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Discrete beta-adrenergic mechanisms regulate early and late erythropoiesis in Epo-resistant anemia

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Abstract

Background—Anemia of critical illness is resistant to exogenous erythropoietin (Epo). pRBC transfusions being the only treatment option; despite related cost and morbidity, presses the need for alternate strategies. Erythrocyte development can be divided into Epo-dependent and Epo-independent stages. We have previously shown that Epo-dependent development is intact in burn patients and epo-independent early commitment stage is compromised, which is regulated by beta1/ beta2-adrenergic mechanisms. Utilizing scald burn injury model, we studied Epo-independent late maturation stages and the effect of beta1/beta2, beta-2, or beta-3 blockade in burn mediated Epo-resistant anemia.

Methods—Burn mice were randomized to receive daily injections of propranolol (non selective beta1/beta2 antagonist), Nadolol (long acting beta1/beta2 antagonist), Butoxamine (selective beta2 antagonist) or SR59230A (selective beta3 antagonist) for 6 days after burn. Total bone marrow (TBM) cells were characterized as non-erythroid cells; early and late erythroblasts; nucleated orthochromatic erythroblasts (Ortho-Es) and enucleated reticulocyte subsets using CD71, Ter119 and Syto-16 by flow cytometry. Multi potential progenitors were probed for MafB expressing cells.

Results—Although propranolol improved early and late erythroblasts, only butoxamine and SR59230A positively reflected in the peripheral blood Hgb and RBC counts. While burn impeded early commitment and late maturation stages; beta1/beta2 antagonism increased the early erythroblasts through commitment stages via beta2 specific MafB regulation. Beta3 antagonism was more effective in improving overall RBCs through late maturation stages.

Conclusions—Study unfolds novel beta2 and beta3 adrenergic mechanisms orchestrating erythropoietin resistant anemia after burn, which impedes both early commitment stage as well as late maturation stages respectively.

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Keywords

Anemia; erythropoietin; erythropoiesis; enucleation; propranolol; beta1/2 selective blocker; SR59230A; beta3-selective blocker; reticulocytes

Introduction

Acute anemia of critical illness is ubiquitously seen in the intensive care unit (ICU) (1). Anemia cannot be ignored as an “innocent bystander” considering its impact on patients with diverse conditions (2, 3). Majority of the patients (80%) admitted in the ICU develop anemia progressively within the first 72 hours. Despite significant decrease in hemoglobin and hematocrit levels (1), many of them are resistant to erythropoiesis-stimulating agents (4) pressing the need for transfusions. About 12 million units of packed RBCs are utilized per year in the United States alone. Among severely burned patients, more than half of all transfusions is attributed to anemia of critical illness; correlating with the initial severity and duration of critical illness based on APACHE II score and number of ventilator days respectively (5). Even though anemia of thermal burns was first noticed in 1946 (6), to this date there is no other alternative to transfusion (7). A paradox emphasizing both anemia and transfusion are associated with increased morbidity and mortality particularly in surgical/trauma/burn patients (3, 8, 9) accentuate the need for detailed characterization of the mechanisms causing anemia of critical illness to develop novel treatment strategies that will reduce number of transfusions.

Our previous studies indicated a reprioritization in bone marrow myelo-erythroid commitment (10) due to perturbed MafB axis following burn injury (11, 12), which is shown to be under beta-adrenergic control in burn patients (12). Therefore, it is essential to understand the deficiencies beyond commitment stage of bone marrow erythropoiesis for efficient targeted strategic endeavors to improve red cell production. In spite of elevated erythropoietin (Epo) levels, which is the key hormone for RBC production, burn patients suffer from persistent anemia (13). Mechanisms of Epo-resistant anemia are poorly understood. Epo is a kidney-derived cytokine induced under hypoxic conditions, which acts synergistically with several growth factors (SCF, GM-CSF, IL-3, and IGF-1) to cause differentiation and proliferation of erythroid progenitor cells (primarily colony-forming unit-E) (14). While Epo is essential for effective bone marrow erythropoiesis, all erythroblasts do not express Epo receptors (15). Only CFU-E, Pro-erythroblasts and basophilic-erythroblasts at early stages express receptors that bind Epo; polychromatic and orthochromatic erythroblasts at late stages do not express receptors for Epo (16). Therefore, endogenous Epo is critical only for the survival, proliferation and differentiation of erythroid progenitors during early- to mid-stage erythropoiesis (17, 18). Our previous work using human PBMCs for erythroid differentiation in an *ex vivo* culture demonstrated Epo dependent stages of erythroblast proliferation and differentiation is unaffected in burn patients (19). Furthermore, exogenously administered Epo did not increase reticulocyte numbers in the bone marrow of burn mice (10). Reticulocytes are the precursors of RBCs, the only two cell types without a nuclear body. It is this uniqueness of mature RBCs (being enucleated) that enables their elasticity and deformability to withstand shear forces as they travel through the

microvasculature. Until the orthochromatic erythroblasts (Ortho-E) eject out their nucleus during final stages of erythropoiesis to become reticulocytes, they cannot leave the bone marrow. Like all other hematopoietic cells, RBCs originate in the bone marrow from a nucleated hematopoietic progenitor.

Hematopoietic stem cell niche and mobilization is regulated by beta-adrenergic receptor signaling in the bone marrow (20–22). While beta 1 and 2 adrenergic receptors are predominantly expressed in heart, skeletal muscle and immune cells; beta3 adrenergic receptor sub type is expressed in adipocytes and bone marrow stromal cells (23, 24). It is well known that increased catecholamine levels is a hallmark in pediatric and adult burn patients (25). We recently found increased catecholamine synthesis in mid brain of mice subjected to 15% TBSA scald burn injury. Moreover, propranolol treatment following burn injury rescued erythroid-committed progenitors in the bone marrow by mitigating burn induced MafB in multipotent progenitors (26). While the above mentioned study underpins the mechanism of myelo-erythroid reprioritization following burn injury that our laboratory has previously established (10), the actual read out would be Hgb levels and RBC counts in peripheral blood that is dependent on maturation of late stage erythroblasts. Based on recent findings and background, we hypothesized that if propranolol is efficacious in both early commitment and late erythroblast maturation, then we should see an increase in peripheral RBCs and Hgb levels. In the absence of it, we speculate defects in enucleation affecting the maturation stage of late erythroblasts independent of beta 1 beta2 receptors. The sub type of beta-adrenergic receptors involved will be validated using selective and non-selective specific beta-adrenergic blockers.

