20% suppression of CFU-GEMM, BFU-E, and CFU-E colonies compared to LCHS alone (Figure 1B–D). Daily BB administration following LCHS and LCHS/CS significantly improved bone marrow CFU-GEMM, BFU-E, and CFU-E colony growth by 9–39% as compared to untreated counterparts (Figure 1B–D). Daily BB administration following LCHS and LCHS/CS also improved bone marrow erythroid function.

Propranolol Improves Anemia

Seven days following LCHS alone there was a mild anemia but the addition of CS to LCHS resulted in a moderate persistent anemia compared to naïve (LCHS: 12.9 ± 1.0 ; LCHS/CS: 10.8 ± 0.8 ; Naïve: 14.3 ± 0.4 g/dL). Daily BB administration following LCHS alone significantly improved hemoglobin levels (Figure 2). After LCHS/CS, the model that produced the most significant persistent anemia, the use of daily BB administration improved hemoglobin levels by 20% versus untreated counterparts (LCHS/CS+BB: 13.7 ± 0.4 vs. LCHS/CS: 10.8 ± 0.8 g/dL) (Figure 2).

The Impact of Propranolol on Ferroportin, Transferrin, TFR-1

Ferroportin is the sole exporter of iron to all cells, especially erythroblasts, which need iron for maturation.^{10, 18} Compared to naïve, liver ferroportin expression following LCHS and LCHS/CS was significantly reduced by 38% and 69% respectively (Figure 3A). The use of daily BB following LCHS increased liver ferroportin expression by nearly 50% (Figure 3A). The daily use of BB following LCHS/CS significantly increased ferroportin expression by 75% (Figure 3A).

Iron uptake in erythroblasts is facilitated by transferrin and its receptor, TFR-1.²³ Seven days after LCHS and LCHS/CS, bone marrow transferrin production was significantly decreased

by 48% and 88% respectively compared to compared to naïve (Figure 3B). The daily use of BB following LCHS and LCHS/CS significantly increased bone marrow transferrin expression compared to untreated counterparts (Figure 3B).

Seven days following LCHS, TFR-1 expression was similar to that of naïve animals (Figure 3C). Following LCHS/CS, TFR-1 expression decreased 41% compared to naïve (Figure 3C). Bone marrow TFR-1 expression increased 21% with use of BB following LCHS and 45% with use of BB following LCHS/CS (Figure 3C). Propranolol administration following LCHS and LCHS/CS restored iron uptake and delivery to erythrocytes.

The Effect on Propranolol on the EPO-Hepcidin Axis

Seven days following LCHS and LCHS/CS animals exhibited mild-moderate anemia and there was persistent elevation of plasma EPO (Figure 4A). Seven days after LCHS+BB, plasma EPO levels were decreased compared to LCHS (LCHS+BB: $26.2 \pm 8.3^{**}$ vs. LCHS: 42.4 ± 5.3 pg/mL). Daily use of BB following LCHS/CS resulted in a 61% reduction in plasma EPO levels compared to LCHS/CS (Figure 4A).

Systemic iron homeostasis is mediated by hepcidin.²⁸ Figure 4B illustrates that plasma hepcidin levels were significantly decreased following LCHS and LCHS/CS. With daily administration of BB, plasma hepcidin increased 85% following LCHS and 86% following LCHS/CS (Figure 4B). Daily use of BB following LCHS and LCHS/CS restored the EPO-hepcidin axis.

Discussion

Following severe trauma in humans and in a rodent model of injury/shock/stress, the persistent elevation of NE is associated with bone marrow dysfunction that manifests clinically as a persistent injury-associated anemia.^{13, 14} Previous work demonstrated that daily propranolol administration after LCHS/CS decreased the prolonged mobilization of hematopoietic progenitor cells from the bone marrow and decreased plasma G-CSF levels.¹⁵ However, the impact of propranolol following LCHS/CS on bone marrow erythroid cell function and iron homeostasis has not been previously described. This study supports previous work demonstrating the benefits of propranolol on bone marrow erythroid function, by employing a unique rodent model of lung contusion/hemorrhagic shock followed by chronic stress. The use of propranolol after LCHS/CS improved bone marrow cellularity, bone marrow erythroid colony growth, and hemoglobin levels. In addition, daily propranolol use after LCHS and LCHS/CS increased expression of liver ferroportin and bone marrow transferrin, and bone marrow TFR-1 expression. In the LCHS/CS group, once the anemia has resolved with the use of propranolol, the EPO-hepcidin axis is also restored.

