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“Role of Adenosine Receptor A_{2A} in Traumatic Optic Neuropathies”

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14. ABSTRACT Our goal is to develop an early therapeutic intervention before the progression of traumatic optic neuropathy (TON), a vision-threatening complication in head injury, becomes irreversible. Under the stress of TON, extracellular levels of adenosine increase due to its increased formation by ecto-5'-nucleotidase (CD73) or decreased metabolism by the intracellular adenosine kinase (AK). Intracellular adenosine is then released through equilibrative nucleoside transporter (ENT). Extracellular adenosine activates an anti-inflammatory pathway through adenosine receptor A _{2A} AR. TON is likely due to an imbalance in adenosine formation and metabolism. We report here how we determine whether AK or CD73 contributes in causing this imbalance. We have demonstrated that hypoxia-induced microglia activation is inhibited by inhibitors of MAP Kinases (ERK and P38) or AK. We have also shown that hypoxia-induced CD73 up-regulation suppresses microglia activation. We now tested the hypothesis that in an <i>in vivo</i> model of TON at least adenosine metabolism by AK contributed to this imbalance.						
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INTRODUCTION

Our goal is to understand the mechanism of traumatic optic neuropathy (TON) in order to prevent vision loss. Following traumatic insults to the optic nerve, retinal microglial cells are activated through MAPKinase pathways and increased cytotoxic activity that lend toward damage and death of neighboring and otherwise unharmed retinal ganglion cells (RGCs), further exacerbating the degenerative process. Under the stress of TON, extracellular concentration of adenosine is likely to increase due to its increased formation by ecto-5'-nucleotidase (CD73) (Ernst *et al.*, 2010) or decreased metabolism by the intracellular adenosine kinase (AK) (Löffler *et al.*, 2007). The accumulated intracellular adenosine is then released through equilibrative nucleoside transporter (ENT). Extracellular adenosine activates an anti-inflammatory pathway through adenosine receptor A_{2A}AR (Bong *et al.*, 1996; Ralevic and Burnstock, 1998). TON-induced retinal inflammation is likely due to an imbalance in adenosine formation and metabolism. However, we do not know whether AK or CD73 contributes more in causing this imbalance. We have demonstrated that hypoxia-induced microglia activation was inhibited by inhibitors of MAPKinases (ERK and P38) and AK. We have also shown that hypoxia-induced CD73 up-regulation suppressed microglia activation. Based on these findings, we tested the hypothesis in an *in vivo* model of TON that at least adenosine metabolism by AK contributed to this imbalance.

BODY

A. Statement of work

We seek to understand the mechanism of inflammation in TON in an effort to control RGC death.

Task 1: To test the hypothesis that A_{2A}AR-cAMP signaling is anti-inflammatory in TON.

Task 1 studies scheduled for Year 1 were described in the first annual report.

Task 2: To test the hypothesis that anti-inflammation by A_{2A}AR-cAMP signaling is impaired in TON.

B. Hypotheses to be tested

Hypothesis 1. We hypothesize that a mechanism of anti-inflammation mediated by adenosine receptor A_{2A} (A_{2A}AR) signaling exists in retinal microglial cells. In the setting of TON, however, this process is overwhelmed by the pro-inflammatory state. We further hypothesize that a selective A_{2A}AR agonist effective in reducing inflammation in other disease processes is of utility in TON.

Hypothesis 2. We hypothesize that an imbalance in adenosine formation and metabolism in the retinal microglia may contribute to retinal complications in the setting of TON.

Hypothesis 3. We hypothesize that an imbalance in adenosine formation and metabolism in the retinal microglia participated by AK may contribute significantly to retinal complications in the setting of TON.

C. Experimental Design and Results

Hypothesis 1. We hypothesize that a mechanism of anti-inflammation mediated by A_{2A}AR signaling exists in retinal microglial cells. In the setting of TON, however, this process is overwhelmed by the pro-inflammatory state. We further hypothesize that a selective A_{2A}AR agonist effective in reducing inflammation in other disease processes is of utility in TON.

Methods. Mice were anesthetized according to standard protocol and bilateral limbal conjunctival peritomy was performed posteriorly to the optic nerve in each mouse. Compression by forceps was performed on the right optic nerve in each mouse with the left optic nerve serving as a control. Compression was released at 10 seconds and pupillary dilation was noted. Mice were treated with or without an A_{2A}AR agonist, CGS21680 (25µg/kg; i. p.) every other day for 7 days. All retinas were then harvested.

Results. Increased expression of Iba1 (Figure 1, 2), TNF α (Figure 3), and A_{2A}AR (Figure 4, 5) were shown in nerve crush model. In subgroup of nerve crush model that was treated with CGS, lower expression of these antigens was noted. Also, administration of CGS showed no effect in control model.