This is the first study reporting the novel role of beta3 adrenergic receptor action in regulating terminal stage of erythropoiesis in burn injury model and provides a platform to further explore the mechanism of Epo resistant anemia prevalent in burn patients.

Methods

Animal protocol

All procedures were performed according to NIH Guidelines for Use of Laboratory Animals and approved by the Loyola IACUC. Six–eight week old B₆D₂F₁ male mice weighing approximately 25 grams were purchased from Jackson Laboratories (Barr Harbor, ME). Mice were housed in our Comparative Medicine Facility with a 12 h light/dark cycle with controlled temperature (20–22° C). The mice were allowed to acclimate to our facility for 7 days prior to use. As the focus of this study is to elucidate adrenergic influence, we excluded female mice because estrous cycle variability may impact burn-induced hormonal responses further complicating data interpretation.

Mice were randomly divided into sham and burn groups. Mice were anesthetized using ketamine and xylazine (100mg/kg, 2.5 mg/kg respectively; i.p) and their dorsal hair was removed by shaving. A 15% total body surface area full thickness scald burn along the dorsum was administered by immersion in a 100°C water bath for 9 seconds (27). All animals were resuscitated with 2 ml of normal saline (i.p.) immediately after the injury protocol. The sham groups also received anesthesia, shaving and resuscitation but were not

subjected to burn injury. Beta-blocker treatments were started at 0.5 days after injury and continued until a day before harvest as explained in the following sections and outlined in Fig 1A. For time course studies, experiments were terminated on post burn days (PBD) 3, 7, 14 and 21. For dose response studies of propranolol and selective beta-blocker comparisons, we chose PBD 7 for terminal read outs. Maximum reduction in erythroid markers and a positive response with propranolol occurred on PBD 7. For erythropoietin (Epo) responsiveness studies, drug delivery mode and time of harvest were altered slightly from the above methods. We implanted alzet pumps and allowed two weeks of treatment with continuous infusion. Mice were euthanized, blood was obtained by cardiac puncture and bilateral femurs were harvested. During the seven days post-burn period no mortality was associated with any experimental groups. None of the animals showed any evidence of wound infection or sepsis. Burn wounds were not treated with any topical agents.

Erythropoietin administration following continuous propranolol infusion to mice

The burn procedure was followed as described earlier. Starting on post burn day (PBD) 1, sham and burn groups were reassigned to vehicle (saline) or propranolol treatments. Preloaded Alzet mini osmotic pump (model 1002; DURECT Corporation, Cupertino, California) was implanted subcutaneously to each sham and burn mouse to deliver either saline or propranolol (5mg/kg bw/day; Sigma) such that there were 4 treatment groups: sham vehicle, burn vehicle, sham propranolol and burn propranolol. After a period of 10 days, each group was reassigned to human recombinant Epo (12.5U/day; i.p), or vehicle (saline), which was administered exogenously for two days on PBD 11 and PBD 12 followed by a day of rest (10) and proceeded with terminal experiments on PBD 14.

Daily injections of beta-adrenergic antagonists to burn mice

Starting after 6 hours on the day of burn injury, burn mice were randomized to receive once daily subcutaneous injections of either vehicle or the following beta-blockers at specified doses for 2, 6, 13, and 20 days or 6 days only post burn according to each experiment. SR59230A (125µg/mouse/day) (28) is a selective beta3 antagonist, propranolol (625µg or 1.2 mg/mouse/day) (29) is a non-selective beta 1 beta 2 antagonist with short half life, nadolol (625µg/mouse/day) is a non-selective beta 1 beta 2 antagonist with longer half life, butoxamine (125µg/mouse /day) is a selective beta 2 antagonist. The sham groups were given vehicle (saline) injections along with the rest of the burn group.

Analysis of bone marrow single cell suspensions for flow cytometry

Bone marrow cells from the bilateral femurs of each mouse were eluted into McCoy's medium (Invitrogen, Carlsbad, CA, USA) using a 1 ml syringe fitted with a 25⁵/₈ -gauge needle. Aliquots of primary cell suspensions were used as needed in various experiments.

Briefly, 1×10^{-6} cells were stained with fluorochrome-conjugated monoclonal antibodies specific for mouse PE-CD71 (BD Bioscience), PerCP.Cy5.5-Ter119 (eBioscience), and 60nM Syto16 (Molecular Probes), which is a nuclear stain to identify live nucleated cells (30). After a 30-minute incubation at 4°C in the dark, cells were washed and resuspended in PBS, and immediately analyzed with a FACS Canto II (BD Biosciences, San Jose, California).

Amnis ImageStreamX

Staining of total bone marrow cells were followed as explained above, but data acquisition was performed using ImageStreamX Imaging Flow Cytometer (Amnis Corporation, Seattle, WA) equipped with INSPIRE software. A 60x magnification was used for all samples. A minimum of 10,000 cells was analyzed for each sample. Data analysis was performed using the IDEAS software (Amnis Corporation). PacBlue (CD71), was excited with a 70nm of 405nm laser. FITC (Syto16) and PerCP-Cy5.5 (Ter119) were excited with a 100 mW of 488 nm lasers. Pac Blue, FITC and PerCP-Cy5.5 fluorescence were collected on channel one (430–505 nm), channel two (505– 560 nm), and channel five (640–745 nm) respectively. Intensity adjusted bright field images were collected on channel four.

Morphological examination

Approximately 2×10^5 total bone marrow cells were diluted in 250µl iscove's modified delbecco medium (IMDM) containing 20% fetal bovine serum and cytospun on glass slides using the Shandon Cytospin 2. The slides were stained with May-Grünwald (Sigma MG500) solution for 5 minutes, rinsed in 25 mM Tris buffer (pH 7.4) for 1.5 minutes, and then stained with a 1:20 diluted Giemsa solution (Sigma GS500) for 20 minutes. The cells were imaged in bright field using Evos microscope.