Previously, the addition of daily chronic stress to LCHS has resulted in bone marrow dysfunction due to prolonged hematopoietic progenitor cell mobilization from the bone marrow, decreased bone marrow cellularity, and decreased erythroid colony growth.^{13, 14} In this study, we demonstrate the benefit of daily propranolol use after LCHS and LCHS. This is most apparent in the LCHS/CS model, in which moderate anemia is resolved with use of propranolol. These results parallel the persistent anemia seen in critically ill trauma patients,

which is also improved with the use of propranolol early after resuscitation.¹² The physiologic response severe trauma includes sympathetic activation followed by a catecholamine surge.^{1, 3} However, when the sympathetic response is prolonged this leads to end organ dysfunction, most commonly studied in the cardiac system.² Previous work has revealed that NE and G-CSF mediate the mobilization of hematopoietic progenitor cells from the bone marrow to sites of injury, decreasing the number of erythroid progenitor cells available for maturation.^{5, 15} In addition, the use of daily clonidine following LCHS/CS prevented prolonged hematopoietic progenitor cell mobilization, improved bone marrow function, and restored hemoglobin levels.²⁹ Clonidine is a central sympatholytic that inhibits the release of NE. Based on the current study and previous work by Alamo et al.²⁹, NE is likely the key mediator of bone marrow dysfunction after trauma.

The liver is well innervated by the sympathetic nervous system and is a central component of iron production. This study sought to determine if prolonged elevation of NE had negative effects on iron metabolism. Seven days following LCHS and LCHS/CS, liver ferroportin expression was decreased and this finding correlated with decreased iron levels after trauma.¹² Similarly, Naz et al.¹¹ demonstrated a 30–50% decrease in liver ferroportin expression at 24 hours in a rodent model of tissue injury. Also, *in vitro* hepatocytes treated with interleukin-6 reduced liver ferroportin by 90% at 24 hours.¹¹ In this study, daily propranolol use following LCHS and LCHS/CS significantly improved liver ferroportin expression. Ferroportin expression is impacted by inflammatory states such as severe trauma and chronic stress as well as hemorrhagic shock. The proposed relationship between a NE-mediated hypercatecholamine state, the release of pro-inflammatory cytokines, and iron metabolism is illustrated in Figure 5A.

In addition to the presence of ferroportin, bone marrow transferrin and TFR-1 are necessary for iron delivery into developing erythroid cells.²³ Previous work by Fitzsimons et al.³⁰ demonstrated that patients with anemia of chronic disease have low serum iron levels, and erythroblast TFR-1 expression is reduced by 39%. Seven days following LCHS/CS, both bone marrow transferrin and TFR-1 expression are reduced. Sandrini et al.²⁰ showed that human serum transferrin incubated with NE caused decreased iron-transferrin signaling. In Figure 5B, we hypothesize that NE disrupts the iron-transferrin complex by decreasing iron availability, decreasing transferrin, or both. The use of propranolol following LCHS and LCHS/CS increased transferrin and TFR-1 expression by competitively blocking NE from binding to its beta-receptors.

Immediately following blood loss, effective erythropoiesis requires that EPO is upregulated while hepcidin is reduced.^{31, 32} Critically injured patients often have moderate to severe persistent anemia despite elevation of EPO.^{25, 33} Naz et al.¹¹ demonstrated that that severe tissue injury in rodents suppressed ferroportin before hepcidin levels were detectable. In our study, liver ferroportin expression was reduced despite low plasma hepcidin levels. This may explain why plasma iron levels are low following trauma and why these patients remain anemic despite the use of exogenous iron.³⁴ The use of propranolol after LCHS/CS improved hemoglobin levels and this correlated with a decrease in plasma EPO levels while plasma hepcidin levels improved. The effects of daily propranolol treatment on plasma EPO levels were most likely secondary to improved hemoglobin levels. This finding demonstrates

an inverse relationship between EPO and hepcidin following severe trauma and chronic stress. The etiology of persistent injury-associated anemia is multifactorial but there is strong evidence that chronic stress and NE play a significant role.

