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14. ABSTRACT

In year 2 of this project, we determined whether Compound 49b, a novel beta-adrenergic receptor agonist, prevented increased inflammatory and apoptosis proteins in insulin-like growth factor binding protein 3 (IGFBP-3) knockout (KO) mice after exposure to ocular blast. Eyes from IGFBP-3 KO mice were exposed to a blast of air from a paintball gun at 26psi. Eyes were collected at 4, 24, and 72 hours after blast exposure. In a subset of mice, Compound 49b eye drops (1mM dose) were applied within 4 hours, 24 hours, or 72 hours after blast. Three days after exposure to blast, all mice were sacrificed. One eye was used for protein analyses of TNFα, IL-1β, Bax, Bcl-xL, caspase 3, and cytochrome C. We found that ocular exposure to 26psi of air pressure led to a significant increase in both inflammatory and apoptotic proteins. When Compound 49b was applied within 4 or 24 hours after blast, it mitigated the increase in inflammatory and apoptotic proteins. Ocular blast produces a significant increase in inflammatory and apoptotic proteins in the retina, specifically in retinal ganglion cells. These proteins are reduced after treatment with a topical beta-adrenergic receptor agonist. This data suggests local application of beta-adrenergic receptor agonists may be protective in ocular blast.

15. SUBJECT TERMS
inflammation, apoptosis, ocular blast, Compound 49b

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Introduction

Ocular trauma constitutes one of the most common causes of unilateral morbidity and blindness in the world today [1]. Due to improvements in body protective gear, the rates of combat-based morbidity and mortality have decreased; however, the number of ocular injuries has increased (from 0.57% during the Civil War to 13% in Desert Storm[1, 2]. Ocular damage occurring in more recent wars is often caused by explosions with fragmentary munitions and represents the 4th most common injury in Operation Iraqi Freedom [2]. Despite improvements in eye protective wear, soldiers report injuries even while wearing eye protection in 24% of cases; in most instances use of eyewear is undocumented [2]. Thus, despite advances in military protective wear, the blast produced by many improvised explosive device (IEDs) pose a significant threat of closed and open globe injuries through the fragmentary munitions. With a goal of improving treatment for these types of injuries, we propose studies using a rodent eye-blast model designed to 1) identify the molecular/cellular pathways within ocular tissue that are activated in response to injury and 2) test the efficacy of a new drug which holds promise as a mitigator of these damage-triggered responses.

Damage to neuronal tissues of the brain and eye has been addressed in several previous studies. Results using various blast models, primarily to whole body, have demonstrated significant neuronal and glial damage, with increased levels of inflammatory markers in the brain after blast exposure [3]. Other work has demonstrated that blast exposure damages the visual system tracts in the brain, producing scotomas and general blindness, Retinal hemorrhages were also reported but there was no in-depth retinal analysis. In a model of head blast injury, axonal injuries to the cerebellum, corticospinal system, and optic tract were noted [4]. Using a similar model, another group found a significant increase in inflammation in the brain following blast exposure [5]. Taken together, these findings suggest that blast injuries may trigger 1) inflammation by activation of specific inflammatory pathways, and 2) cell death by activation of apoptotic or other cell death pathways.

To better characterize direct retinal responses to injury in the absence of indirect effects from distant sites, our collaborator, Dr. Tonia Rex developed an eye-specific blast model. In preliminary studies, Dr. Rex found that her model generates an open waveform primary blast with a pressure level that can be carefully controlled by altering input pressures or distance from the eye. Use of this model produced some of the changes noted in IED blast warriors [6]. We will use the eye-specific blast model to test two hypotheses: 1) We will test the hypothesis that principle retinal changes produced in this model include an activation of inflammatory pathways (associated with increased levels of inflammatory markers, specifically TNFα and IL-1β) and apoptotic pathways (linked to increased apoptotic markers, specifically Bax, Bcl-xL, cytochrome C, Fas, and Fas ligand). 2) We will test the hypothesis that treatment with a novel anti-apoptotic and anti-inflammatory agent, Compound 49b [7], within 1 day of blast injury will protect against retinal damage. We will further determine if the protective actions of Compound 49b involve insulin-like growth factor binding protein-3 (IGFBP-3) pathways, as we have shown this to be the case in its protection against apoptosis in an in vitro damage model using human retinal endothelial cells.

Keywords: inflammation, apoptosis, IGFBP-3, β-adrenergic receptor agonist

Body

Statement of Work-Aim 1. Using the eye blast model, establish the major effects of various blast intensities on retinal structure and function at three time points: immediately after injury (4 hours), shortly after injury (24 hours), or days after injury (3 days), In particular, we will focus on blast-induced changes in markers of apoptosis (Bax, Bcl-xL, cytochrome C, Fas, and Fas ligand) and inflammatory mediators (TNFα, IL-1β). Our goal is to establish biomarkers that are predictive of the severity and extent of retinal damage and thus will be useful in assessing the efficacy of our proposed protective treatment described in Aim 2. Based on a significant amount of preliminary data, we foresee few problems in completing these experiments within year 1 of the award.

This aim has been completed and was published in the Journal of Neuroinflammation on July 30, 2013. Briefly, we found that a blast of 26Psi significantly increased inflammatory and apoptotic mediators in mice. This was mitigated if topical Compound 49b was added 4 hours or 24 hours after blast. Application of Compound 49b seventy-two hours after blast reduced levels of both inflammatory and apoptotic markers, but not to control levels.

Figure 1

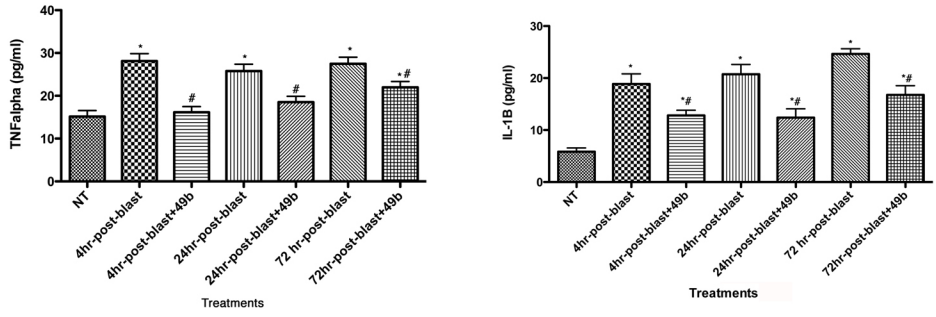

Figure 1. ELISA results for TNFα (left) and IL-1β (right) in mouse retina without exposure to blast (NT) or exposure to blast for 4, 24, or 72 hours or exposure to blast+Compound 49b for 4, 24, or 72 hours. *P<0.05 vs. NT. #P<0.05 vs. blast only at the same time point. N=5 mice for each group.

