Stimulation of A₂B adenosine receptors protects against trauma–hemorrhagic shock-induced lung injury

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Abstract Inflammation is responsible for secondary organ failure after trauma and hemorrhagic shock (T/HS). Adenosine, acting through four G protein-coupled cell surface receptors, A_1, A_{2A}, A_{2B}, and A_3, exerts a number of tissue protective and anti-inflammatory effects. The goal of the present study was to test the effect of A_{2B} adenosine receptor stimulation on T/HS-induced organ injury and inflammation in rats. Rats after T/HS were resuscitated with Ringer's lactate containing the A_{2B} receptor agonist BAY 60–6583 or its vehicle. We found that BAY 60–6583 decreased T/HS-induced lung permeability and plasma creatine kinase levels but failed to affect T/HS-induced lung neutrophil infiltration and $I\kappa B\alpha$ expression and plasma alanine aminotransferase levels. Thus, we conclude that stimulation of A_{2B} receptors protects against T/HS-induced lung and muscle injury.

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Background

Organ failure following trauma and hemorrhage is a consequence of immune dysfunction and cellular damage. Trauma patients who survive the early post-injury period can develop a wide range of conditions, such as acute lung injury, acute respiratory distress syndrome, systemic inflammatory response syndrome, and multiple organ dysfunction syndrome [1, 2].

The endogenous purine nucleoside adenosine has been implicated in the regulation of the development of multiple organ dysfunction following trauma. Adenosine has long been known as a cytoprotective and anti-inflammatory agent that accumulates in response to shock, trauma, ischemia, and inflammation [3, 4]. Cells release adenine nucleotides such as adenosine triphosphate (ATP) or adenosine diphosphate (ADP) in response to stress by a number of mechanisms, including membrane damage, connexin/pannexin channels, and hormone transporting vesicles [3]. Extracellular ATP and ADP are then degraded to adenosine by ecto-nucleoside triphosphate diphosphohydrolase 1 (CD39) and ecto-5′-nucleotidase (CD73) enzymes that are located on the surface of various cell types [5]. Adenosine is also released directly from cells such as neutrophils or endothelial cells to the extracellular space via nucleoside transporters [5, 6]. Extracellular adenosine signals through four G protein-coupled cell surface receptors, A_1, A_{2A}, A_{2B}, and A_3 adenosine receptors (ARs) [7]. ARs are expressed on virtually all cell types that are involved in orchestrating an inflammatory/immune response, and these cell types include polymorphonuclear neutrophil (PMN)s, monocytes/macrophages, endothelial cells, lymphocytes, and dendritic cells [8–10]. While all adenosine

receptors have immunomodulatory functions, stimulation of $A_{2A}ARs$ and $A_{2B}ARs$ is primarily anti-inflammatory, whereas A_1ARs and A_3ARs have both pro- and anti-inflammatory effects [8].

We have previously shown the protective effects of pre-treatment of rats with an $A_{2A}AR$ agonist against organ injury in trauma/hemorrhagic shock (T/HS) and resuscitation, a clinically relevant model comprising global ischemia and reperfusion [11]. The goal of present study was to evaluate the effect of $A_{2B}AR$ stimulation on T/HS-induced organ injury in rats.

Materials and methods

Drugs and reagents

The selective $A_{2B}AR$ agonist 2-[6-amino-3,5-dicyano-4-[4-(cyclopropylmethoxy) phenyl]pyridin-2-ylsulfanyl] acetamide (BAY 60–6583) was provided by Bayer Healthcare [12]. Stock solutions of BAY606583 were pre-pared using dimethylsulfoxide (Sigma Aldrich, St. Louis, MO, USA). Ringer's lactate (RL) was obtained from Fisher Scientific (Pittsburgh, PA, USA).

