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Inhibition of tPA-induced hemorrhagic transformation involves adenosine A2b receptor activation after cerebral ischemia

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Abstract

Tissue plasminogen activator (tPA) is administered after ischemic stroke to dissolve intravascular clots, but its use can lead to hemorrhagic transformation (HT). Therapeutic strategies to reduce hemorrhagic complications of tPA might be of benefit for stroke patients. Adenosine A2b receptor (A2bR) plays pivotal roles in regulating vascular protection in peripheral organs. This study explored whether A2bR agonist BAY 60-6583 reduces hemorrhage risk after tPA usage. Using a rat transient middle cerebral artery occlusion model, we showed that mRNA and protein expression of A2bR increased to a greater extent after ischemia-reperfusion than did expression of the other three adenosine receptors (A1, A2a, and A3). tPA administration reduced A2bR expression in ischemic brain microvessels. Post-treatment with BAY 60-6583 (1 mg/kg) at the start of reperfusion reduced lesion volume in the absence or presence of tPA (10 mg/kg) and attenuated brain swelling, blood-brain barrier disruption, and tPA-exacerbated HT at 24 h. Additionally, BAY 60-6583 mitigated sensorimotor deficits in the presence of tPA. BAY 60-6583 inhibited tPA-enhanced matrix metalloproteinase-9 activation, probably through elevation of tissue inhibitor of

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Author contributions

Y.Z., R.C.K., J. Z., and J.W. conceived and designed the research; Q.L., X. Han, X.L., X. Hong, T.L., Y.G., and Qian L. performed the experiments; Q.L., R.C.K., Y.Z., J.Z., Q.Y., and J.W. analyzed data and wrote the paper. Y.Z., R.C.K., J.Z., and J.W. obtained funding. All authors read and approved the final draft.

matrix metalloproteinases-1 expression, and thereby reduced degradation of tight junction proteins. These effects would likely protect cerebrovascular integrity. A2bR agonists as an adjuvant to tPA could be a promising strategy for decreasing the risk of HT during treatment for ischemic stroke.

Keywords

Adenosine receptor; Cerebral ischemia; tPA; Hemorrhagic transformation

1. Introduction

The thrombolytic agent tissue plasminogen activator (tPA) is underutilized in ischemic stroke, in part because of its limited therapeutic window and hemorrhagic complications (Kleindorfer et al., 2008; Wang et al., 2015d). tPA can injure the blood-brain barrier (BBB) by activating matrix metalloproteinases (MMPs) and can increase BBB permeability by degrading extracellular matrix components and tight junction proteins on endothelial cells (Tsuji et al., 2005; Yepes et al., 2009). These effects can lead to hemorrhagic transformation (HT) and aggravate brain damage after reperfusion (Kelly et al., 2006; Mishiro et al., 2012). Identifying an adjuvant drug that reduces the risk of hemorrhage could increase the applicability of tPA.

Adenosine is a potent neuromodulator that acts through four G-protein-coupled receptors (A1, A2a, A2b, and A3). A1 and A2a are high-affinity receptors that are active under normal physiologic conditions. A2b receptors (A2bRs) are activated by micromolar concentrations of adenosine in tissues that experience ischemia, trauma, inflammation, or other types of stressful insults (Eltzschig, 2009). When ischemic conditions trigger a robust and sustained increase in cerebral extracellular levels of adenosine, activation of low-affinity A2bR might protect against ischemic injury.

Studies have shown that the selective A2bR agonist BAY 60-6583 reduces myocardial infarct size in rabbit and mouse hearts when it is administered before or at the onset of reperfusion (Eckle et al., 2007; Kuno et al., 2007; Methner et al., 2010). Moreover, vascular permeability was increased in peripheral organs of A2bR-null mice that were subjected to hypoxia (Eckle et al., 2008). BAY 60-6583 treatment reversed vascular leakage induced by hypoxic stress and luminal mechanical injury (Bot et al., 2012; Eckle et al., 2008; Yang et al., 2008). Further, adenosine and adenosine receptors modulate MMP-9 expression and activity in macrophages. By reducing MMP-9 expression, A2bR activation might contribute to the vasoprotection that was observed in a femoral artery injury model (Chen et al., 2011). Collectively, these studies indicate that A2bR can regulate vascular leakage. Indeed, because A2bR expression is selectively increased under pathologic conditions, the effectiveness of an A2bR agonist might be augmented (Eckle et al., 2008; Eckle et al., 2007; Hart et al., 2009). Thus, we postulate that activation of A2bR has the potential to protect against BBB disruption induced by tPA after ischemic stroke.