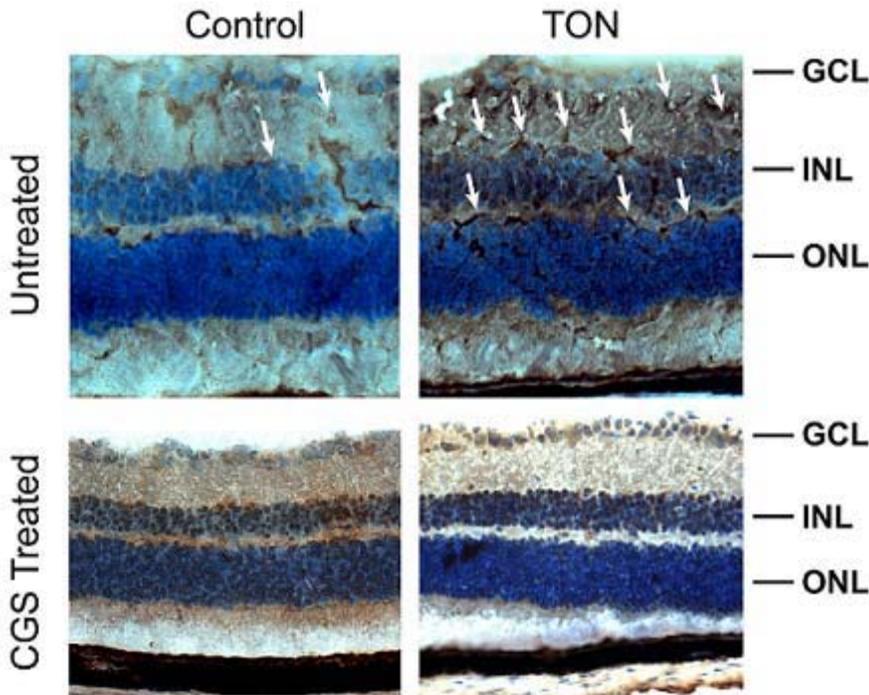


Figure 1. Microglial cell activation assessed by Iba 1 expression in mouse model of TON is inhibited by A_{2A}AR agonist— immunohistochemistry.

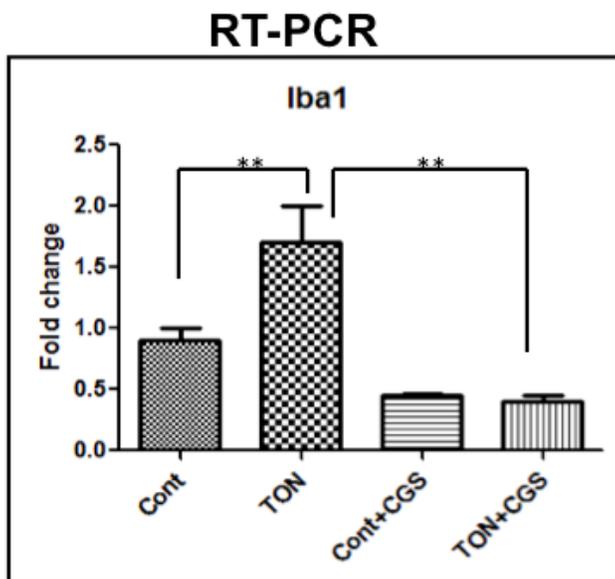


Figure 2. Microglial cell activation in mouse model of TON is inhibited by A_{2A}AR agonist— RT-PCR (QPCR) (n = 4; *P < 0.05; **P < 0.01).

RT-PCR

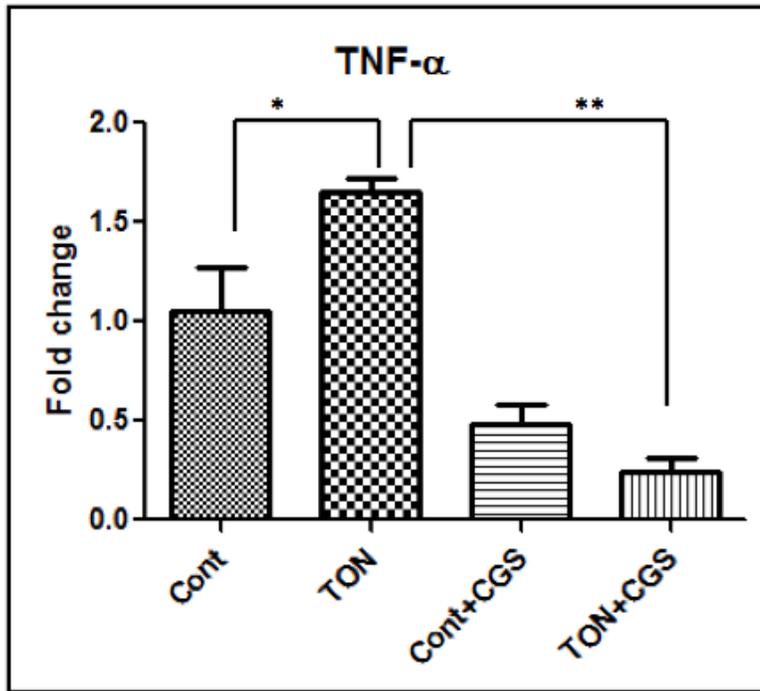


Figure 3. TNF- α in mouse model of TON is inhibited by A_{2A}AR agonist— QPCR (n = 4; **P* < 0.05; ***P* < 0.01).