Bone marrow multipotent progenitors (MPPs) and flow cytometric analysis

Total bone marrow cells pooled from both femurs of each mouse were labeled with biotin conjugated lineage specific primary antibodies: anti-CD86, anti-CD11c, anti-Ter119, anti CD19, anti-B220, anti-CD11b, anti-CD90, anti-CD8a, anti-Gr1 and anti-CD3e (BD Biosciences, San Diego, CA, USA) followed by incubation with anti-biotin magnetic beads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Magnetic cell separation was carried out using the AutoMACS separator (Miltenyi Biotec) referring to the AutoMACS User Manual applying the separation program 'depletes'. The enriched lineage negative (lin^{neg}) fraction was surface stained with PerCP-Cy5.5-Sca1 (BD Biosciences), APC-CD117 (c-Kit receptor), efluor 450-CD34 and Pe-Cy7-FcγR (eBioscience, San Diego, CA, USA) and taken for FACS analysis to either identify MEPs ($\text{lin}^{\text{neg}}\text{Sca1}^{\text{neg}}\text{cKit}^+ \text{CD34}^{\text{neg}}\text{Fc}\gamma^{\text{neg}}$), GMPs ($\text{lin}^{\text{neg}}\text{Sca1}^{\text{neg}}\text{cKit}^+ \text{CD34}^+ \text{Fc}\gamma^+$), LSKs ($\text{lin}^{\text{neg}}\text{Sca1}^+ \text{cKit}^+$), or to sort MPPs ($\text{lin}^{\text{neg}}\text{cKit}^+$).

Intra-cellular MafB expression

Sorted MPPs were then fixed using BD Cytofix/Cytoperm (BD Biosciences, USA) for 20 minutes in the dark at room temperature. Cells were permeabilized by adding perm buffer (PBS, 0.1% (w/v) D-Glucose, 0.1% (w/v) BSA, 0.09% (w/v) sodium azide, 0.1% (w/v) saponin) and incubated for an additional 10 minutes. Cells were then washed with Perm wash (PBS, 0.1% (w/v) glucose, 0.09% (w/v) sodium azide, 0.1% (w/v) saponin). The cells were then incubated with anti-MafB-FITC (FITC was conjugated to Abcam's anti-MafB Ab using FITC conjugation kit from Abcam) in perm buffer and incubated at 4°C overnight on shaker. Cells were washed in perm wash and analyzed with a fluorescence activated cell sorting (FACS) Canto II and data were obtained using Flow Jo software (Tree Star, Ashland, OR).

Peripheral blood

Heparinized blood samples from study groups were examined on a veterinary hematology analyzer (HemaTrue, Heska, Loveland, CO, USA), RBC counts and hemoglobin levels were recorded.

Statistical analysis

Results from all experiments are expressed as mean $\pm$ SEM. KaleidaGraph statistical program version 4.1.0 (Synergy Software, Reading, PA, USA) was used for analysis of variance with Tukey's post hoc test for comparisons between multiple treatment groups. Statistical significance was set at $P < 0.05$.

Results

Strategies for quantification of early and late erythroblasts from TBM cells

As shown in the flow chart (Fig 1A), total bone marrow cells (TBM) were first divided as erythroid and non-erythroid cells. Those cells which lack both transferrin receptor (CD71) and glycophorin A (Ter119) were classified as non-erythroid cells and those that express Ter119 were counted as erythroid cells. TBM cells were further reassigned as early erythroblasts (CD71⁺) and late erythroblasts (CD71⁺ Ter119⁺). Among the late erythroblasts, nucleated (Syto16⁺) cells were categorized as Ortho-E and enucleated (Syto16^{neg}) cells were categorized as reticulocytes. We performed sort purification (by FACS Aria) followed by May Grunwald giemsa staining to confirm their maturation stage by morphological determination (Fig 1B). The gating strategy was also verified by Amnis ImagestreamX analysis (Fig 1C). We also did total nucleated cell count from bilateral femurs after trypan blue exclusion. We did not find any difference in cellularity either with burn injury or with beta-blockers. Bar graphs in Fig 1D is representative of typical nucleated TBM counts over the course of burn injury. PBD 14 is slightly lower compared to PBD 0 but not with other time points.

Time course with propranolol treatment influences bone marrow but not peripheral blood responses

Bone marrow erythroid cells were higher in burn mice treated with propranolol (1.2mg/mouse/day; sub. cu.) for 6, 13 and 20 days post burn. As shown in Figures 2A and 2B, the number of erythroid cells (Ter119^{pos}) and early erythroblasts (CD71^{pos}) in bilateral femurs decreased significantly from PBD 7 in both burn groups ($P < 0.0001$ vs Sham-Veh) respectively. However, administering propranolol improved bone marrow erythroid cells and early erythroblasts with statistical significance on PBD 7 and PBD 14 ($p < 0.001$ vs Burn - Veh). In agreement with early erythroblasts over the course of burn, we also could reproduce our previously published data on megakaryocyte erythrocyte progenitor (MEP) response to propranolol (12) which is shown as inset. In contrast, propranolol failed to impart parallel responses in peripheral blood Hgb and RBC counts. The line graphs in Figures 2C and 2D represent mean RBC count and Hb levels respectively. Both parameters were significantly lower starting from PBD 7 and remained low for the entire study period irrespective of

treatment suggesting a plausible b1, b2 independent defects in late erythroblast maturation stage.

Burn injury impedes bone marrow late erythroblast maturation stage non responsive to propranolol

Next, in order to delineate various stages of erythropoietic paradigm not influenced by propranolol that was affected by burn, two different doses of propranolol (625ug/mouse/day and 1.2mg/mouse/day) were administered to burn mice. Non-erythroid cells remained higher in all burn groups compared to sham irrespective of propranolol treatments (Fig 3A). Nonetheless, propranolol administration improved the erythroid cells in the bone marrow with statistical significance at the higher dose (Fig 3B; $p < 0.001$ vs Burn+saline). Interestingly, both low and high doses of propranolol were significantly effective in increasing early erythroblasts in the bone marrow (Fig 3C; low = $P < 0.05$ and high = $p < 0.0001$ compared to Burn+saline). Consequently the dual positive (Ter119^{pos} CD71^{pos}) late erythroblasts followed the same trend as Ter 119^{pos} erythroid cells with significantly higher levels upon high dose propranolol treatment (Fig 3D; high = $p < 0.0001$ compared to Burn +saline). Next, when we categorized late erythroblasts into reticulocytes and orthochromatic erythroblasts, all burn groups were significantly lower than sham (Fig 3E and 3F; $p < 0.0001$ versus Sham+ saline) but did not vary between propranolol doses. Similarly, the mean values of maturation index calculated as the ratio of reticulocytes: OrthoE were not influenced by propranolol at both doses, which was reduced by burn injury ($P < 0.0001$ compared to Sham +saline).