Figure 2

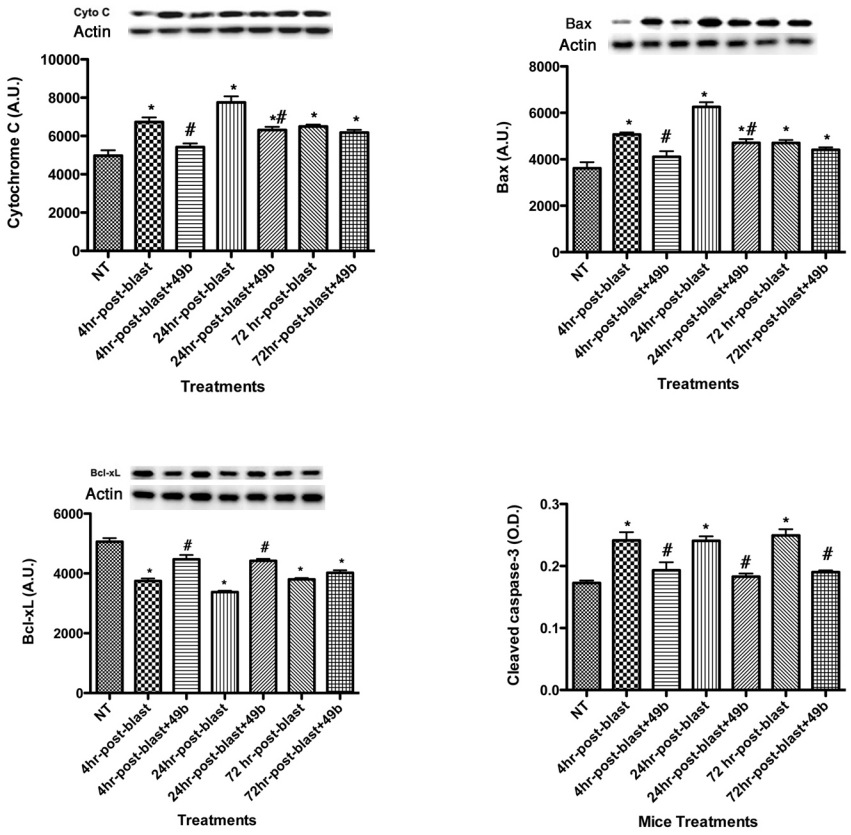

Figure 2. Western blot results for key pro-apoptotic proteins (Cytochrome C and Bax—top) and anti-apoptotic protein BcL-xL (bottom left). ELISA results for cleaved caspase 3. *P<0.05 vs. NT. #P<0.05 vs. blast only at the same time point. N=5 mice for each group.

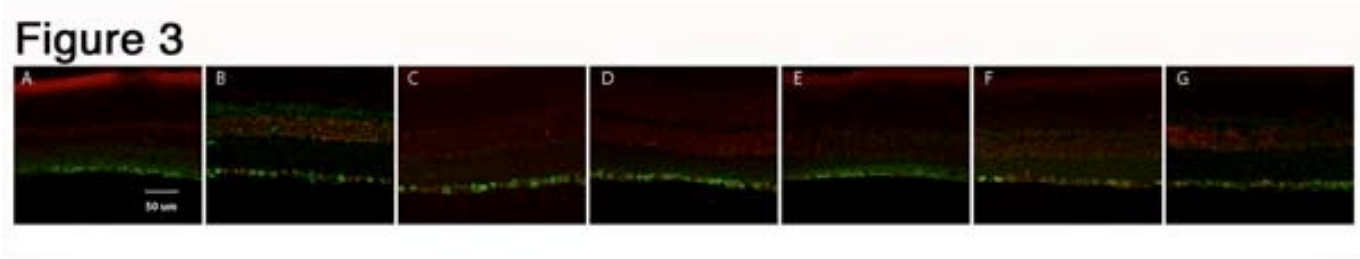

Figure 3. TUNEL labeling and NeuN (retinal ganglion cell marker) in untreated mice (A), 4 hours post blast (B), 4 hours post-blast+49b (C), 24 hours post-blast (D), 24 hours post-blast+49b (E), 72 hours post-blast (F) and 72 hours post-blast+49b (G). TUNEL labeling is green with NeuN staining is red. Scale bar is 50um.

Statement of Work-Aim 2. Determine if topical treatment with Compound 49b provides protection against blast injury to retinal structure and function compared to baseline data obtained in Aim 1. We will further assess if our proposed biomarkers are predictive of the degree of protection provided. Finally, we will examine the role of IGFBP-3 (a downstream

target of Compound 49b's actions) in providing retinal protection. The goal of these experiments is to establish the efficacy of our proposed treatment with Compound49b and its likely mechanism of action and downstream targets, which in turn would provide additional treatment strategies or drug targets.

As shown above, Compound 49b is effective at mitigating the increased inflammatory and apoptotic protein levels observed after blast with 26Psi pressure. We are currently working on the actions of IGFBP-3 in regulation of response to blast injury.

The work in Aim 2 is recently published in the journal Cytokine on July 28, 2014. We found similar results in the IGFBP-3 KO mice as we did in wildtype mice, in that Compound 49b was able to significantly reduce inflammatory and apoptotic markers in these mice.

Figure 1

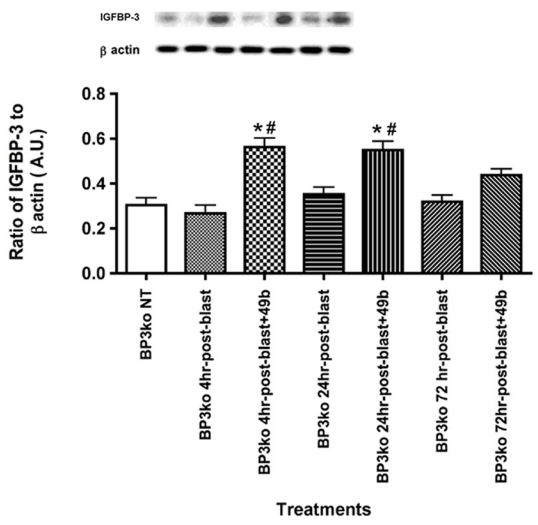

Figure 1. IGFBP-3 protein levels after ocular blast and Compound 49b treatment. Western blot results of IGFBP-3 KD mice without exposure to blast, IGFBP-3 KD mice exposed to blast for 4, 24, and 72 hours, and IGFBP-3 KD mice exposed to ocular blast + topical 1mM Compound 49b within 4, 24, or 72 hours after blast exposure. *P<0.05 vs. BP3 NT (not treated); # P<0.05 vs. BP3 KD+blast only at the same time point. N=6 mice in each group.

Figure 2

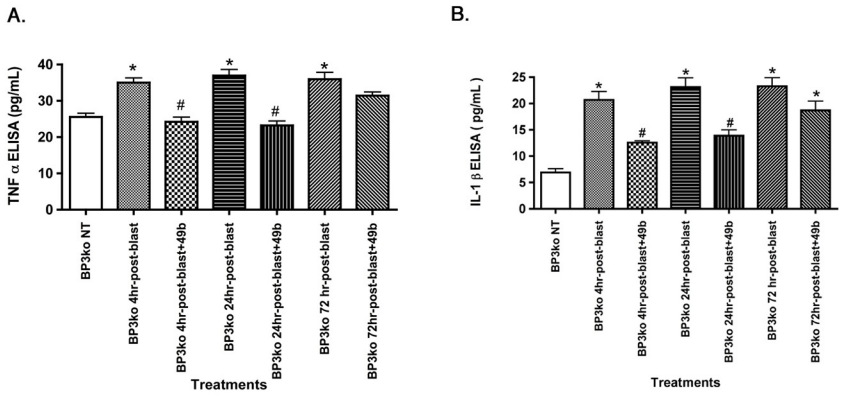

Figure 2. Ocular blast increased TNFα and IL-1β. ELISA results for TNFα (left) and IL-1β (right) in IGFBP-3 KD mice without exposure to blast, IGFBP-3 KD mice exposed to blast for 4, 24, and 72 hours, and IGFBP-3 KD mice exposed to ocular blast + topical 1mM Compound 49b within 4, 24, or 72 hours after blast exposure. *P<0.05 vs. BP3 NT (not treated); # P<0.05 vs. BP3 KD+blast only at the same time point. N=6 mice in each group.