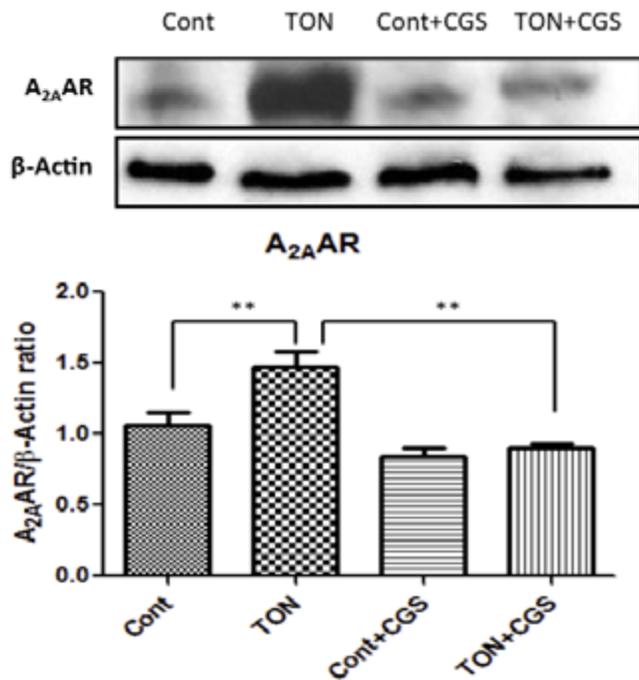


Figure 4. A_{2A}AR expression in mouse model of TON is inhibited by A_{2A}AR agonist— Western analysis (n = 4; **P* < 0.05; ***P* < 0.01).

RT-PCR

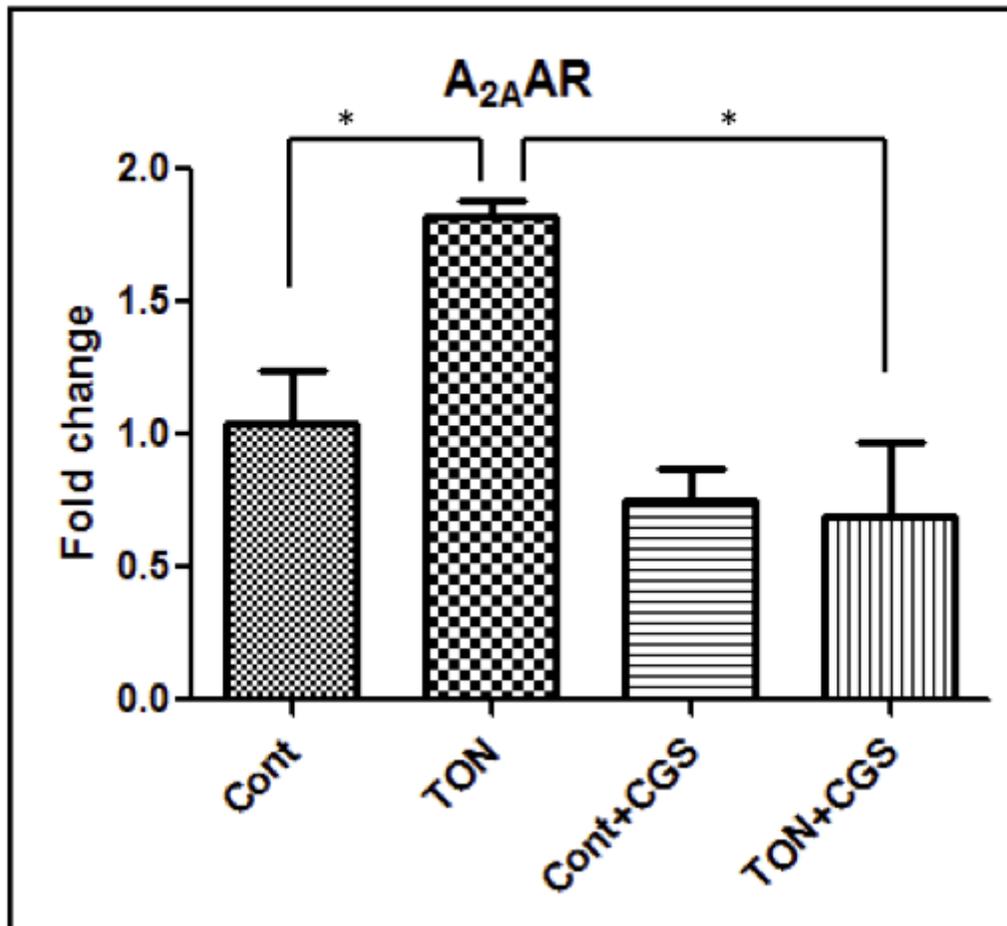


Figure 5. A_{2A}AR expression in mouse model of TON is inhibited by A_{2A}AR agonist—QPCR (n = 4; *P < 0.05; **P < 0.01).