Experimental animals and T/HS

Male Sprague Dawley rats weighing 300–400 g obtained from the Jackson Laboratory (Bar Harbor, ME, USA) were used for the experiments. Rats were anesthetized with intraperitoneal sodium pentobarbital (50 mg/kg), and their right femoral arteries and jugular veins were isolated using aseptic tech-niques and were cannulated with polyethylene (PE-50) tubing or 50-gauge silicone catheter containing 0.1 ml of heparinized saline (10 U/ml), respectively. A laparotomy was then performed on each rat, and the abdominal cavity was closed with a running 3–0 silk surgical suture. The arterial catheter was attached to a continuous blood-pressure monitoring sys-tem (Powerlab 8/30, ADinstruments, Colorado Springs, CO, USA) during the experiment. The jugular vein catheter was used for blood withdrawal, volume resuscitation, and Evan's blue dye (Sigma Aldrich, St. Louis, MO, USA) administra-tion. Rectal temperature was monitored throughout the shock period and was maintained at 37 °C by a heat lamp positioned over the animal. T/HS rats were exsanguinated to a mean arterial pressure of 30–35 mmHg through the internal jugular vein (at a rate of 1 ml/min) and were maintained at that level for 90 min by withdrawing or reinfusing shed blood (kept at 37 °C) as needed. Trauma/sham-shock (T/SS) rats underwent a laparotomy and vascular cannulation, but no blood was withdrawn. At the end of the 90-min shock or sham-shock period, the animals were resuscitated with RL containing either 0.5 mg/kg BAY 60–6583 or its vehicle [13, 14]. All

rats were maintained in accordance with the recommendations of the "Guide for the Care and Use of Laboratory Animals," and the experiments were approved by the Institutional Ani-mal Care and Use Committee of the New Jersey Medical School.

Measurement of lung permeability

Three hours after the end of the 90-min shock period, the rats were injected with 10 mg of Evan's blue dye through the internal jugular catheter. After 5 min, to allow for complete circulation of the dye, a blood sample (1.5 ml) was withdrawn from the femoral artery catheter, and the plasma was used to determine the plasma Evan's blue dye concentration. Twenty minutes after injection of the dye, the rats were killed and the lungs harvested. Bronchoalveolar lavage was performed on the excised lungs by lavaging the lungs three times with 5 ml aliquots of physiological saline. The recovered bronchoalve-olar lavage fluid (BALF) was then centrifuged at $1,500 \times g$ at 4 °C for 20 min to remove any cells. The supernatant fluid was assayed spectrophotometrically at 620 nm to measure the concentration of the Evan's blue dye in the BALF. The con-centration of Evan's blue dye in the BALF was expressed as the percentage of that present in the plasma.

Assessment of myeloperoxidase activity

For measuring myeloperoxidase (MPO) activity in lung, tissue samples were homogenized in extraction buffer (20 mM acetate buffer (pH 4.7) containing 0.2 M NaCl, 0.5 % cetyltrimethylammonium bromide, 10 μg/ml of phenylmethylsulfonyl fluoride, and 1 mM EDTA). MPO activity was measured using the NWLSS Myeloperoxidase Activity Assay (Northwest Life Science Specialties, Van-couver, WA, USA) and according to the instructions provid-ed with the kit.