Maturation of orthochromatic erythroblasts is not influenced by exogenous erythropoietin in propranolol treated mice

We carried out long term (for 2 weeks) propranolol infusion via alzet pump followed by exogenous Epo administration in mouse model of burn injury to study the effect on late erythropoiesis. By qualitative visual demonstration of total bone marrow pellet, Epo seemed to increase redness in all three groups and similarly propranolol alone also developed the red coloration indicating overall improvement in erythropoiesis (Fig 4A top panel). However, representative FACS plots (Fig 4A bottom panel) reveal that within the late erythroblasts (CD71⁺ Ter119⁺ cells) in the bone marrow, nucleated Ortho-Es were increased than enucleated reticulocytes in burn mice with saline or propranolol implants compared to non-burned shams. Propranolol implants did not demonstrate any improvement in the percentage of reticulocytes in burn mice. Similarly, Epo administration to any of the three groups including shams did not restore enucleated reticulocytes. Therefore, maturation index did not improve with administration of exogenous Epo to either sham mice or burn mice with or without propranolol treatment as seen in bar graph (Fig 4B). Similarly, peripheral blood RBC numbers were significantly decreased in all burn groups compared to sham irrespective of propranolol administration ($P < 0.001$; Fig 4C). Additionally, Epo administration did not have any effect in burn groups with a slight improvement only in sham group ($P < 0.05$). These results are in line with bone marrow maturation index explaining why propranolol cannot relieve impaired terminal maturation defects in burn.

Together, evaluation of erythroblasts in late stages (Ortho-E and reticulocytes) proves Epo-independency during the maturation (enucleation) process and ascertains a good model (15% TBSA burn) to study erythropoietin-resistant anemia.

Myelo-erythroid shift is orchestrated by beta2 and not beta 3 adrenergic mechanisms

Next, to test whether a long acting non-selective drug will be more effective than a short acting propranolol (half life =4 hours), we administered Nadolol (half life =20–24 hours). To decipher the subtype-specific action of beta-receptors, we administered a selective b2 antagonist (Butoxamine - 125µg) or a selective b3 antagonist (SR59230A - 125µg) and compared with vehicle treatment for six days after burn injury. In line with our previous report that propranolol mitigates MafB expression in burn patients and burn mice (12), we observed significant reductions in MafB expressing multi potent progenitors (MPPs; lineage^{neg} Sca1^{neg} cKit⁺) after nadolol, propranolol and butoxamine administrations. But SR59230A treatment was ineffective in mitigating burn-induced MafB expressing MPPs. The representative histograms are shown as the overlay of MafB⁺ MPPs from the respective treatment groups (Fig. 5A). But, much to our surprise, TBM from SR59230A treated mice expressed more erythroid cells (Ter119⁺), similar to butoxamine and comparable with propranolol treatments whereas, nadolol treated mice had no improvement in erythroid cells, despite lower MafB expressing MPPs. The representative histograms in Fig. 5B is the overlay of Ter119⁺ total bone marrow cells between all five experimental groups and shams.

Following burn injury, beta2 specific mechanisms orchestrate erythro-myeloid shift in the bone marrow progenitors, which is restored by butoxamine treatment and not by SR59230A treatment.

Late maturation stage is regulated by beta3 AR mechanisms following burn

It is still not clear how SR59230A treatment could improve erythroid cell number bypassing the myeloid commitment bias observed with MafB⁺ MPPs. Therefore, we tested the possibility of beta3 mediated maturation of late erythroblasts. Representative FACS plots of the percentages of nucleated Ortho-E and enucleated reticulocytes within the late erythroblasts (CD71⁺ Ter119⁺) characterized by Syto16⁺ and Syto16⁻ expressions from all the four beta antagonists treatments are shown in Fig. 5C. Only SR59230A and not propranolol, nadolol or butoxamine improved the proportion of reticulocytes (enucleation stage) in late erythroblasts, which is significantly from vehicle treatment.

Mean values of the total erythroid cells and early erythroblasts from nadolol, butoxamine and SR59230A treatments are shown in bar graphs (Fig 6A and 6B). Administering Butoxamine and SR59230A for 6 days after burn injury proved to be more effective in restoring the erythroid cells (P<0.001 and P<0.0001 respectively) and early erythroblasts (P<0.0001 and P<0.001 respectively) compared to saline treatment, and were still significantly reduced compared to sham irrespective of any treatments (P<0.0001).

Nucleated Ortho-Es were also significantly reduced in burn groups treated with vehicle, nadolol or SR59230A compared to sham+vehicle (Burn+vehicle= P<0.001; nadolol=p<0001 and SR= p<0.001). However, within the burn groups, butoxamine had higher OrthoE numbers than burn+vehicle group (P< 0.001) and slightly lower than Sham levels (not

significant) [Fig 6C]. On the other hand, enucleated reticulocytes were significantly decreased in all burn groups compared to Shams ($P<0.001$, Fig 6D). Both butoxamine and SR59230A treatments significantly increased the number of reticulocytes compared to burn +vehicle ($P<0.001$). SR59230A selectively improved the maturation index showing efficacy at the late stage of erythropoiesis.

Peripheral blood responses to beta-adrenergic receptor blockers

Hemoglobin level and RBC count were significantly decreased in the peripheral blood of burn vehicle group at PBD 7 ($P<0.0001$ vs sham). Treatments with non-selective beta1/beta2 blockers (propranolol or nadolol) did not change these parameters compared to burn vehicle. Whereas, in line with TBM results, selective b2 (butoxamine) and b3 (SR59230A) antagonist treatments significantly improved red cell parameters (Hgb, $P<0.001$ and RBCs, $P<0.05$, vs. burn vehicle; Hgb, $P<0.001$ and RBCs, $P<0.01$; vs. burn + propranolol or burn +nadolol) as shown in Table 1.