Problem Area We have successfully tested the hypothesis that a mechanism of anti-inflammation in TON is mediated by adenosine-A_{2A}AR signaling. However, it is not known why there is limited adenosine availability in TON.

Hypothesis 2. We hypothesize that an imbalance in adenosine formation and metabolism in the retinal microglia may contribute to retinal complications in the setting of TON.

Methods. Primary rat retinal microglial cells were treated in 1% oxygen (hypoxia) or normoxia at 37°C for 4 hours, then in normoxia for 24 hours. TNF- α levels were measured in the presence and absence of inhibitors for MAPKinases (ERK and P38), AK, and CD-73.

Results. Microglia activation by hypoxia as assessed by TNF- α release is inhibited by inhibitors of MAPKinases (ERK and P38) and AK (**Figure 6**). In addition, hypoxia-induced CD73 upregulation suppresses TNF- α release, which is reversed by CD73 inhibitor (**Figure 7**).

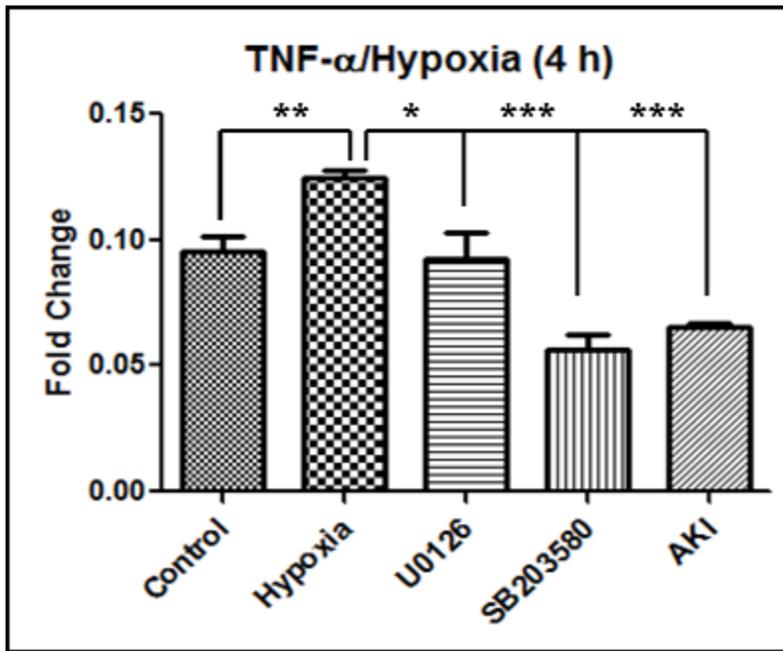


Figure 6. Microglia activation by hypoxia as assessed by TNF- α release is inhibited by inhibitors of MAPKs (U0126 for ERK, and SB203580 for P38) and AK. (n = 4; * P < 0.05; ** P < 0.01; *** P < 0.005).

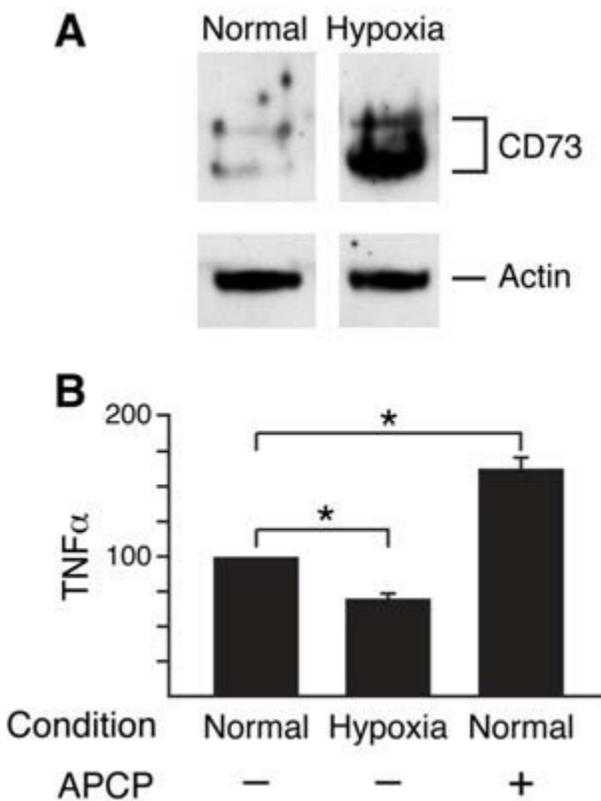


Figure 7. Hypoxia-induced CD73 upregulation (A) suppresses TNF- α release (B) in microglia. This suppression is relieved by CD73 inhibitor, APCP (n = 4; * P < 0.01).

Problem Area We do not know whether the limited adenosine availability in stressed cells or in TON is due to increased adenosine metabolism by AK or decreased adenosine formation by CD73, or both.

Hypothesis 3. We hypothesize that an imbalance in adenosine formation and metabolism in the retinal microglia participated by AK may contribute significantly to retinal complications in the setting of TON.