Figure 3

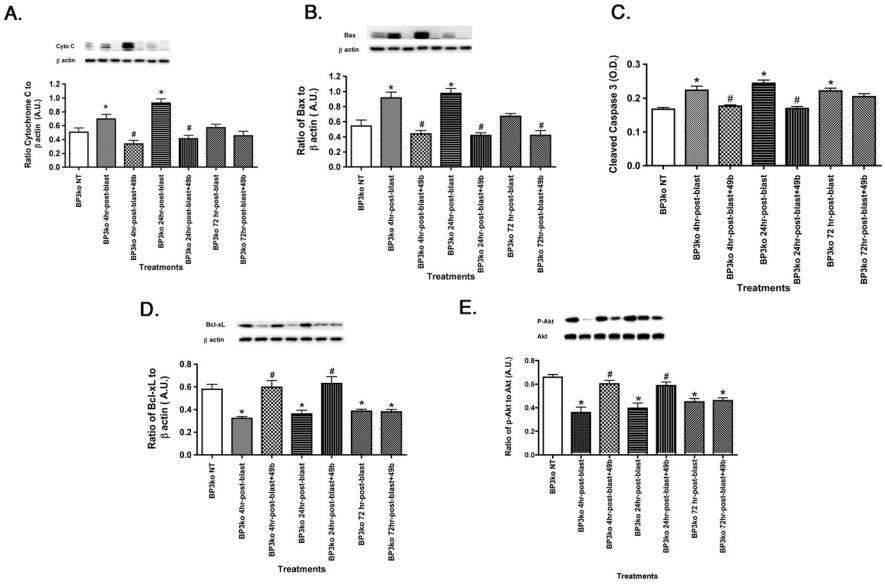

Figure 3. Ocular blast increased apoptotic factors in IGFBP-3 KD mice. Western blot results for key pro-apoptotic proteins Cytochrome C (A) Bax (B) and anti-apoptotic proteins BcL-xL (D) and Akt (E). ELISA results for cleaved caspase 3 (C) in IGFBP-3 knockdown mice without exposure to blast, IGFBP-3 KD mice exposed to blast for 4, 24, and 72 hours, and IGFBP-3 KD mice exposed to ocular blast + topical 1mM Compound 49b within 4, 24, or

72 hours after blast exposure. *P<0.05 vs. BP3 NT (not treated); # P<0.05 vs. BP3 KD+blast only at the same time point. N=6 mice in each group.

Our current plan for the no-cost extension phase is to follow up on these studies to investigate potential interactions of IGFBP-3 and TNFα through 49b. We know that Compound 49b reduces TNFα, while increasing IGFBP-3, but it is not clear whether both are required or if they occur through independent actions. This is key to development of Compound 49b as a therapeutic for warriors.

Key Research Accomplishments

- Demonstration that 26Psi blast induces increased TNFα, IL-1β and increases apoptotic protein levels
- Demonstration that topical Compound 49b can inhibit the increased inflammatory and apoptotic protein levels if administered within 24 hours of blast in both wildtype or IGFBP-3 KO mice

Reportable Outcomes

- Manuscript in J. of Neuroinflammation-- Compound 49b protects against blast-induced retinal injury
- Abstract on data in manuscript presented at Association for Research in Vision and Ophthalmology in Seattle, WA in May 2013
- Manuscript in Cytokine--Insulin-like Growth Factor-1 Binding Protein 3 (IGFBP-3) Promotes Recovery From Trauma-induced Expression of Inflammatory and Apoptotic Factors in Retina

Conclusions

In conclusion, exposure to ocular blast, similar to closed globe injuries observed in soldiers, increases key inflammatory and apoptotic proteins for up to 72 hours after blast exposure. This response occurred primarily in the cells of the ganglion cell layer of the retina. Compound 49b, a novel β-adrenergic receptor agonist, was able to mitigate the increased inflammatory and apoptotic markers, with optimal responses observed when treatment was initiated within at least 24 hours of blast exposure. Since Compound 49b has little observed toxicity and is an eye drop, it may offer a new therapy to protect the retina of soldiers after exposure to explosive devices in the combat field.

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Appendices 1

Compound 49b protects against blast-induced retinal injury

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Running Title: Beta-adrenergic receptor agonist protects retina

Abstract

BACKGROUND: To determine whether Compound 49b, a novel beta-adrenergic receptor agonist, can prevent increased inflammatory and apoptotic markers in mice after exposure to ocular blast.

METHODS: Eyes from C57/BL6 mice were exposed to a blast of air from a paintball gun at 26psi. Eyes were collected 4 hours, 24 hours, and 72 hours after blast exposure. In a subset of mice, Compound 49b eye drops (1mM) were applied within 4 hours, 24 hours, or 72 hours of blast. Three days after exposure of blast, all mice were sacrificed. One eye was used for measurement of retinal proteins (TNFα, IL-1β, Bax, BcL-xL, caspase 3 and cytochrome C). The other eye was used for TUNEL labeling of apoptotic cells, which were co-labeled with NeuN to stain for retinal ganglion cells.

RESULTS: We found that ocular exposure to 26psi air pressure led to a significant increase in apoptotic and inflammatory mediators within 4 hours, which lasted throughout the time period investigated. When Compound 49b was initiated within 4 hours or 24 hours of blast injury, levels of apoptotic and inflammatory mediators was significantly reduced. Application of Compound 49b within 72 hours of blast injury reduced inflammatory mediators, but not to untreated levels.

CONCLUSIONS: Ocular blast injury produces a significant increase in key inflammatory and apoptotic markers in the retina as early as 4 hours after blast exposure. These inflammatory and apoptotic markers are significantly reduced if a beta-adrenergic receptor agonist is applied within 24 hours of blast exposure. Data suggest that local application of beta-adrenergic receptor agonists may be beneficial to reduce inflammatory and apoptotic markers.

Keywords: beta-adrenergic receptor agonists; apoptosis, cytokines

Background

Ocular trauma constitutes one of the most common causes of unilateral morbidity and blindness in the world today [1]. During recent wars, many ocular injuries are caused by explosions with fragmentary munitions and are the 4th most common injury in Operation Iraqi Freedom [2]. Due to improvements in body protective gear, the rates of combat-based morbidity and mortality have decreased, while the number of ocular injuries has increased (from 0.57% during the Civil War to 13% in Desert Storm)[1, 8]. While all soldiers agree that eyewear is important, many are non-compliant because the eyewear becomes foggy, is bulky, or is unstylish [2]. In addition to the compliance issue, the ability of eye protective gear is at present, limited. Even with improved eye protective wear, injuries still occur in 24% of blast injury cases. Thus, despite advances in military protective wear, the blast produced by many improvised explosive device (IEDs) is associated with closed, as well as open globe injuries, through the fragmentary munitions. Due to other life-threatening injuries that may occur after IED blast exposure, ocular repair and treatment is often delayed for as long as 3-4 days after the initial injury [2, 8].