Protein isolation and Western blot

For Western blot analysis, lungs were homogenized in a Dounce homogenizer in modified radioimmunoprecipitation assay buffer (50 mM Tris HCl, 150 mM NaCl, 1 mM EDTA, 0.25 % sodium deoxycholate, 1 % Nonidet P-40, 100× diluted proteinase inhibitor cocktail mix). Then the lysates were centrifuged at $15,000 \times g$ for 15 min, and the supernatant was recovered. The Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA) was used to determine the protein concentrations. Protein samples (40 μg from each organ) were separated on 8–12 % Tris-glycine gel (Invitrogen Life Technologies, Carls-bad, CA, USA) and transferred to nitrocellulose membrane. The membranes were probed with a monoclonal antibody against IκBα (Cell Signaling Technology, Danvers, MA, USA), which was followed by incubating with a secondary
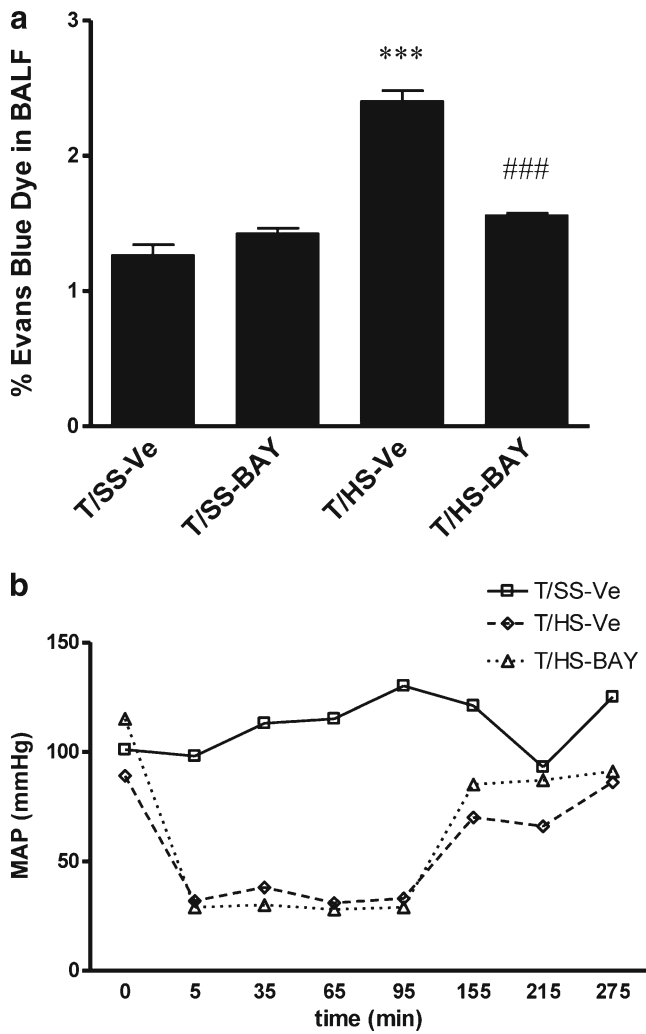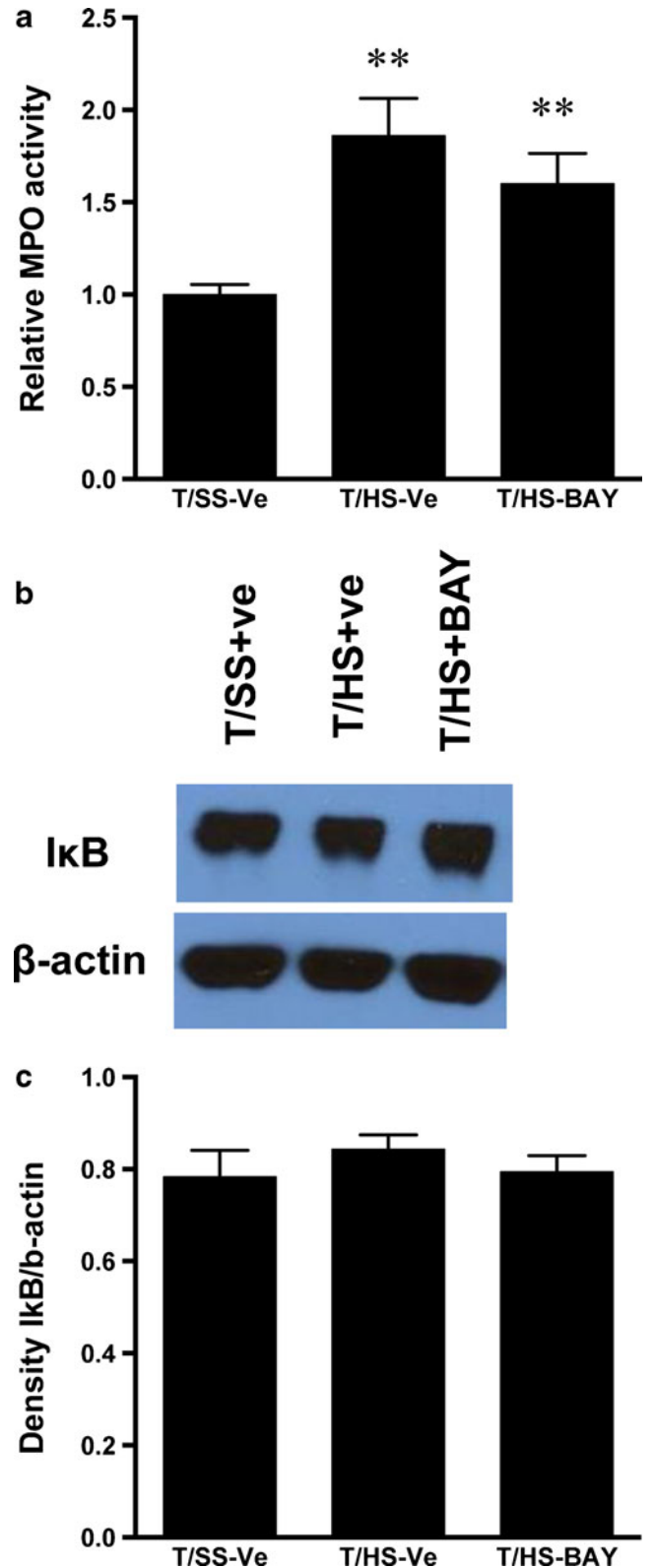
Fig. 1 Effect of BAY 60–6583 on lung permeability and mean arterial pressure (*MAP*) in rats subjected to trauma/hemorrhagic shock (*T/HS*). **a** Trauma/sham shock (*T/SS*) or T/HS rats were resuscitated with Ringer's lactate (*RL*) containing 0.5 mg/kg BAY 60–6583 or its vehicle. Lung permeability was evaluated using Evan's blue dye. ***$p<0.001$ vs T/SS-vehicle, ###$p<0.001$ vs. T/HS-vehicle. Data shown are mean±SEM (*n*=7 to 14). **b** MAP of T/SS rats resuscitated with RL containing vehicle and T/HS rats resuscitated with RL containing vehicle or 0.5 mg/kg BAY 60–6583. Data shown are representative of seven experiments in each group