Methods. Mice were anesthetized according to standard protocol and bilateral limbal conjunctival peritomy was performed posteriorly to the optic nerve in each mouse. Compression by forceps was performed on the right optic nerve in each mouse with the left optic nerve serving as a control. Compression was released at 10 seconds and pupillary dilation was noted. Mice were treated with or without an AK inhibitor (AKI), ABT702 (25µg/kg; i. p.), every other day for 7 days. All retinas were then harvested for RNA preparation. Gene expression was determined by Real-Time PCR analysis.

Results. In a series experiments with Real-Time PCR, increased expression of TNF- α (Figure 8), Iba1 (Figure 9), and caspase3 (Figure 10) were shown in nerve crush model. In subgroup of nerve crush model that was treated with AKI, lower expression was noted. It was also noted that administration of AKI showed no effect in the control.

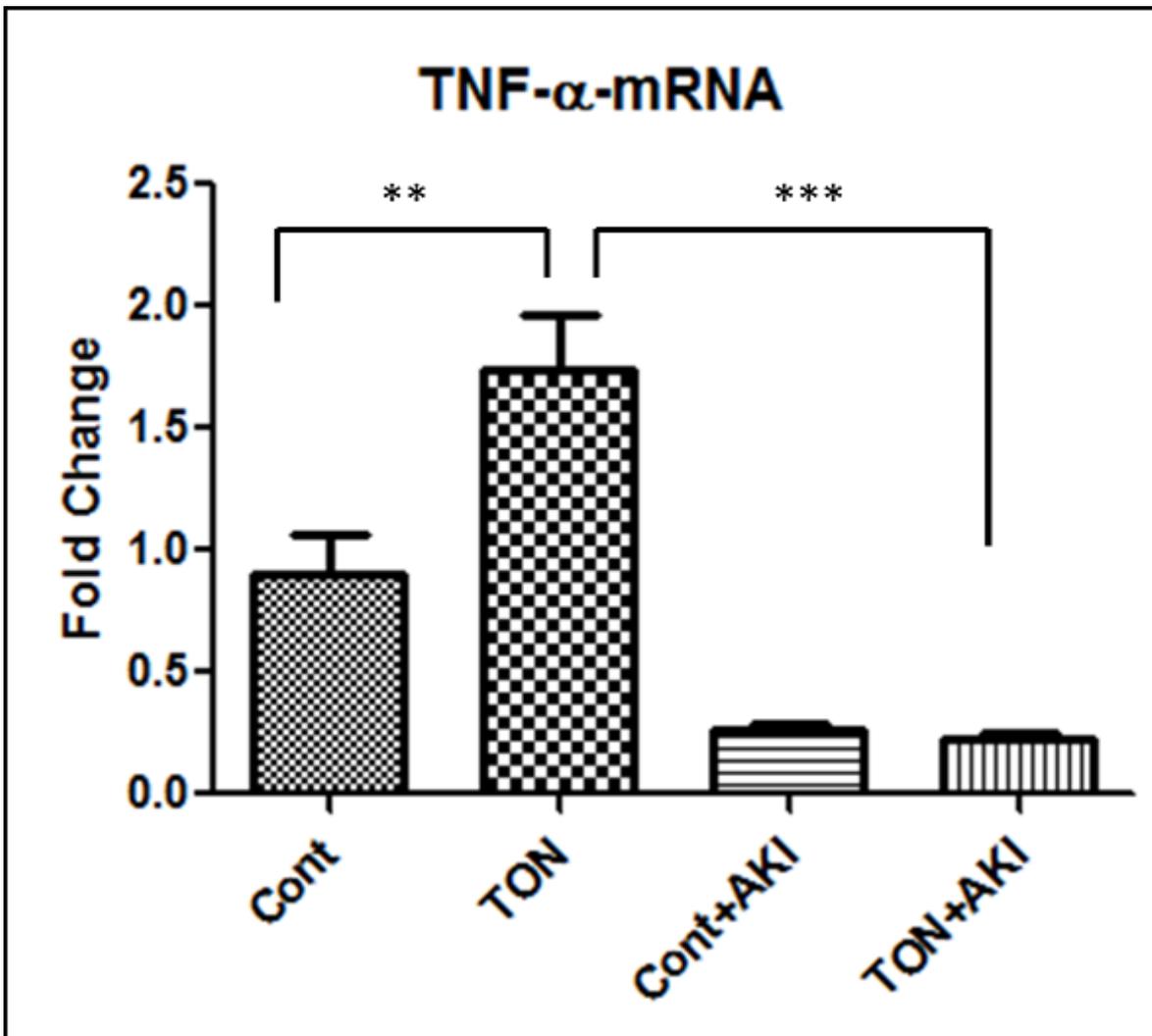


Figure 8. Microglia activation in TON mouse model as assessed by TNF- α mRNA is inhibited by AK inhibitor. (n = 4; ** P < 0.01; *** P < 0.005).