Here, we subjected rats to transient middle cerebral artery occlusion (tMCAO) and examined how tPA administration, in the presence of A2bR agonist BAY 60-6583, affects BBB

permeability and risk of HT. We hypothesized that administration of BAY 60-6583 as an adjuvant drug would reduce MMP-9 activity, BBB permeability, and HT and improve neurologic function compared with administration of tPA alone.

2. Material and methods

2.1. Transient focal cerebral ischemia

Animal procedures were approved by the Johns Hopkins University Animal Care and Use Committee and were conducted in accordance with the National Institutes of Health Guidelines and the ARRIVE (Animal Research: Reporting In Vivo Experiments) guidelines (www.nc3rs.org.uk/arrive-guidelines). In total, we used 163 adult, male Sprague-Dawley rats (300–350 g; Charles River Laboratories, Frederick, MD), which were anesthetized with isoflurane (5% for surgical induction, 2–2.5% for maintenance) in oxygen-enriched air (30% oxygen/70% air) during surgical procedures. Transient focal cerebral ischemia was produced by inserting a silicone-coated 4–0 nylon filament into the left middle cerebral artery. Cerebral blood flow (CBF) was monitored by laser-Doppler flowmetry, as described previously (Li et al., 2014). Successful occlusion was defined as the decrease in surface CBF to <20% of baseline (Wang et al., 2015b). After 2 h of occlusion, reperfusion was initiated by withdrawal of the filament. Physiologic parameters, including mean arterial blood pressure (MABP), blood gases, blood glucose, and body temperature, were measured immediately before the initiation of MCAO and right after reperfusion. We chose the filament stroke model to test our hypothesis in this study because the reperfusion time after tMCAO can be manipulated, and the infarct size is relatively consistent compared with those produced by the thrombo-embolic and autologous blood clot models.

2.2. Experimental design

A sample size calculation based on our previous study (Wang et al., 2015e) and pilot data indicated that 6 rats/group would provide at least 80% power for detecting a 20% decrease in hemoglobin content at $\alpha = 0.05$ (2-sided). Rats were randomly allocated into study groups by using the website Randomization.com (Cheng et al., 2016). Animals underwent tMCAO or sham surgery. Rats that underwent tMCAO were randomly assigned to treatment with vehicle (0.1% dimethyl sulfoxide [DMSO]), tPA alone (Genentech, San Francisco, CA, USA), BAY 60-6583 alone (R&D Systems, Inc., Minneapolis, MN), or a combination of BAY 60-6583 and tPA. At the start of reperfusion, rats were administered 10 mg/kg tPA, with 10% given as a bolus and the remaining 90% delivered as a constant infusion over 30 min. BAY 60-6583 (1 mg/kg, i.v.) (Bot et al., 2012; Maas et al., 2010) was infused immediately before tPA treatment. All drugs were administered in a blinded manner. At 6 h after tMCAO, the fibrin degradation product (FDP) D-D dimer was measured in blood samples with an automatic biochemical analyzer (Hitachi 7020, Tokyo, Japan).

2.3. Real-time quantitative reverse-transcriptase PCR

Total RNA was prepared with the RNeasy Lipid Tissue Mini Kit (Qiagen, Valencia, CA) (Li et al., 2017). The concentration was determined by a spectrophotometer (NanoDrop 1000, Thermo, Wilmington, DE). One microgram of total RNA was reversely transcribed with the SuperScript VILO cDNA Synthesis Kit (Invitrogen, Grand Island, NY). The primers and

probes for A1R (Rn00567668_m1), A2aR (Rn00583935_m1), A2bR (Rn00567697_m1), A3R (Rn00563680_m1), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Rn01775763_g1) were obtained from Applied Biosystems (Grand Island, NY). The amplification was performed in an ABI 7500 Fast Real-Time PCR system (Applied Biosystems) using the TaqMan Universal 2× PCR Master Mix (Applied Biosystems). Reactions (total volume 20 µl) were incubated at 50 °C for 2 min and at 95 °C for 10 min, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. mRNA levels of candidate genes were normalized to the endogenous control GAPDH in the same sample. The 2^{-C_t} method was used for relative quantification of gene expression as described (Wang et al., 2015a).