In order to better understand the damage to the eye after exposure to ocular blast, a good model needs to be developed. Unfortunately, prior to this year, little has existed. Whole body models of blast injury have been used to investigate the effects of blast on the major organ systems [3] or blast injuries to the brain [5]. These studies demonstrated that Kevlar protection is effective to protect internal organs from injury, but that the brain and eyes are still affected by the blast wave. Furthermore, work in the brain blast model demonstrated damage to the visual tracts of the brain; the retina itself was not fully examined in this study. [4]. To better mimic ocular trauma and allow for thorough characterization of retinal responses, a new model has been developed using an air blast from a paintball gun as the primary inducer of trauma [9]. Using this model, Hines-Beard et al (2012) demonstrated that a pressure of 23-26psi produced a number of anterior with few

posterior ocular injuries, using high-resolution optical coherence topography, gross pathology, and optokinetics [9]. In this study of various blast pressures, the authors found only 1 eye after exposure to 26psi to have retinal, choroidal, or retinal pigmented epithelium (RPE) changes. This corresponds well with previous studies in Veterans [6]. Despite the lack of gross pathology, it is probable that the posterior eye is still altered after exposure to blast, including increased levels of inflammatory or apoptotic markers. In order to investigate changes in retinal inflammatory and apoptotic mediators after blast, we employed the same model as described in [9] and measured protein levels of key proteins within 4 hours, 1 day and 3 days after blast exposure.

Additionally, we have previously reported that β-adrenergic receptor agonists, particularly a novel drug, Compound 49b, have anti-apoptotic and anti-inflammatory properties in retinal endothelial cells and in a diabetic retinopathy model [7, 10]. Compound 49b was based on the chemical structure of isoproterenol with chemical modifications to increase its ocular potency as a topical treatment. Chemical properties of Compound 49b are in Table 1. The chemical structure of Compound 49b is patent pending (WO 2011112243 A2). Our hypothesis in this study was that topical application of Compound 49b within 24 hours of blast injury would prevent blast-induced increases in inflammatory mediators and apoptotic markers.

Methods

Mice. C57/BL6 mice were purchased from Charles River (Wilmington, MA) at 2 months of age. Mice were exposure to an ocular blast of 26psi to both eyes using an air blast from a paintball gun [9]. Pressures were measured before and after exposure of each eye to a blast. The pressures were measured and analyzed using Labview software (National Instruments, Austin, TX).

Both eyes of the mice were exposed to the ocular blast. In one subset of mice, eyes were collected at 4 hours post-blast, 24 hours post-blast, 72-hours post blast or at 72 hours without exposure to the blast. In the second subset of mice, both eyes were blasted; however, a novel β-adrenergic receptor agonist, Compound 49b (1mM), was applied topically within 4 hours, 24 hours or 72 hours post-blast. For the Compound 49b-treated mice, mice received daily Compound 49b treatment for up to 3 days. For example, for the 4 hour treatment group, the mice received the first treatment within 4 hours post-blast, then another treatment 24 hours, 48 hours, and 72 hours post-blast (for a total of 4 treatments), while 72 hour post-blast mice only received 1 treatment of Compound 49b prior to sacrifice. All mice were sacrificed 3 days post-blast for the Compound 49b treated mice. Ten mice were used at each time point for all experiments.

Western Blotting. At the appropriate time after blast or Compound 49b treatments, one eye was used for protein analyses. For Western blot analyses, retinal lysates were collected into lysis buffer containing the protease and phosphatase inhibitors and scraped into the tubes. Retinal extracts were prepared by sonication. Equal amounts of protein from the cell or tissue extracts were separated on the pre-cast tris-glycine gel (Invitrogen, Carlsbad, CA), blotted onto a nitrocellulose membrane. After blocking in TBST (10mM Tris-HCl buffer, pH 8.0, 150 mM NaCl, 0.1% Tween 20) and 5% (w/v) BSA, the membrane was treated with appropriate primary antibodies followed by incubation with secondary antibodies labeled with horseradish peroxidase. Antigen-antibody complexes were detected by chemilluminescence reagent kit (Thermo Scientific). Primary antibodies used were Cytochrome C, Bax, and Bcl-xL, NFkB, and phosphorylated NFkB (all purchased from Cell Signaling, Danvers, MA).

ELISA Analysis. A cleaved caspase 3 ELISA (Cell Signaling, Danvers, MA) was used to measure levels of the active apoptotic marker in whole retinal lysates. TNFα and IL-1β protein concentrations were measured using a TNFα and IL-1β ELISA, respectively (ThermoFisher, Pittsburgh, PA). All ELISAs were done according to manufacturer's instructions with equal protein loaded into all wells.

Terminal deoxynucleotidyl transferase mediated dUTP Nick End Labeling assay (TUNEL). TUNEL was done on 10um cryosections of mouse retina according to manufacturer's instructions using the ApopTag-FITC kit (Millipore, Bilerica, MA) to localize apoptotic cells in the retina at the various time points. Co-localization of TUNEL positive cells with NeuN antibody (Abcam) was done to demonstrate which cell types were undergoing apoptosis.

Statistics. For all analyses, all experiments were done in triplicate. Data is presented as mean ± SEM, with statistical analyses using Kruskal-Wallis non-parametric testing, followed by Dunn's test.

Results.

Blast exposure increases TNFα and IL-1β levels in retinal lysates as early as 4 hours, which is mitigated by topical Compound 49b. Since a thorough analyses of inflammatory and apoptotic markers after blast exposure has not been reported, we chose to investigate levels of TNFα and IL-1β, both key proteins in other retinal diseases [11, 12], within 4 hours, 24 hours and 72 hours of exposure to 26psi blast. We found that protein levels of both TNFα and IL-1β are significantly increased within 4 hours of blast exposure and remain elevated for the 72 hours investigated (Figure 1). Additionally, we found that if topical Compound 49b (1mM) was applied within 4, 24 or 72 hours of blast exposure, the β-adrenergic receptor agonist

was able to significantly reduce levels of both TNFα and IL-1β (Figure 1). In the case of TNFα, treatments within 4 or 24 hours of blast exposure were able to return TNFα levels to a level not significantly different than an eye not exposed to blast at all.

Compound 49b reduces apoptotic proteins after exposure to ocular blast. Because activation of inflammatory mediators often leads to apoptosis [13, 14], we evaluated key apoptotic proteins (Bax, Cytochrome C, cleaved caspase 3) and an anti-apoptotic marker (BcL-xL) after exposure to blast alone or blast+Compound 49b at 4, 24, and 72 hours post-blast. Blast significantly increased pro-apoptotic markers and reduced BcL-xL. Compound 49b effectively reduced Bax and Cytochrome C and increased BcL-xL if applied within 24 hours of blast. Compound 49b was effective in reducing cleaved caspase 3 at all time points investigated. Taken together, blast induces a strong apoptotic response, which is mitigated by application of Compound 49b, best applied within 1 day of blast exposure.

Ocular blast induces apoptosis of cells in ganglion cell layer. In order to visualize which cells were undergoing apoptosis after ocular blast exposure, we performed TUNEL labeling of retina sections with co-localization with NeuN to label retinal ganglion cells after blast or blast+Compound 49b. It is clear that apoptosis is occurring in cells of the ganglion cell layer as early as 4 hours after blast, becoming more pronounced over the 72 hours of analyses. Compound 49b reduces this apoptosis, which is in agreement with the apoptotic protein marker analyses.

Discussion.

Ocular trauma is a leading cause of vision loss for soldiers, as well as the general public [8, 15]. Unfortunately, little is known of the effects of exposure of blast pressure to the

retina. Using the same model as used for this work, Hines-Beard only observed changes to the RPE in 1 eye [9]. While the morphology of the retina may not have changed, the proteins within the retina likely have become activated and initiated changes to be manifested in the morphology in the future. We found that within 4 hours of exposure to ocular blast, a significant increase in key inflammatory and apoptotic markers could be observed. This was associated with increased TUNEL labeling within the cells of the ganglion cell layer, which became more pronounced with additional exposure time of inflammatory and apoptotic markers.