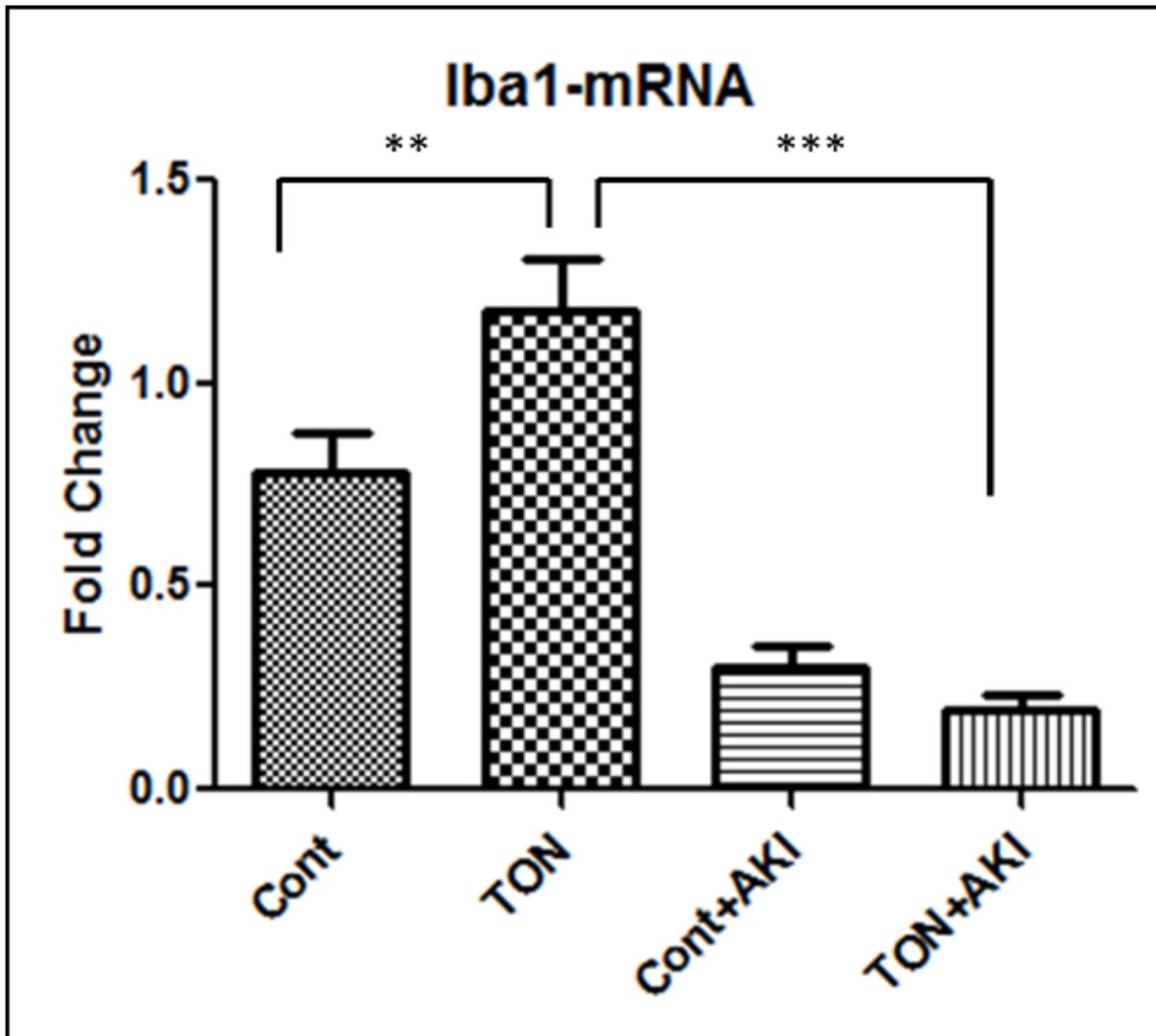


Figure 9. Microglia activation in TON mouse model as assessed by Iba1 mRNA is inhibited by AK inhibitor (n = 4; ** $P < 0.01$; *** $P < 0.005$).