Qualitative morphological evidence

In line with quantitative results, we also present the qualitative morphological evidence using May-Grünwald Giemsa staining of total bone marrow cells (TBM). In comparison to Sham animals, we noticed a marked decrease in enucleated reticulocytes and red blood cells after burn injury with a concomitant increase in myeloid cells represented by lobulated nucleus. While treatment with a non-selective beta1, beta2 blocker (PR) seemed to increase the early erythroblasts, administration of a selective beta 2 blocker (Butoxamine) increased the late erythroblasts (OrthoE) and beta3 blocker (SR59230A; SR) resulted in a marked increase in reticulocytes and red blood cells compared to burn mice. In contrast, we noticed reticulocytes scattered sparingly with more obular nucleated myeloid cells predominating over the early erythroblasts (Fig 7A).

Discussion

In this study we report some basic concepts of Epo-resistant anemia. We discovered defective enucleation of late erythroblasts following burn injury and a selective beta3 AR blocker (SR59230A) was effective in improving late stage erythropoiesis. We found that burn-induced erythro-myeloid shift in the bone marrow is dependent on specific beta 2 adrenergic mechanisms via MafB. While propranolol (a nonselective beta1/2 blocker) improved only the early and late erythroblasts in the bone marrow of scald burn injured mice, both butoxamine and SR59230A were effective in improving peripheral blood RBC production and uncovers a previously unknown beta3-adrenergic mechanism while holding promise as a therapeutic strategy for anemia of critical illness (Schematic summary in Fig. 7B). More importantly, a long acting non-selective beta1 beta2 blocker nadolol, though effective in mitigating MafB⁺ MPPs, was found to be ineffective in improving overall erythropoiesis. Data suggests a more complex interplay between the choices of beta-adrenergic antagonists due to the discrete subtype-specific actions at stage-specific erythroblast development following burn injury.

Given that erythropoiesis begins with an uncommitted hematopoietic stem cell that expresses functional beta2 receptors (31), we chose to administer a selective b2 antagonist to delineate b2 action in myelo-erythroid commitment. It is reasonable to speculate that a ten fold increase in catecholamine levels resulting from burn injury (25) could have beta-3 mediated implications as well because, high dose isoproterenol is known to activate beta-3 adrenergic receptors in dogs (32). Although we have not measured absolute plasma catecholamine levels in mice, we have recently found that sympathetic activity is significantly increased after a 15% TBSA burn injury (26). Here we show evidence that blocking the action of burn-induced catecholamines with a selective beta2 or beta3 antagonist significantly improved the peripheral RBC count, and hemoglobin levels compared to burn mice. The absolute numbers of Hgb and RBCs in burn mice may not appear to be predominantly low as in humans, but are at levels considered as anemic for rodents (33). Moreover, we have recently reported a graded anemic response with burn size validating the model (11).

In the current study, both dose response (Fig3) and time course (Fig2) with the highest dose of propranolol failed to reflect in peripheral blood RBCs and Hgb levels though effective in improving the bone marrow erythroid progenitors as well as erythroblasts. Similarly, even with continuous propranolol administration via alzet pump (14 days); the visual representation of bone marrow pellets appear to indicate an increase in erythropoietic activity after either propranolol or Epo administration or both in burn group with increasing red cell mass as seen previously with six day administration of propranolol (26). Intriguingly, this did not reflect in peripheral blood RBC count thus probing to understand the discrepancy. One of the reasons could be due to an increase in late stage erythroblasts in burn mice with propranolol implants compared to saline implants, in which nuclear condensation and hemoglobination begins to occur. Determining the enucleation by measuring bone marrow maturation index in Epo treated mice allowed us to further understand that enucleation of ortho-E is an Epo-independent mechanism. Moreover, in a randomized double-blind placebo-controlled trial, critically ill anemic adult patients were administered 40,000 IU of epoetin alfa once a week for 4 weeks with no apparent differences in mean hemoglobin level compared to placebo group indicating no correlation between exogenous Epo and hemoglobin levels in critically ill patients (34). While supporting Epo resistant anemia observed in burn patients, this also explains why propranolol administration did not reflect in Hgb levels.

In the current study with mice, propranolol was delivered for 13 days via alzet pump to minimize stress caused by subcutaneous injections. Administering exogenous Epo at PBD11 should improve Epo responsiveness of the regenerated MEP populations from propranolol treatment based on recent report (26). Our results indicate that despite increased MEPs, exogenous Epo fails to act at the terminal maturation phase resulting in stagnation of late nucleated erythroblasts. This observation is in line with our earlier study where exogenous Epo administration to burn mice did not improve reticulocyte count in TBM (10) and further supports Epo resistance seen in burn patients (35, 36). Building on our earlier observation that propranolol can mitigate burn-induced myelo-erythroid commitment in burn patients (26), here we further substantiate that erythropoietin resistance cannot be relieved by a combination therapy with propranolol and Epo to improve burn induced anemia at least in

animal model negating our previous notion. In contrast, mice treated with butoxamine and SR59230A showed a marked improvement in early and late erythroblasts as well as reticulocytes exhibiting novel and discrete actions in late stage erythroblast maturation.

In order to study burn-induced beta-adrenergic stimulation on erythroblast maturation stage, we examined the percentage of bone marrow cells expressing CD71 and Ter119 as differential expressions of these receptors aid in the characterization of erythroblast subpopulations (37). Because, CD71 is expressed on erythroid precursors and early erythroblasts (38), a decrease in CD71⁺ cells in the TBM after burn injury will show a reduction in erythroid precursors. However, administration of beta blockade (propranolol, butoxamine and SR59230A) compared to saline treatment increased CD71⁺ cells revealing an increase in early erythropoiesis, which is in line with our earlier finding with MEP regeneration consequent to propranolol administration (26). While transferrin receptors (CD71) are important for iron uptake and hemoglobin synthesis (39, 40), Ter119 is a glycoprotein associated with glycophorin-A, which begins to be expressed from pro-erythroblast onwards through mature erythrocytes (41). On this pretext, burn mice exhibited a marked reduction in Ter119⁺ cells in the TBM along with a corresponding increase in CD71^{neg}Ter119^{neg} non-erythroid cells, which is consistent with our earlier observations (10, 11) further confirming hematopoietic reprioritization away from erythroid lineage. Interestingly, butoxamine and not SR59230A treatment significantly reduced the MafB expressing multi potent progenitors mimicking the action of non-selective beta1/beta2-blockers (propranolol and nadolol). These results further strengthen our earlier observations in burn patients treated with propranolol implicating a common reciprocal mechanism (12) predominantly via beta2 receptor action in regulating MafB. However, whether this effect is directly imposed by hematopoietic cells or indirectly influenced by bone marrow microenvironment is not known. Bone marrow consists of cell types like adipocytes, macrophages, osteoblasts, osteoclasts, endothelial cells and stromal cells of distinct function and origin secrete soluble regulatory factors and serve as extracellular matrix which play a key role in regulation of erythropoiesis (42, 43). Bone marrow stromal cells, leukocytes and adipocytes express beta3 ARs (21, 24). Moreover, beta3 AR and not beta2 AR activation of stromal cells influenced transcription factors and chemokines (22, 24). While this could explain the differential action of SR59230A in late stage erythropoiesis we observed in this study, the specific role of beta3 AR signaling in the context of burn injury is not known. Moreover as SR59230A can also have alpha 1 antagonistic effect, the current data does not rule out the possibility of alpha 1 action.