Little is known on the cellular changes following exposure to ocular blast or in closed-globe ocular injuries. For most ocular trauma studies, work has focused on corneal burns or trauma. However, since it is likely that other ocular targets are affected following exposure to the blast, literature on cellular changes in these targets may be relevant. Mice receiving a chemical burn to the cornea had significantly increased levels of TNFα and IL-1β, as well as macrophage migration inhibitory factor [16]. In a review of animal models of retinal injury, retinal ganglion cell apoptosis and inflammation are key points of discussion [17]. While the majority of the discussion in the work by Blanch [17] was focused on axotomy of retinal ganglion cells or the optic nerve, the findings are similar to our observations after exposure to ocular blast, with increased levels of inflammatory mediators and apoptotic rates. In a subsequent paper on retinal changes in a closed globe injury model, authors describe increased TUNEL labeling and apoptosis of photoreceptors in the retina, after injury was induced by firing an air gun pellet or ball bearing into the mouse eye [18]. In this model involving projectiles into the eye, photoreceptor apoptosis and necrosis were observed, but specific apoptotic proteins or inflammatory proteins were not investigated. It is clear that further work on the cellular changes in the retina after injury is warranted.

Our findings of increased inflammatory and apoptotic markers after exposure to ocular blast agree with work from corneal burns or other closed globe models. A recent

report from British soldiers in Iraq and Afghanistan investigated ocular injuries, with the primary injury being trauma from exposure to a foreign body. In that work, the authors concluded that treatments could be safely delayed for 24 hours to allow for treatment of more life-threatening injuries [19]. Our results with Compound 49b eye drop therapy support this conclusion, demonstrating that Compound 49b can reduce both inflammatory mediators and apoptotic markers for up to 72 hours after exposure to blast. We have previously reported that Compound 49b is effective in reducing TNFα and apoptotic proteins in diabetic animals up to 6 months, when applied daily [7]. Isoproterenol was equally effective at reducing TNFα and apoptosis, but it had unwanted cardiovascular effects [10]. Future work will focus on the mechanisms by which Compound 49b can reduce inflammatory and apoptotic markers induced by ocular blast exposure.

In conclusion, exposure to ocular blast, similar to closed globe injuries observed in soldiers, increases key inflammatory and apoptotic proteins for up to 72 hours after blast exposure. This response occurred primarily in the cells of the ganglion cell layer of the retina. Compound 49b, a novel β-adrenergic receptor agonist, was able to mitigate the increased inflammatory and apoptotic markers, with optimal responses observed when treatment was initiated within at least 24 hours of blast exposure. Since Compound 49b has little observed toxicity and is an eye drop, it may offer a new therapy to protect the retina of soldiers after exposure to explosive devices in the combat field.

Abbreviations

TNFα: tumor necrosis factor alpha; IL-1β:interleukin-1beta; TUNEL:Terminal deoxynucleotidyl transferase mediated dUTP Nick End Labeling

Competing Interests

YJ, DM, and JS are inventors of Compound 49b for blast injury

Author Contributions

YJ completed all experiments and analyzed the data. LL completed the immunohistochemical staining. DM and JP designed Compound 49b. JS designed the experiments, assisted with data analyses, and wrote the paper. All authors have read and approved the final version of the manuscript.

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Table 1

Compound 49b:

Molecular Formula (in salt)	$C_{19}H_{26}ClNO_6$
Molecular Weight (in salt)	399.87
Molecular Formula (in Neutral)	$C_{19}H_{25}NO_6$
Molecular Weight (in Neutral)	363.40
Log P	1.92
pKa	~ 9

Molecular weight and Chemical Formula of the Compound 49b in salt form is 399.87 and $C_{19}H_{26}ClNO_6$. In Neutral form Molecular Weight: 363.40; Chemical Formula: $C_{19}H_{25}NO_6$.
Compound 49b pKa is around 9. We also have measured the octanol-water partition coefficient (*Log P*) of compound 49b (1.92) as measure of their lipophilicity. *Log P* was calculated using ChemDraw Ultra version 8.0 (CambridgeSoft Corporation, Cambridge, MA)

Figure Legends.

Figure 1. ELISA results for TNFα (left) and IL-1β (right) in mouse retina without exposure to blast (NT) or exposure to blast for 4, 24, or 72 hours or exposure to blast+Compound 49b for 4, 24, or 72 hours. *P<0.05 vs. NT. #P<0.05 vs. blast only at the same time point. N=5 mice for each group.

Figure 2. Western blot results for key pro-apoptotic proteins (Cytochrome C and Bax—top) and anti-apoptotic protein BcL-xL (bottom left). ELISA results for cleaved caspase 3. *P<0.05 vs. NT. #P<0.05 vs. blast only at the same time point. N=5 mice for each group.

Figure 3. TUNEL labeling and NeuN (retinal ganglion cell marker) in untreated mice (A), 4 hours post blast (B), 4 hours post-blast+49b (C), 24 hours post-blast (D), 24 hours post-blast+49b (E), 72 hours post-blast (F) and 72 hours post-blast+49b (G). TUNEL labeling is green with NeuN staining is red. Scale bar is 50um.

Figure 1

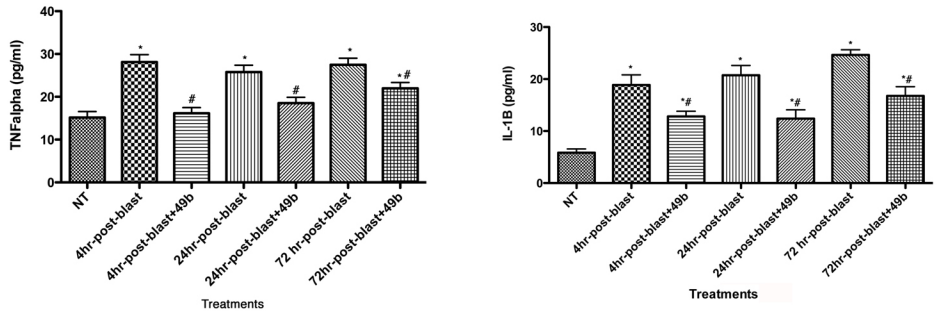


Figure 2

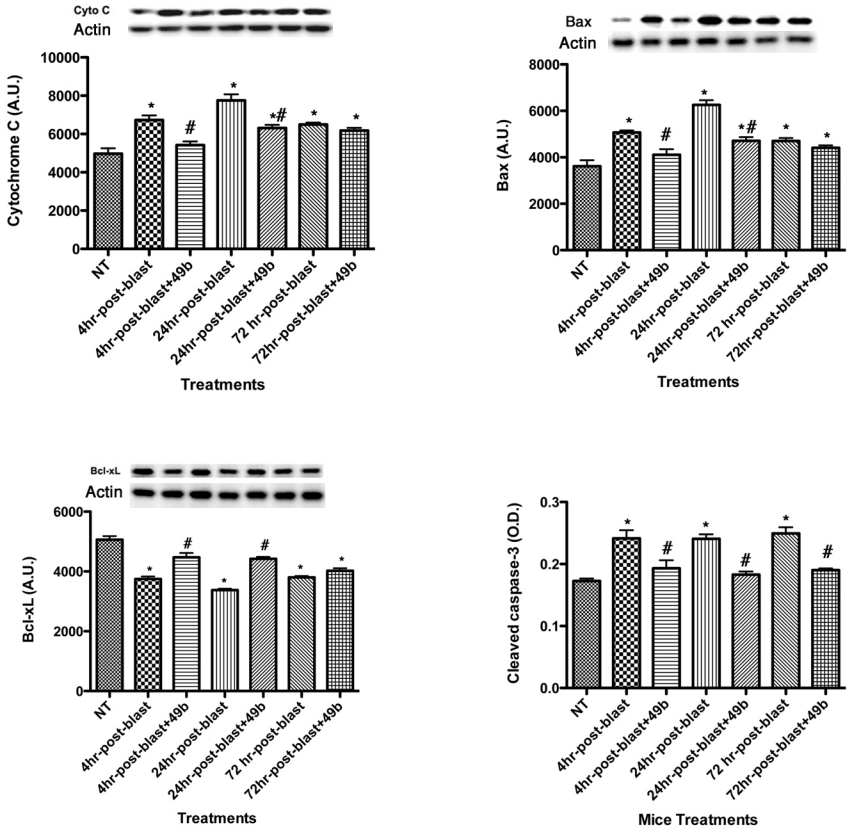
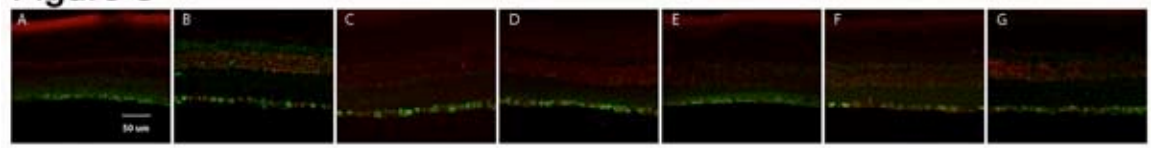