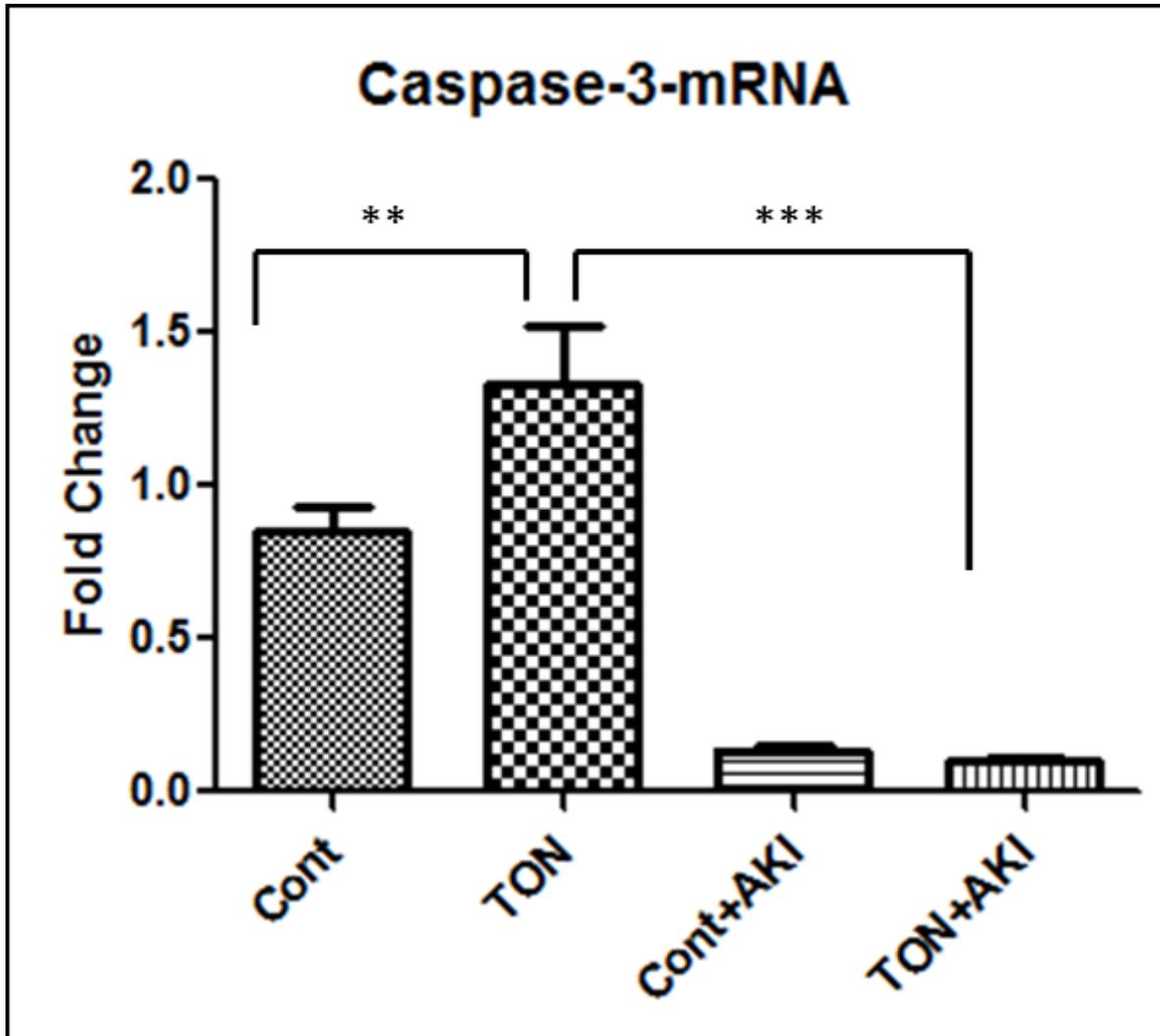


Figure 10. Retinal cell apoptosis in TON mouse model as assessed by caspase3 mRNA is inhibited by AK inhibitor (n = 4; ** $P < 0.01$; *** $P < 0.005$).

Problem Area Although the results suggest that the limited adenosine availability in TON is due to the activity of AK, we do not know whether CD73 also plays a role.

KEY RESEARCH ACCOMPLISHMENTS

1. Our results suggest that TON can be effectively treated with selective adenosine receptor agonist which ameliorates inflammation by activating A_{2A}AR, thereby reducing microglial activity.
2. Our results suggest that TON can also be effectively treated with selective inhibitors for MAPKinases, which ameliorate inflammation by reducing microglial activity.
3. Our results suggest that TON can also be effectively treated with an inhibitor for AK, which ameliorates inflammation by reducing microglial activity.

REPORTABLE OUTCOMES

This research has resulted in one presentation in a scientific meeting (ARVO).

CONCLUSION

Optic nerve injury-induced retinal degeneration can be effectively treated with selective adenosine receptor agonists, selective inhibitors for MAPKinases, or inhibitor for AK. All of these ameliorate inflammation by reducing microglial activity.

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APPENDICES

Adenosine Agonists Combating Inflammation in Traumatic Optic Neuropathy (TON)

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Background

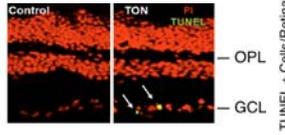
Traumatic optic neuropathy is an irreversible vision-threatening complication often in head injury. Following optic nerve trauma, the body's innate immune cells scavenge the trauma site for debris while releasing cytokines that cause additional damage and cell death beyond that of the initial trauma. While neuronal cell loss stemming directly from the initial insult is irreversible, the secondary inflammation from cytokine release may be prevented. The purpose of our study is to further elucidate mechanisms by which exogenous agonists can effect anti-inflammation and ultimately curb the damage from traumatic optic neuropathy before it is irreversible. Under stress or ischemia such as TON, the local tissue concentrations of adenosine are likely to increase due to the release of ATP and its conversion to adenosine by ectonucleotidases. The released adenosine is anti-inflammatory by stimulating the adenosine receptor $A_{2A}AR$. We tested the hypothesis that $A_{2A}AR$ agonist is of therapeutic utility in TON.