In contrast to propranolol and nadolol, both butoxamine and SR59230A treated mice showed marked improvement in early and late erythroblasts as well as reticulocytes, which primarily occur by enucleating. This result is further reinforced by the increased maturation index significant with SR59230A and not with either butoxamine or propranolol or nadolol treatments. Moreover, improvement in bone marrow reticulocyte count in the SR59230A and butoxamine treated mice was positively correlated with significant restoration in the peripheral RBC count and hemoglobin levels compared to burn mice, which did not happen with propranolol treatment. We were quite surprised at the response (inaction) by nadolol, despite our anticipation to perform better than propranolol. Given the longer half-life, the only positive action of nadolol was to mitigate MafB expressing cells. Specific beta1

adrenergic blockers such as Nebivolol can augment beta 3 mediated responses by acting as beta3 agonists while simultaneously blocking beta1 receptors (44). Therefore, we can only speculate that by the extended antagonism on both beta2 and beta1 receptors, nadolol while increasing MEPs (through beta 2 receptors), also exacerbated beta3 agonist action synergistically following burn injury thereby inhibiting late erythropoiesis. Based on this concept, we speculate that the maturation index was blunted with higher dose of propranolol compared to the lower dose (Fig. 3D) and insists caution while dosing propranolol in patients. The speculation of beta3 agonism specifically by blocking beta1 and not beta2 receptors also justifies why butoxamine was more effective in replenishing the peripheral RBC count despite comparable maturation index to that of propranolol and nadolol treatments.

Furthermore, our results are supported by similar observation in rats subjected to trauma/hemorrhagic shock where treatment with SR59230A and not with butoxamine or atenolol improved Hgb levels after seven days of treatment (45). In a separate sequential study, the authors observed an increase in Hgb levels with propranolol. (46). The latter model varies with a superimposed chronic stress to the hemorrhagic shock where the bone marrow cellularity was altered. Therefore, in the absence of information about the development stage of erythropoiesis, it can be interpreted that increase in Hgb by propranolol could be due to increase in overall bone marrow cellularity. Moreover in our model of burn injury we did not see any change in bone marrow cellularity but only a shift in myeloid and erythroid lineages. Nonetheless, we acknowledge that butoxamine and not propranolol administration following burn injury was effective in increasing peripheral Hgb levels in par with SR59230A even in the absence of effective terminal maturation.

Results not only emphasize the importance of Ortho-E maturation to enucleated reticulocytes in the bone marrow, but also indicates compromised enucleation may perhaps alter the deformability and elastic properties of circulating RBCs affecting oxygen delivery to tissues. Nonetheless, further studies are required to substantiate the functional implications of impaired terminal maturation of erythroblasts following burn injury.

This is the first study implying the role of beta3 AR blockade on improving terminal erythropoiesis in burns. At this point it is not exactly clear how terminal erythropoiesis is regulated by beta3 AR signaling but it can be speculated that beta3 AR signaling influences bone marrow hematopoietic niche, which is innervated by autonomic sympatho-adrenergic efferent nerve fibers (47). Recent investigations in trauma model have shown the effects of non-selective (propranolol) and selective beta-blocker (SR59230A, a beta3 AR blocker) in preventing hematopoietic progenitor cell mobilization into the peripheral blood (21). However, only selective beta3 AR blocker reduced plasma G-CSF levels (48). G-CSF administration has been reported to negatively influence erythropoiesis (49–51) supporting our results that late stage erythropoiesis may be rescued with SR59230A by sequestering the cytokine, which is elevated in burn patients (52).

Limitations

Here we show evidence that beta2 and beta3 AR mechanisms differentially regulate erythropoiesis at early and late stages. Even though we have used selective antagonists in

burn mice, similar studies in beta1, beta2 and beta3 knock out mice will further strengthen this concept. However, beta1 beta2 double knock out mice did not survive a burn injury due to lack of baroreceptor function, which is a major limitation. While we utilized alzet pump to study Epo-independency, solubility of SR590230A in organic solvent precluded us from using implants in subsequent experiments explaining why there is discrepancy in mode of propranolol administration. One other limitation is that SR590230A is not a drug approved for human use; which impedes from investigating its efficacy using a direct translational approach to improve terminal erythropoiesis in humans. On the other hand, Myrbetriq is a beta-3 adrenergic agonist being administered as a prescription drug for treating urinary incontinence. Therefore, future studies to explore selective beta-3 as well as beta-2 adrenergic receptor pathways in erythropoiesis and hematopoiesis independent of burn injury are warranted.

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Abbreviations

BX	Butoxamine (selective b2-antagonist)
Epo	Erythropoietin
EB	Erythroblasts
ICU	Intensive Care Unit
MEP	Megakaryocyte Erythrocyte Progenitors
ND	Nadolol (non selective long acting b1/b2-antagonist)
Ortho-E	Orthochromatic erythroblasts
PR	Propranolol (non selective short acting b1/b2-antagonist)
SR	SR59230A (selective b3-antagonist)
TBM	Total bone marrow

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Figure 1. Study of bone marrow erythropoiesis in a mouse model of burn injury