HRP conjugated anti-rabbit antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). To assess equal protein loading, HRP-conjugated polyclonal goat anti-β actin antibody was used from Santa Cruz Biotechnology. Bands were detected

Fig. 2 BAY 60–6583 fails to protect against T/HS-induced neutrophil infiltration and inflammation in the lung. **a** Myeloperoxidase (*MPO*) level in lungs of T/SS rats resuscitated with RL containing vehicle or T/HS rats resuscitated with RL containing vehicle or 0.5 mg/kg BAY 60–6583. Data shown are mean±SEM of fold increase relative to T/SS-vehicle. **$p<0.01$ vs. T/SS-vehicle (*n*=8–10). **b** Expression of IκBα protein in lungs of T/SS rats resuscitated with RL containing vehicle, T/HS rats resuscitated with RL containing vehicle or 0.5 mg/kg BAY 60–6583. Data shown are representative of seven experiments in each group. **c** Densitometric analysis of IκBα protein expression. Data shown are mean±SEM (*n*=4)

using Chemiluminescent HRP Detection Reagent (Denville Scientific, South Plainfield, NJ, USA).

Determination of alanine aminotransferase and creatine kinase levels in blood of T/HS rats

Plasma concentrations of alanine aminotransferase (ALT) and creatine kinase (CK) were analyzed using a clinical chemistry analyzer system (VetTest8008, IDEXX Laboratories).