Figure 3



Appendices 2

Insulin-like Growth Factor-1 Binding Protein 3 (IGFBP-3) Promotes Recovery From Trauma-induced Expression of Inflammatory and Apoptotic Factors in Retina

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Running Title: IGFBP-3 blunts trauma-induced retinal inflammatory and apoptosis

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Abstract

Ocular trauma affects 20% of Americans in their lifetime and can cause permanent visual system damage. We have used a mouse model of ocular trauma (exposure to an air blast from a paintball gun) to examine pathways that trigger the resulting retinal damage and

to develop treatment strategies that might ameliorate the deleterious effects of trauma on retinal tissue. Our previous studies have shown that ocular blast causes an increase in protein levels of inflammatory mediators and apoptotic factors, including tumor necrosis factor alpha (TNFα) and interleukin-1-beta (IL-1β), as well as the apoptotic markers, Bax, cytochrome C, and cleaved caspase 3. Furthermore, topical treatment by eye drop application of a β-adrenergic receptor agonist, Compound 49b, was shown to decrease these inflammation/apoptosis markers and thus ameliorate the effects of blast trauma. We postulate that the protective effect of Compound 49b may be linked to its demonstrated ability to activate the β-adrenergic receptor and in turn trigger production of insulin-like growth factor binding protein 3 (IGFBP-3). In the current study, we tested this hypothesis using mice with minimal IGFBP-3 activity (IGFBP-3 knockdown mouse) vs. wildtype mice. We found that ocular blast alone did not affect IGFBP-3 levels in retinas of wild type or knockdown mice and surprisingly, the lower levels of IGFBP-3 in knockdown animals did not exacerbate the blast-induced increase in protein levels of inflammation/apoptosis markers. Nevertheless, the levels of IGFBP-3 were significantly increased in knockdown mouse retina by treatment with Compound 49b 24 hours post-trauma and as expected, the increase in IGFBP-3 was linked to a decrease in inflammation/apoptosis markers. We conclude that while lowered IGFBP-3 may not make the retina more vulnerable to blast injury, an increase in IGFBP-3 post-trauma may play an important role in limiting trauma-induced inflammatory and apoptotic pathways leading to retinal damage. Eye drop application of the β-adrenergic receptor agonist, Compound 49b, provides a promising treatment strategy for increasing IGFBP-3 levels to promote recovery from retinal inflammation and apoptosis after ocular blast.

1.0 Introduction.

Ocular trauma affects 20% of Americans during their lifetime and up to 1% may experience retinal damage [1]. The most commonly affected people are young males, manual workers, and members of the military [2]. As many of 13% soldiers experience eye injuries in the battlefield, with 80% of the eye injuries related to blast exposure [2]. A study

that included a representative sample of 46 Iraq and Afghanistan war veterans showed up to 28% had changes in the posterior eye causing visual damage [3]. While it is clear that exposure to ocular blast produces damage, an animal model to determine the cellular mechanisms was lacking until recently.

In order to dissect potential pathways involved in retinal damage after exposure to blast, multiple approaches have recently been developed. Work in a shock wave tube using moderate open-field blast waves showed damage to multiple organs, including a significant effect on long axon tracts of the central nervous system [4]. Blanch et al. (2012) developed two different animal models of blast: a weight drop model and a low-velocity ballistic trauma model [1]. While weight drop did not cause retinal injury, the low-velocity ballistic trauma model, in which ball bearings are shot from an air gun at a known pressure and velocity, did show apoptosis of retinal cells and disruption of photoreceptor cells, as well as changes in the electroretinogram [1]. A modification of the low-velocity ballistic trauma model was developed by Rex et al. [5], where an air gun is set to a specific pressure to induce a closed globe injury to the mouse eye. While Rex et al. only found some visual acuity reductions at pressures of 26psi, we recently reported increased protein levels of key inflammatory markers including tumor necrosis factor alpha (TNFα), and interleukin-1-beta (IL-1β), as well as apoptotic markers in retinal lysates from mice exposed to 26psi blast [6]. We also reported that a novel β-adrenergic receptor agonist, Compound 49b, could reduce the levels of inflammatory and apoptotic markers after exposure to 26psi blast.

In order to fully evaluate the treatment potential for Compound 49b as a promising therapy for individuals such as warriors, who are vulnerable to ocular blast, we wanted to ascertain a potential mechanism of action. We have previously reported that Compound 49b can regulate TNFα in other retinal damage models, specifically the streptozotocin-induced type 1 diabetic retinopathy model and retinal endothelial cells cultured in high

glucose [7]. In those models, it was observed that another protein is down-regulated in response to high glucose, namely insulin-like growth factor-1-binding protein 3 (IGFBP-3). We recently showed that treatment of diabetic rats with IGFBP-3 plasmid could significantly reduce TNFα levels, as well as apoptotic markers [8]. Because IGFBP-3 can reduce both inflammatory and apoptotic markers in models of diabetic retinopathy, we hypothesize that IGFBP-3 may play a similar protective role in retinal damage after ocular blast.

To investigate the role of IGFBP-3 in ocular blast, we exposed IGFBP-3 knockdown (KD) mice to 26psi blast using the method previously described [6]. At 4, 24, and 72 hours post blast exposure, mice were treated with topical Compound 49b, to determine if the treatment increased endogenous IGFBP-3 levels concomitant with decreases in inflammatory and apoptotic markers.

2.0 Methods

2.1 Mice. IGFBP-3 KD mice were generously provided by Dr. John Pintar (Rutgers University). Although we and others originally designated these mice as IGFBP-3 knockout, our current data demonstrates low, but measurable levels of endogenous IGFBP-3 protein levels despite no measureable mRNA levels [9]. Thus we recognize that the IGFPB-3 gene is still minimally active in these animals. All mice were blasted and treated with Compound 49b at 2 months of age. All animal studies confirm to ARVO Guidelines for Use of Animals in Research.