(A) Overall study design of the beta-adrenergic blocker treatment regimen to burn mice. Following 15% TBSA injury, mice were administered with various beta-adrenergic blockers: -propranolol (non-selective beta1 and beta2 antagonist with half-life of 4 hours), Nadolol (non-selective beta1 and beta2 blocker with long action of 20 hours), Butoxamine (selective beta2 blocker) and SR59230A (selective beta3 blocker) via daily injections sub.cu. Only for Erythropoietin (Epo) response studies, propranolol was administered with continuous infusion via alzet pump followed by Epo injections on days 11 and 12 and harvested on PBD 14 to evaluate late erythropoiesis readout. Except for time course, all other studies were terminated on PBD 7. After euthanasia, blood and bilateral femurs were harvested. Blood was used for RBC count and Hgb measurement while total bone marrow cells (TBM) from bilateral femurs were analyzed for MafB expressing multi potential progenitors (MPPs). Stage specific alterations in TBM of mice treated according to experimental design are depicted next. TBM cells were marked with antibodies specific for Ter119 and CD71 and nuclear dye Syto16. Ter119^{pos} cells were gated as erythroid cells whereas CD71^{neg} Ter119^{neg} cells as non-erythroids. Of the TBM cells, CD71^{pos} cells were gated as early erythroblasts and those positive for both markers (CD71^{pos} Ter119^{pos}) were gated as late erythroblasts, which was again subdivided into nucleated orthochromatic erythroblasts (Ortho-Es, CD71^{pos} Ter119^{pos} Syto16^{pos}) and enucleated reticulocytes (CD71^{pos} Ter119^{pos} Syto16^{neg}) based on the presence or absence of nuclear dye (cell

permeable Syto16) respectively. **(B)** Ortho-E and reticulocyte gating was further confirmed by may-grunwald giemsa staining of FACS sorted cells. **(C)** Amnis ImageStreamX analysis (60X magnification) for immune cytological and morphological affirmation combined in one. Syto16 (green) shows nuclear positive Ortho-E versus nuclear negative reticulocytes while brightfield images reconfirm the erythroblast stage based on size. Yellow and red represent CD71 and Ter119 expression in late erythroblasts. **(D)** TBM cell count from bilateral femurs/mouse. Box plots representative of typical nucleated TBM counts over the course of burn injury.

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Figure 2. Time course of propranolol treatment to burn mice: bone marrow and peripheral blood responses

Propranolol (PR) was administered at 1.2mg/mouse/day dose to burn mice for 2, 6, 13 and 20 days via subcutaneous injections. Blood and bilateral femurs were harvested on PBD3, 7, 14 and 21, as shown in Y-axis. **(A)** Number of erythroid cells in TBM of bilateral femurs was significantly decreased from PBD7 until PBD 21 and was improved with propranolol administration until PBD 14. **(B)** Number of early erythroblasts was significantly decreased from PBD7 until PBD 21 and was improved with propranolol administration until PBD 14. The inset represents megakaryocyte erythrocyte progenitors (MEP), which reflects the same pattern is reproduced from previous publication (12). **(C)** RBC count and **(D)** Hgb levels were decreased with burn injury from PBD 7 to PBD 21. However, propranolol had an exacerbated reduction at PBD 14 and PBD 21 or PBD 14 respectively. N=6 mice per group were analyzed per experiment and repeated two other times with similar results. Line graphs represent mean $\pm$ SEM analysed by ANOVA using tukey's post hoc test. ***P<0.0001 vs. sham-veh; ##P<0.001, vs. burn-veh. (In the inset, * P<0.001 vs. sham-veh; #P<0.001 vs. burn-veh)

Figure 3. Dose response of propranolol to burn mice

Propranolol was administered to burn mice at 625µg/mouse/day and 1.2mg/mouse/day and TBM erythroid cells at different stages of development were analyzed by cell surface antibodies; anti Ter119 and anti CD71. **(A)** Bar graphs representing Ter119^{neg} CD71^{neg} non-erythroid cells **(B)** Bar graphs representing Ter119^{pos} erythroid cells **(C)** Bar graphs representing CD71^{pos} early erythroblasts **(D)** Bar graphs representing Ter119^{pos} CD71^{pos} late erythroblasts (EBs). **(E)** Bar graphs representing late EBs were further categorized as orthochromatic EBs (Ter119^{pos} CD71^{pos} Syto16^{pos}) and **(F)** reticulocytes (Ter119^{pos} CD71^{pos} Syto16^{neg}). **(G)** Maturation index calculated as the ratio of reticulocyte number per million live bone marrow cells to OrthoE number per million live bone marrow cells. In each panel, Y-axis represents $\times 10^3$ cells per million of TBM cells eluted from bilateral femurs. Bar graphs represent mean $\pm$ SEM values analyzed by ANOVA using tukey's post hoc test ^a*P<0.05, ^a**P<0.01, ^a***P<0.001 vs. sham. N=6 mice per group were analyzed per experiment and repeated two other times with similar results.

Figure 4.

Intraperitoneal administration of exogenous erythropoietin (Epo) to burn mice fails to increase mature reticulocytes. Burn mice were treated with propranolol at 5mg/kg bw/day for 14 days through continuous infusion via Alzet pumps. 12.5 units of Epo were administered on PBD11 and 12 followed by a day of rest before the harvest. Bilateral femurs were harvested and total bone marrow (TBM) cells were eluted. **(A) Top panel.** Pictures of the pelleted TBM cells from bilateral femurs depicting the changes observed with various treatment strategies. Sham mice treated with Epo show a darker red color indicating increased erythroblast population, thereby showing the efficacy of Epo. Whereas combination treatment with PR and Epo show darker red pellet demonstrating improved erythropoiesis in the burn mice compared to individual Epo and PR treatments. **Bottom panel.** Representative contour plots from flow cytometry analysis showing reticulocyte and Ortho-E distribution of CD71^{pos}Ter119^{pos} cells in TBM delineates the late erythroblast stage specific changes resulting from various treatments. **(B)** Maturation index characterized by reticulocyte/ortho-Es ratio show a stunted maturation of ortho-E to reticulocytes in all burn groups. **(C)** Peripheral blood was drawn by cardiac puncture and analyzed on a veterinary hematology analyzer. White bars are sham group, black bars are burn group and grey bars are burn+propranolol treated group (+ and – represents Epo or vehicle treatment respectively). All animals were harvested on PBD14. Bars represent mean $\pm$ SEM values analyzed by ANOVA using tukey's post hoc test ^a*P<0.05, ^{a***}P<0.001 vs. sham. N=4–6

mice per group were analyzed per experiment and repeated two other times with similar results.

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Figure 5.