Currently, blood transfusions remain the mainstay of treatment for anemia and despite transfusion restrictive practices, trauma patients are more likely to be transfused than nontrauma patients.³⁵ Outcomes for those receiving transfusions has been linked to increased hospital stay, infection, and lung injury.³⁶ Both the use of intravenous iron and erythropoiesis stimulating agents in critically ill trauma patients have failed to show morbidity benefit or transfusion reduction.^{34, 37, 38} The search for alternative therapies for anemia treatment remains a priority, as professional societies and other organizations work to develop hospital wide blood management programs to improve outcomes and reduce costs. This study demonstrates that following severe trauma and chronic stress decreased bone marrow erythroid progenitor cell growth is due in part to decreased iron availability. It is likely the reduction of NE rather than the blockage of a specific beta receptor that causes a disruption in the iron transport system. Daily use of propranolol following severe trauma and chronic stress restored bone marrow function, improved iron homeostasis, and restored the EPO-hepcidin axis. Thus, future investigations targeting the prolonged stress response after trauma may be prove to be beneficial in treating critically injured patients with persistent anemia.

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Figure 1.

Seven days following LCHS and LCHS/CS, daily administration of BB restored bone marrow function. **A.** BB treatment after LCHS and LCHS/CS significantly improved bone marrow cellularity. **B–D.** The addition of BB after LCHS and LCHS/CS improved all erythroid progenitor colony growth, including CFU-GEMM, BFU-E and CFU-E. BM=bone marrow; BB=propranolol; LCHS= lung contusion followed by hemorrhagic shock; LCHS= LCHS followed by daily chronic stress; *p <0.05 vs. naïve; **p <0.05 vs. untreated counterpart

Figure 2.

Seven days following LCHS and LCHS/CS, daily BB administration improved hemoglobin levels. BB=propranolol; LCHS= lung contusion followed by hemorrhagic shock; LCHS/CS= LCHS followed by daily chronic stress; *p < 0.05 vs. naïve; **p < 0.05 vs. untreated counterpart

Figure 3.

A. BB use after LCHS and LCHS increased liver ferroportin expression. **B–C.** Seven days following LCHS and LCHS/CS, bone marrow transferrin and TFR-1 expression was significantly reduced. The use of daily BB after LCHS and LCHS/CS increased bone marrow transferrin and TFR-1 expression. BM=bone marrow; BB=propranolol; LCHS=lung contusion followed by hemorrhagic shock; LCHS= LCHS followed by daily chronic stress; * $p < 0.05$ vs. naïve; ** $p < 0.05$ vs. untreated counterpart

Figure 4.

A. Following LCHS and LCHS/CS, daily BB administration reduced plasma EPO levels. **B.**

Daily BB administration after LCHS and LCHS/CS increased plasma hepcidin levels.

EPO=erythropoietin; BB=propranolol; LCHS= lung contusion followed by hemorrhagic shock; LCHS= LCHS followed by daily chronic stress; *p < 0.05 vs. naïve; **p < 0.05 vs.

untreated counterpart

A

B

Figure 5.

A. Norepinephrine stimulates IL-6 which stimulates hepcidin and inhibits iron export by reducing ferroportin which leads to reduction in systemic iron availability. **5B.** Norepinephrine mediates a decrease in iron availability to erythroid cells in the bone marrow by either disrupting the bond between iron and transferrin in the peripheral blood or by reducing TFR-1 in the bone marrow. EPO=erythropoietin; EPOr=erythropoietin receptor; TFR1=transferrin receptor, NE=norepinephrine.