2.2 Blast Procedure. Both eyes of the mice were exposed to the ocular blast. In one subset of mice, eyes were collected at 4 hours post-blast, 24 hours post-blast, 72-hours post blast or at 72 hours without exposure to the blast. In the second subset of mice, both eyes were blasted; however, a novel β-adrenergic receptor agonist, Compound 49b (1mM), was

applied topically within 4 hours, 24 hours or 72 hours post-blast. For the Compound 49b-treated mice, mice received daily Compound 49b treatment for up to 3 days. For example, for the 4 hour treatment group, the mice received the first treatment within 4 hours post-blast, then another treatment 24 hours, 48 hours, and 72 hours post-blast (for a total of 4 treatments), while 72 hour post-blast mice only received 1 treatment of Compound 49b prior to sacrifice. All mice were sacrificed 3 days post-blast for the Compound 49b treated mice. Six mice were used at each time point for all experiments.

2.3 Western Blotting. At the appropriate time after blast or Compound 49b treatment, both eyes were removed for analyses. For Western blot analyses, eyes were used for retinal lysates and collected into lysis buffer containing protease and phosphatase inhibitors, followed by sonication. Equal amounts of protein from tissue extracts were separated on the pre-cast tris-glycine gel (Invitrogen, Carlsbad, CA), blotted onto a nitrocellulose membrane. After blocking in TBST (10mM Tris-HCl buffer, pH 8.0, 150 mM NaCl, 0.1% Tween 20) and 5% (w/v) BSA, the membrane was treated with appropriate primary antibodies followed by incubation with secondary antibodies labeled with horseradish peroxidase. Antigen-antibody complexes were detected by chemilluminescence reagent kit (Thermo Scientific). Primary antibodies used were Cytochrome C, Bax, and Bcl-xL, Akt, phosphorylated Akt^{Ser473} (all purchased from Cell Signaling, Danvers, MA). β-actin antibody was purchased from Santa Cruz (Santa Cruz, CA).

2.4 ELISA Analysis. A cleaved caspase 3 ELISA (Cell Signaling, Danvers, MA) was used to measure levels of the active apoptotic marker in whole retinal lysates. Equal protein was loaded to allow for statistics based upon optical density measurements. TNFα and IL-1β protein concentrations were measured using a TNFα and IL-1β ELISA, respectively with

equal protein loaded into each well to eliminate changes in protein due to amount of protein added (ThermoFisher, Pittsburgh, PA). All ELISAs were done according to manufacturer's instructions.

2.5 Statistics. For all analyses, all experiments were done in triplicate. Data is presented as mean ± SEM, with statistical analyses using Kruskal-Wallis non-parametric testing, followed by Dunn's test.

3.0 Results

3.1 Blast did not affect IGFBP-3 levels, while Compound 49b increased IGFBP-3 in IGFBP-3 KD mice at 4 and 24 hours post-blast. We have previously reported diabetes significantly reduces IGFBP-3 levels [7], leading to increased TNFα levels and apoptosis in whole retina and in retinal endothelial cells (REC). Additionally, we have shown that ocular blast leads to a significant increase in TNFα, IL-1β and a number of apoptotic markers [6], which was negated by treatment with Compound 49b. This led us to hypothesize that Compound 49b protected the retina through increasing IGFBP-3 levels. In Figure 1, we demonstrate that IGFBP-3 KD mice expressed very low levels of endogenous IGFBP-3 and that exposure to ocular blast did not affect these levels. Nevertheless, Compound 49b treatment of the IGFBP-3 KD mice was able to significantly increase protein levels if administered within 24 hours post-blast. Compound 49b given 72 hours after blast had no effect on IGFBP-3 levels. This suggests that despite very low levels of endogenous IGFBP-3 in these knockdown mice, Compound 49b is still able to substantially increase protein levels of IGFBP-3.

3.2 TNFα and IL-1β were increased after blast exposure in IGFBP-3 KD mice, which was negated by treatment with Compound 49b. Exposure of IGFBP-3 KD mice to ocular blast

increased TNFα levels by approximately 40% (Figure 2A) and IL-1β by approximately 3-fold (Figure 2B), which is somewhat similar to data obtained after ocular blast exposure to wild type mice [6]. However, the increase in IL-1β after blast exposure in IGFBP-3 KD mice was greater than that noted in wildtype mice, suggesting that IGFBP-3 is a particularly potent regulator of IL-1β. For both TNFα and IL-1β, Compound 49b was able to significantly reduce protein levels of both inflammatory mediators, TNFα and IL-1β, when administered within 24 hours of blast. Unlike the effects of Compound 49b on wildtype mice [6], Compound 49b treatment did not reduce TNFα and IL-1β levels in IGFBP-3 KD mice at 72 hours post-blast (Figure 2).

3.3 Ocular blast increased pro-apoptotic factors, which were reduced with Compound 49b if given by 24 hours after blast exposure. In wildtype rats, exposure to ocular blast increased pro-apoptotic factors, while reducing anti-apoptotic factors [6]. Similar results were obtained in IGFBP-3 KD mice (Figure 3). Unlike the data in wildtype mice, Compound 49b was only effective in IGFBP-3 KD mice if administered within 24 hours of blast exposure. In wildtype mice, cleaved caspase 3 levels were still reduced at 72 hours post-blast with Compound 49b treatment [6]. This is not the case in IGFBP-3 KD mice, as Compound 49b has no effect on either pro- or anti-apoptotic factors when administered at 72 hours post-blast, with the exception of Bax (which was reduced with Compound 49b at 72 hours; however baseline Bax levels were not increased 72 hours after blast).

4.0 Discussion.

Exposure to ocular blast and the resulting trauma puts our soldiers and other vulnerable individuals at risk for long-term visual damage. In order to better understand the retinal damage caused by closed-globe blast injury, multiple models have been developed

to examine the mechanism for the retinal changes, as well as test novel therapies [4, 10]. We recently reported that ocular blast produced a significant increase in TNFα, IL-1β, and apoptotic factors, which could be reduced by treatment with a novel β-adrenergic receptor agonist, Compound 49b, when administered within 24 hours [6]. Blast produced a much greater increase in IL-1β levels (>3-fold) compared to TNFα levels (1.5-fold), but Compound 49b was more effective in reducing TNFα levels than IL-1β in both wildtype mice [6] or IGFBP-3 KD mice exposed to ocular blast (Figure 2).

One potential pathway by which Compound 49b may reduce inflammatory and apoptotic factors is through increasing IGFBP-3. Despite very low endogenous IGFBP-3 levels in the IGFBP-3 KD mice, Compound 49b was still highly effective in increasing IGFBP-3 levels (Figure 1). Based upon on previous work in retinal endothelial cells, Compound 49b likely increases IGFBP-3 through activation of DNA-PK pathways [11]. Increased levels of IGFBP-3 in the knockdown mice can significantly reduce TNFα levels through activation of the Jun pathway (Zhang et al, in revision). Additionally, Compound 49b-induced increases in IGFBP-3 levels after ocular blast likely reduced pro-apoptotic factors, Bax, cytochrome C, and cleaved caspase 3, through activation of its receptor [12]. Thus, we conclude that Compound 49b is effective in protecting the retina against blast-induced injury through increasing IGFBP-3 levels, as well as reducing TNFα signaling. Based on the work by Jeschke et al., as well as our own findings in diabetic retina [8], it appears that IGFBP-3 lies upstream of TNFα and increasing IGFBP-3 levels can antagonize any responses induced by increased TNFα levels. These findings are in agreement with those from other injury models including a thermal model of total body surface burn in which increasing IGFBP-3 is protective to gut mucosal tissue [13]. Similar to our findings in the blast model, in the thermal model IGF-1/IGFBP-3 reduced the effects of thermal burn on gut homeostasis, which was also associated with decreased TNFα and IL-1β levels [13],