Comparative analysis of nadolol, butoxamine, SR59230A, and propranolol in improving the erythroid cells after burn injury. Burn mice were either treated with vehicle (B+vehicle), or with propranolol (B+Prop625µg/mouse/day), long acting beta1 and beta2 blocker (B+nadolol, 625µg/mouse/day), selective beta1 and beta2 blocker (B+Butoxamine, 125µg/mouse/day) or selective beta3 (B+SR59230A, 125µg/mouse/day). **(A)** Histograms representing MafB^{+/−} multi potent progenitors (MPP) in TBM from bilateral femurs. Compared to Sham+ vehicle (Blue) B+ Vehicle (Red) had more MafB⁺ MPPs. Both non-selective beta1 beta 2 blockers (B+Prop; [Peach] and B+Nadolol [Green]) and selective beta 2 blocker (B+ Botoxamine; [Orange]) effectively decreased the proportion of MafB⁺ MPPs induced by burn while selective beta3 blocker (B+SR59230A; [Purple]) was ineffective. **(B)** Histograms representing Ter119⁺ erythroid cells in TBM from bilateral femurs. Compared to Sham+ vehicle (Blue) B+ Vehicle (Red) and B+Nadolol (Green) had the lowest number of erythroid cells. B+Prop; [Peach], B+ Botoxamine;[Orange] and B+SR59230A; [Purple] effectively increased the number of erythroid cells. **(C)** Representative contour plots from flow cytometry analysis showing reticulocyte and Ortho-E distribution in late erythroblasts (CD71^{pos}Ter119^{pos} cells) resulting from various treatments. R= reticulocytes (Ter119^{pos} CD71^{pos} Syto16^{neg}), and OrthoE=orthochromatic EBs (Ter119^{pos} CD71^{pos} Syto16^{pos}).

Figure 6.

Comparative analysis of a long acting beta1 and beta2 blocker (nadolol, 625µg/mouse/day), selective beta2 (butoxamine, 125µg/mouse/day) and selective beta3 (SR59230A, 125µg/mouse/day) AR-blockers in improving the erythroid cells after burn injury. **(A)** Ter119^{pos} erythroid cells, **(B)** CD71^{pos} early erythroblasts, **(C)** orthochromatic EBs (Ter119^{pos} CD71^{pos} Syto16^{pos}), **(D)** reticulocytes (Ter119^{pos} CD71^{pos} Syto16^{neg}), **(E)** Maturation index characterized by reticulocyte/ortho-Es ratio. Butoxamine and SR59230A prove more effective in restoring the erythroid compartment after burn injury compared to nadolol. While both butoxamine and SR59230A are equally effective in improving the CD71+ and Ter119+ cells (A and B), SR59230A selectively improves maturation index showing efficacy at the late stage of erythropoiesis. Graphs represent mean ± SEM analyzed by ANOVA using tukey's post hoc test ^a***P<0.001 vs. sham; ^b*P<0.05, ^b**P<0.01, ^b***P<0.001 vs. burn. N=6–8 mice per group shown, with similar results upon three repetitions.

Figure 7.

(A) Morphological characterization of representative total bone marrow (TBM) cells from distinct study groups. Smears of eluted TBM cells obtained from femurs of mice where, mature red cells and reticulocytes are increasingly seen in sham mice. Whereas, mature erythroid cells are markedly reduced with a concomitant increase in lobular nucleated myeloid cells in PBD7 mice. In propranolol (PBD7+PR ;1.2 mg/mouse/day) treated mice, an increase in early erythroblast population is seen. Nadolol (PBD7+nadolol ;625µg/mouse/day) treatment did not show any change in reticulocytes compared to burn. With selective beta 2 antagonist butoxamine, (PBD7+butoxamine ; 125µg/mouse/day) we see slight improvement in reticulocytes and more early erythroblast population. In selective beta3 blocker (PBD7+SR59230A; 125µg/mouse/day) treated mice, enucleated reticulocyte population is markedly improved. Images were captured by light microscopy at 40X magnification using EVOS FL Cell Imaging System. **(B) Schematic summary.** Discrete beta-adrenergic subtypes play predominant roles during stage-specific erythropoietic development affected by burn injury. HSC: hematopoietic stem cells, MEP: megakaryocyte erythrocyte progenitors, BFU-E: burst forming unit-erythroid, CFU-E: colony forming unit – erythroid, Pro-E: proerythroblast (normoblast), Baso-E: basophilic erythroblast, Poly-E: polychromatic erythroblast, Ortho-E: orthochromatic erythroblast, RBC: red blood cells, β 1:

beta1, β 2: beta2, β 3:beta3, AR : adrenergic receptors, EPO: erythropoietin, epoR:
erythropoietin receptor. : impaired after burn injury, : not impaired after burn injury.

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Table 1
Peripheral blood profile with selective beta adrenergic antagonist treatment for six days following burn injury

Total RBC counts and Hgb levels in peripheral blood as determined by HemaTrue. Propranolol (625 µg/mouse/day), Nadolol (625µg/mouse/day), Butoxamine (625µg/mouse/day), SR59230A (125µg/mouse/day) and Vehicle (Saline) were delivered via subcutaneous injections once a day for 6 days and blood was harvested by cardiac puncture on PBD 7.

Treatment Groups	Hgb (gm/dl)	RBC (10^6 cells/ μ l)
Sham + Vehicle	14.64 $\pm$ 0.12	10.06 $\pm$ 0.09
Burn + Vehicle	13.66 $\pm$ 0.09 ^{a***}	9.46 $\pm$ 0.06 ^{a***}
Burn + Propranolol	13.43 $\pm$ 0.15 ^{a***}	9.18 $\pm$ 0.20 ^{a***}
Burn + Nadolol	13.73 $\pm$ 0.18 ^{a***}	9.38 $\pm$ 0.12 ^{a***}
Burn + Butoxamine	14.2 $\pm$ 0.55 ^{b***, c***}	9.7 $\pm$ 0.39 ^{b*, c**}
Burn + SR59230A	14.48 $\pm$ 0.09 ^{b***, c***}	9.9 $\pm$ 0.18 ^{b*, c**}

Values represent mean $\pm$ SEM. Statistical significance by ANOVA using Tukey's post hoc test.

^{a***} P<0.001 vs. Sham+Vehicle

^{b*} P<0.05

^{b***} P<0.001 vs. Burn+ vehicle;

^{c**} P<0.007

^{c***} P<0.001 vs. Burn + Propranolol