Statistical analysis

Values in the figures are expressed as mean plus or minus of the standard error of mean (SEM) of the indicated number of observations. Statistical analyses of the data were performed using Student t test.

Results

$A_{2B}AR$ stimulation protects against T/HS-induced lung injury

We began our experiments by testing the efficacy of BAY 60–6583 in preventing T/HS-induced lung injury. We found that lung permeability was increased in rats subjected to T/HS in comparison to T/SS. Resuscitation with RL containing BAY 60–6583 provided an almost complete protection against the T/HS-induced increase in lung permeability (Fig. 1a). We also monitored blood pressure and found that BAY 60–6583 did not have any adverse effect on blood pressure (Fig. 1b).

$A_{2B}AR$ stimulation fails to inhibit T/HS-induced lung inflammation

We next studied the effect of $A_{2B}AR$ stimulation on neutrophil infiltration into the lung. Our results showed that BAY 60–6583 failed to decrease T/HS-induced neutrophil infiltration as indicated by MPO activity (Fig. 2a). We then examined the effect of BAY 60–6583 on inflammation in the lung by determining $I\kappa B\alpha$ levels. We found that both T/HS and BAY 60–6583 failed to affect $I\kappa B\alpha$ levels (Fig. 2b, c).

$A_{2B}AR$ stimulation protects against T/HS-induced muscle injury

Finally, we tested the effect of BAY 60–6583 on markers of liver and muscle injury. We found that T/HS induced significant increases in the plasma levels of ALT and CK. While plasma ALT concentration was not affected by BAY 60–6583 (Fig. 3a), BAY 60–6583 reversed the increase in plasma CK levels caused by T/HS (Fig. 3b). Thus, $A_{2B}AR$ stimulation protects against T/HS-induced muscle, but not liver injury.
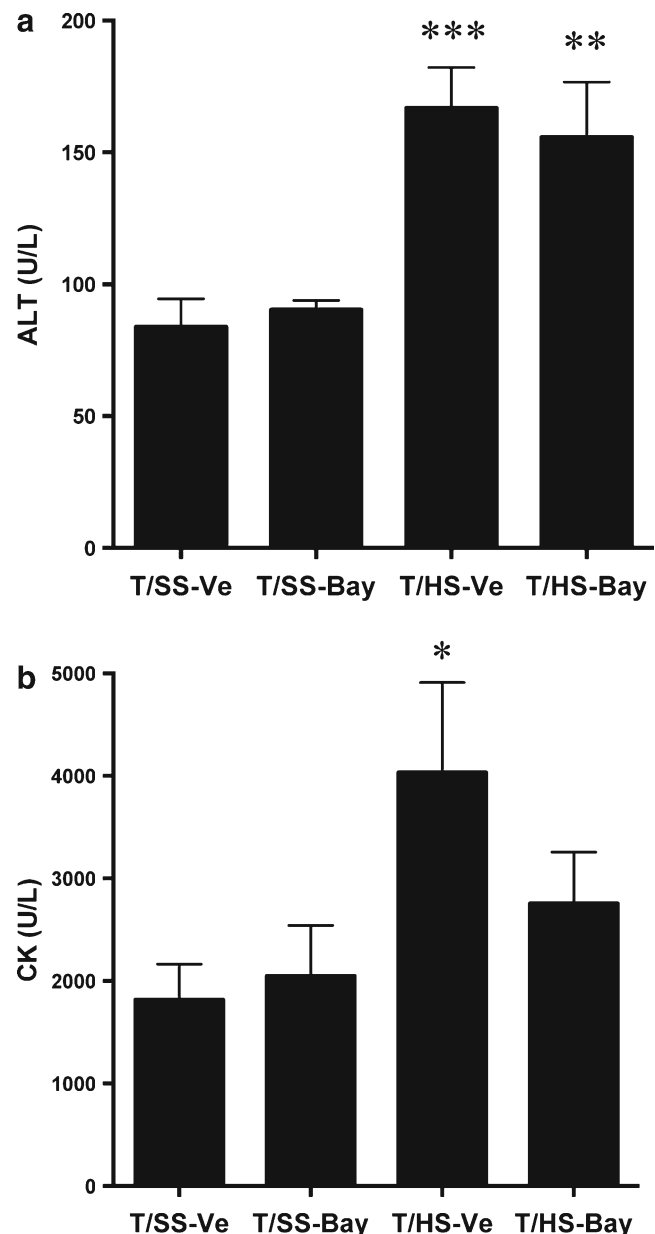