We have previously reported in a mouse model for diabetic retinopathy, that the increased expression of retinopathy markers including TNFα, occurred concomitantly with a decrease in endogenous IGFBP-3 in whole retinal lysates and in retinal endothelial cells [7, 9]. We hypothesize in this case, that the insult of high glucose could have direct effects on reducing β-adrenergic receptor signaling, which in turn decreases IGFBP-3 levels to trigger downstream effects on inflammation and apoptosis in diabetes. Such is not the case in ocular blast injury where IGFBP-3 levels were not affect by blast treatment either in wild type or in IGFBP-3 KD mice. Furthermore, the degree of blast injury, as measured by inflammatory and apoptotic markers, was not more severe in the knockdown mice, suggesting that low levels of IGFBP-3 does not make the retinal more vulnerable to blast injury. We assume that the minimal levels of IGFBP-3 produced by the knockdown are sufficient to maintain homeostasis under normal conditions. However, an increase in IGFBP-3 resulting from after blast treatment with Compound 49b was very beneficial in lowering blast induced increases in inflammation and apoptosis. Unlike diabetic retinopathy, blast injury does not specifically target the β-adrenergic receptor/IGFBP-3 pathway. Nevertheless, stimulation of this pathway after injury promotes recovery. These results suggest that Compound 49b could be an effective treatment for blast-induced injury and thus merits further study as a treatment option for vulnerable patient populations.

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Figure Legends.

Figure 1. IGFBP-3 protein levels after ocular blast and Compound 49b treatment. Western blot results of IGFBP-3 KD mice without exposure to blast, IGFBP-3 KD mice exposed to blast for 4, 24, and 72 hours, and IGFBP-3 KD mice exposed to ocular blast + topical 1mM Compound 49b within 4, 24, or 72 hours after blast exposure. *P<0.05 vs. BP3 NT (not treated); # P<0.05 vs. BP3 KD+blast only at the same time point. N=6 mice in each group.

Figure 2. Ocular blast increased TNFα and IL-1β. ELISA results for TNFα (left) and IL-1β (right) in IGFBP-3 KD mice without exposure to blast, IGFBP-3 KD mice exposed to blast for 4, 24, and 72 hours, and IGFBP-3 KD mice exposed to ocular blast + topical 1mM Compound 49b within 4, 24, or 72 hours after blast exposure. *P<0.05 vs. BP3 NT (not treated); # P<0.05 vs. BP3 KD+blast only at the same time point. N=6 mice in each group.

Figure 3. Ocular blast increased apoptotic factors in IGFBP-3 KD mice. Western blot results for key pro-apoptotic proteins Cytochrome C (A) Bax (B) and anti-apoptotic proteins BcL-xL (D) and Akt (E). ELISA results for cleaved caspase 3 (C) in IGFBP-3 knockdown mice without exposure to blast, IGFBP-3 KD mice exposed to blast for 4, 24, and 72 hours, and IGFBP-3 KD mice exposed to ocular blast + topical 1mM Compound 49b within 4, 24, or 72 hours after blast exposure. *P<0.05 vs. BP3 NT (not treated); # P<0.05 vs. BP3 KD+blast only at the same time point. N=6 mice in each group.

Figure 1

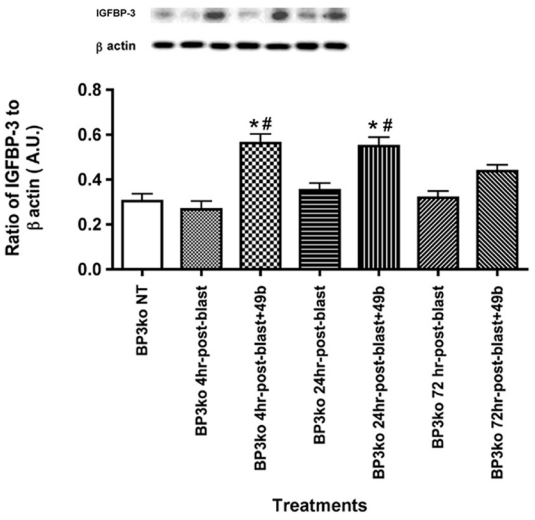


Figure 2

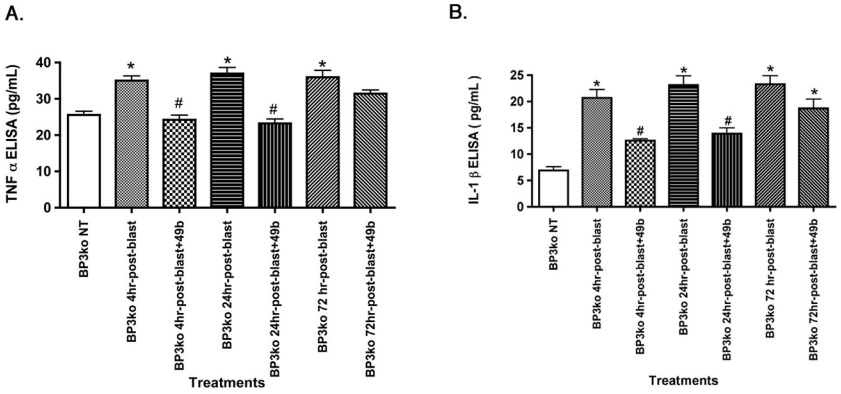
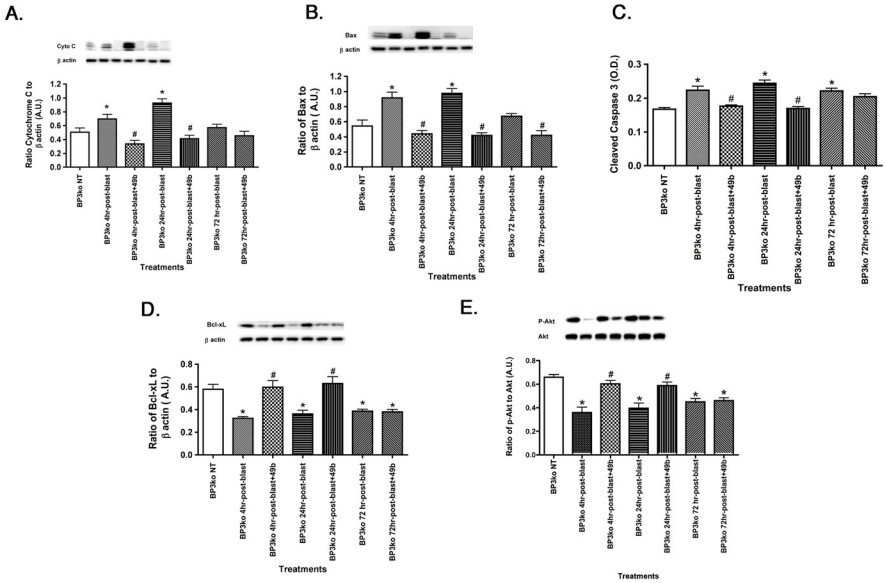


Figure 3


A.
Cyto C
β actin
Ratio Cytochrome C to β actin (A.U.)
BP3ko NT
BP3ko 4hr-post-blast
BP3ko 4hr-post-blast+48b
BP3ko 24hr-post-blast
BP3ko 24hr-post-blast+48b
BP3ko 72 hr-post-blast
BP3ko 72hr-post-blast+48b
Treatments
B.
Bax
β actin
Ratio of Bax to β actin (A.U.)
Treatments
C.
Cleaved Caspase 3 (O.D.)
Treatments
D.
Bcl-xL
β actin
Ratio of Bcl-xL to β actin (A.U.)
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E.
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Akt
Ratio of p-Akt to Akt (A.U.)
Treatments