Hypothesis

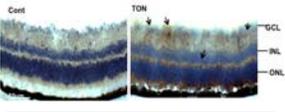
We hypothesize that a mechanism of anti-inflammation mediated by ecto-5'-nucleotidase (CD-73) and adenosine receptor $A_{2A}AR$ signaling exist in retinal microglial cells. In the setting of TON, however, this process is overwhelmed by the pro-inflammatory state. We further hypothesize that selective $A_{2A}AR$ agonist (CGS21680) effective in reducing inflammation in other disease processes may be of utility in TON.

Methods

Mice were anesthetized according to standard protocol and bilateral limbal conjunctival peritomy was performed posteriorly to the optic nerve in each mouse. Compression by forceps was performed on the right optic nerve in each mouse, with the left optic nerve serving as a control. Compression was released at 10 seconds and pupillary dilation was noted. All retinas were then harvested for various procedures including RT-PCR and DCF. Mice were treated with or without an $A_{2A}AR$ agonist, CGS21680.



TUNEL assay of control and TON model demonstrates ganglion cell death in TON. Remainder of retinal layers are relatively well-preserved (above left). A statistically significant increase in TUNEL+ cells in TON compared to control model is shown (above right). Caspase 3 shows increased amounts of apoptosis with focal hyperintensities within ganglion cell layer.



A statistically significant increase is seen in Iba1 in the TON model as compared to control. Iba1 is a marker for microglial cell activation.

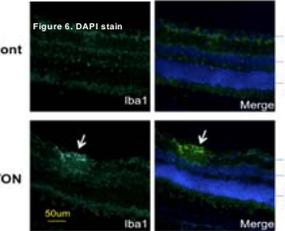
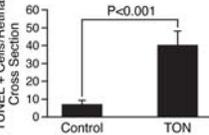
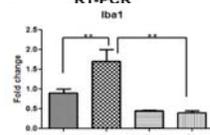


Figure 6. DAPI stain



TUNEL+ Cells/Retinal Cross Section



RT-PCR confirms increased mRNA expression of Iba1 and the reversal effect of CGS21680. Immunohistochemistry illustrates localization of Iba1 within ganglion cell layers

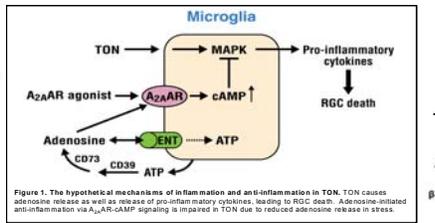
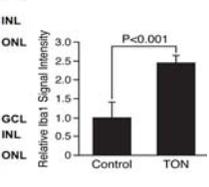
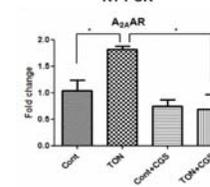


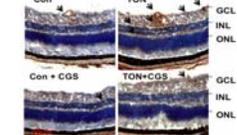
Figure 1. The hypothetical mechanisms of inflammation and anti-inflammation in TON. TON causes adenosine release as well as release of pro-inflammatory cytokines, leading to RGC death. Adenosine-initiated anti-inflammation via $A_{2A}AR$ -cAMP signaling is impaired in TON due to reduced adenosine release in stress.



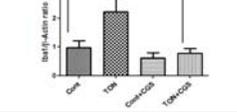
Relative Iba1 Signal Intensity



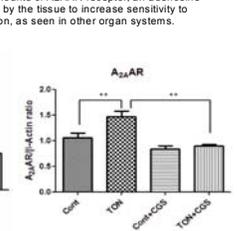
RT-PCR



Iba1



Iba1/β-Actin



A_{2A}AR

Results

RT PCR showed increased mRNA expression of $TNF\alpha$, Iba-1, and CD73 in nerve crush model. In subgroup of nerve crush model that was treated with CGS, lower mRNA expression was noted. Also, administration of CGS showed no effect in control model. Mouse retinas with TON demonstrated higher levels of microglial activity, pro-inflammatory cytokines, reactive oxygen species, and ganglion cell death, as compared with retinas without TON. All the TON-associated retinal damages as well as microglia activity were reduced by treatment with CGS21680, and retinas without TON were apparently without treatment effect.

Conclusion

Traumatic optic neuropathy is a common occurrence in the setting of military combat, with soldiers suffering significant visual loss. Traumatic optic neuropathy causes retinal ganglion cell injury that may trigger microglial cell activation in the mouse retinal model of TON. Microglial cells may continue to be activated to phagocytize degenerating ganglion cells and release pro-inflammatory cytokines, causing further cell death. Therefore, controlling microglial activation may be a way to treat TON. Our results suggest that TON can be effectively treated with selective adenosine receptor agonists which ameliorate inflammation by activating $A_{2A}AR$ thereby reducing microglial activity.

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