2.4. Immunofluorescence staining

Rat brains were cut into 25-µm sections and used for double immunostaining analysis (Han et al., 2016). Brain sections were incubated with primary antibody and then with the appropriate Alexa 488- or 594-conjugated secondary antibody (Molecular Probes, Eugene, OR). We used the following primary antibodies: rabbit anti-A2bR (1:200, Santa Cruz Biotechnology, Dallas, TX), mouse anti-GFAP (1:500, Sigma-Aldrich, St. Louis, MO), mouse anti-NeuN (1:500, Millipore, Billerica, MA), mouse anti- von Willebrand factor (vWF; 1:50, Dako, Carpinteria, CA), and sheep anti-albumin (1:1000, Bethyl Laboratories, Montgomery, TX). Negative controls were treated with a similar procedure, but the primary antibody was omitted (Lan et al., 2017). The coverslips were mounted on glass slides and stained with 4', 6-diamidino-2-phenylindole. The sections were examined under a fluorescence microscope (Eclipse 90i; Nikon, Tokyo, Japan).

2.5. Brain microvessel isolation

Brain microvessels were isolated according to a previously described procedure (Ospina et al., 2002). Brain hemispheres were homogenized in cold phosphate-buffered saline (PBS) and centrifuged at 2000g for 5 min at 4 °C. The pellet was resuspended in PBS and centrifuged at 2000g for another 5 min at 4 °C. The resulting pellet was resuspended in PBS, carefully layered over a 15% dextran density gradient, and centrifuged at 3500g for 35 min at 4 °C. The supernatant was discarded, and the pellet was resuspended in PBS, layered over dextran, and centrifuged for an additional 35 min at 3500g. The resulting pellet was thoroughly washed with ice-cold PBS over a 70-µm nylon mesh. The cerebral vessels were collected and used for Western blot analysis.

2.6. Western blot analysis

Twenty-four hours after ischemia, animals were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneal) and perfused transcardially with saline. The brains were removed and sectioned into seven 2-mm-thick coronal slices (Li et al., 2017). The third and fifth slices located within the core of the lesion were used for Western blotting and the hemoglobin assay (Jeong et al., 2014; Zhang et al., 2017). Equal amounts of protein per lane (30 µg) were subjected to electrophoresis on 10–12% SDS-PAGE gels. Proteins were electro-transferred onto polyvinylidene difluoride membranes (Millipore) and blocked with 5% nonfat dry milk/0.1% Tween-20 in Tris-buffered saline for 2 h at room temperature. Thereafter, membranes were incubated with different primary antibodies, including rabbit anti-A1R (1:1000, Santa Cruz Biotechnology), mouse anti-A2aR (1:1000, Santa Cruz Bio-

technology), rabbit anti-A2bR (1:500, Santa Cruz), rabbit anti-A3R (1:500, Santa Cruz Biotechnology), rabbit anti-zonula occludens-1 (1:1000, Invitrogen), mouse anti-MMP-9 (1:500, Abcam, UK), rabbit anti-tissue inhibitor of matrix metalloproteinases-1 (1:500, Abcam), and sheep anti-albumin (1:20,000, Bethyl Laboratories, Montgomery, TX). Subsequently, membranes were treated with horseradish peroxidase-labeled secondary antibody for 1 h at room temperature. Immunoblots were probed with enhanced ECL substrate (Pierce, Rockford, IL). The chemiluminescence level was assessed with an imaging system (GE Healthcare, Piscataway, NJ), and results were normalized to β -actin.

2.7. Measurement of infarction volume and brain swelling

At 24 h after the induction of tMCAO, the rats were killed and perfused with saline. Their brains were removed immediately, and brain tissue was cut into seven serial 2-mm coronal sections. Brain slices were immersed in a 2% solution of 2,3,5-triphenyltetrazolium chloride in PBS at 37 °C for 15 min. Infarct volume was quantified in the regions that failed to stain and expressed as a percentage of the contralateral area (Pan et al., 2014). The extent of swelling was calculated by using the following equation: extent of brain swelling = (volume of ipsilateral hemisphere – volume of contralateral hemisphere)/volume of contralateral hemisphere (Wu et al., 2017). A person blinded to the treatment groups conducted the measurements and evaluations.

2.8. Behavioral tests

Behavioral tests were conducted in a dedicated, enclosed room at 20–22 °C. An investigator blinded to treatment group carried out a battery of tests on each rat as described below.

Neurologic deficit score—Neurologic deficits were assessed at 24 h post-ischemia with a neurologic deficit grading system as described previously (Wang et al., 2015e). Each animal was scored on a scale of 0 to 5. Higher scores indicated more severe impairment of neurologic function.

Corner test—In the corner test, a rat is placed between two boards that form a 30-degree angle. When the boards brush the rat's whiskers, a healthy rat will turn left or right with equal frequency. However, rats with unilateral brain injury will turn preferentially toward the non-impaired side (in our study, the left side) (Li et al., 2017). We repeated this test 10 times for each rat and recorded the number of left turns.