Fig. 3 BAY 60–6583 protects against T/HS-induced muscle injury. Alanine aminotransferase (*ALT*), an indicator of liver injury (**a**), or creatine kinase (*CK*), an indicator of muscle injury (**b**) of T/SS or T/HS rats resuscitated with RL containing vehicle or 0.5 mg/kg BAY 60–6583. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. T/SS-vehicle. Mean±SEM ($n = 9$–13)

Discussion

Here we have provided evidence that $A_{2B}AR$ stimulation with BAY 60–6583 during resuscitation protects against T/HS-induced lung injury. As we have recently demonstrated, $A_{2A}AR$ activation also protects against acute lung injury following T/HS [11], but there are differences between the mechanisms of action of stimulating A_{2A} vs. A_{2B} receptors.

The protection afforded by $A_{2A}AR$ stimulation was accompanied by a decrease in PMN sequestration in the lung [11]. In contrast, our results suggest that $A_{2B}AR$ stimulation does not provide protection by inhibiting the PMN infiltration to the lung because BAY 60–6583 did not reverse the T/HS-induced increase in lung MPO activity. This finding is surprising because PMNs reportedly express $A_{2B}ARs$ and activation of these receptors suppresses PMN transmigration through both endothelium and epithelium [15, 16]. On the other hand, it has also been shown that $A_{2B}ARs$ are important in moderating vascular injury in systemic endotoxin challenge [17] and have a key role in dampening hypoxia-induced vascular leak in vivo [15, 18]. $A_{2B}AR$ KO mice exposed to ambient hypoxia had increased vascular permeability in the majority of organs tested and $A_{2B}AR$ KO mice suffering from ventilator-induced lung injury demonstrate increased pulmonary vascular leak and edema, which is due to lack of $A_{2B}ARs$ on pulmonary and not hematopoietic cells [18]. These findings together with our results raise the possibility that BAY 60–6583 protects against T/HS-induced lung injury by stimulating the $A_{2B}ARs$ of pulmonary parenchyma cells.

Muscle injury, a marker of which is elevated CK level in plasma, is a well-known consequence of T/HS [19–21]. The role of adenosine signaling in protecting against ischemia–reperfusion induced myocardial injury has been described, and A_1ARs [22], $A_{2B}ARs$ [12, 23], or A_3ARs [24, 25] have all been shown to be protective. Our data showing that resuscitation with BAY 60–6583 reverses T/HS-induced increases in plasma CK levels suggest that $A_{2B}ARs$ also have protective roles against muscle injury following T/HS.

We have previously shown that $A_{2B}AR$ stimulation augments IL-10 expression [26–28]. In addition, recent studies have shown the protective role of IL-10 in hemorrhagic shock induced lung inflammation and injury [29, 30]. Our results together with these data raise the possibility that one of the mechanisms of action of adenosine in protecting against lung injury is through augmenting IL-10 expression. This will be an interesting aspect to explore in the future.

In conclusion, we found that stimulation of $A_{2B}ARs$ during resuscitation following T/HS protects against lung and muscle injury without any adverse effect on blood pressure, suggesting the therapeutic potential of $A_{2B}AR$ agonists in the treatment of hemorrhage.

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