Rotarod test—Spontaneous motor function of the rats was measured on an accelerating rotarod treadmill (Zhu et al., 2017). The speed of the rotor increased from 5 to 40 rotations per minute (rpm) at intervals of 1 rpm every second. The run time was recorded as the performance time. We trained the animals for five trials 1 day before tMCAO to obtain stable baseline values. The animals were then tested at 24 h after tMCAO. We recorded the mean time duration of five trials on the rod. These data are presented as the mean duration on the rod.

2.9. Magnetic resonance imaging (MRI)

All MRI experiments were conducted on a standard 4.7 T horizontal-bore animal imager (Bruker Biospin, Billerica, MA), and images were analyzed with the IDL program (Exelis Visual Information Solutions, Inc., Boulder, CO) (Wang et al., 2015c; Wang et al., 2017; Zhou et al., 2013). To assess BBB permeability changes, we acquired T1-weighted spin-echo images (T1WIs) in the coronal plane (7 slices) before and after gadolinium contrast agent administration (0.4 mmol/kg gadoteridol, Bracco Diagnostics, Princeton, NJ). Contrast-enhanced T1-weighted images (CE-T1WIs) were acquired immediately after contrast injection and again 15 and 30 min later. Abnormalities on the CE-T1WIs were used to highlight regions of gadoteridol extravasation and to analyze the BBB permeability changes. We quantified the gadoteridol-permeable BBB volume (PBV) corresponding to all hyperintense regions in all seven slices on the CE-T1WIs. This volume indicates brain tissue with leaky BBB and the contrast-enhanced ratio (CER; compared to the contralateral normal brain tissue).

2.10. Hemoglobin assay

Hemoglobin concentration of brain tissue was determined with a hemoglobin assay kit (Sigma-Aldrich) (Wu et al., 2015a). Samples were taken from the same tissue as that used for Western blot analysis. Briefly, equal amounts of sample supernatant (300 µg in 50 µl) were added to 200 µl of reagent. After the reaction mixture incubated for 15 min at room temperature, optical density was measured at 400 nm with a spectrophotometer. A standard curve was obtained by using the calibrator reagent in incremental dilutions (0, 1.25, 6.25, 12.5, 25, 37.5, 50, 75, and 100 mg/dl). The data were expressed as milligrams per deciliter.

2.11. MMP zymography

Gelatin gel zymography was carried out as previously reported with some modifications (Wu et al., 2017; Zhao et al., 2015). Briefly, 2 mg of protein in 500 µl was incubated with 50 µl of Gelatin-Sepharose 4B beads (GE Healthcare) for 1 h at 4 °C with gentle rotation. The beads were collected, and the MMP-2 and MMP-9 were eluted by incubation with 20 µl of elution buffer (10% DMSO in PBS) for 30 min at 4 °C with gentle shaking. Equal amounts of sample (20 µl) were electrophoretically separated on 10% zymogram (0.1% gelatin) protein gels (Invitrogen) under nonreducing conditions. Gels were removed and incubated for 1 h at room temperature in 100 ml of 2.5% Triton X-100 (renaturation buffer) on a rotary shaker. Then they were incubated for 48 h with a zymogram developing buffer (Invitrogen) at 37 °C before being stained with 0.5% Coomassie blue R-250 for 30 min at room temperature. Gels were destained with a solution containing 10% acetic acid and 10% methanol until clear bands of gelatinolysis appeared on a dark blue background. Mouse MMP-9 and mouse MMP-2 (R&D Systems, Minneapolis, MN) were used as standards. The activity of MMP-2 and MMP-9 was quantified by Image J software (NIH, Bethesda, MD) (Zhang et al., 2017).

2.12. Statistical analysis

Parametric data are presented as mean ± SD and were compared among groups with one-way ANOVA followed by Bonferroni post hoc test (GraphPad Software, San Diego, CA).

Neurologic deficit scores are presented as median and interquartile ranges and were compared among groups with the non-parametric Kruskal-Wallis test followed by Dunn's post hoc analysis. *p* values <0.05 were considered statistically significant.

3. Results

3.1. Cerebral ischemia induces A2b receptor expression

Adenosine receptor activation is crucial for endogenous protective mechanisms after cerebral ischemia. We investigated the consequences of cerebral ischemia on expression levels of all four adenosine receptors. Our results revealed that the relative increases in mRNA and protein expression levels of A2bR were higher than those of the other three adenosine receptors after cerebral ischemia (Fig. 1A, B). A2bR expression at 24 h was significantly higher in ischemic cortex and striatum than in the corresponding contralateral regions (Fig. 1C). A2bR is expressed in the endothelium and vasculature of multiple organs (Yang et al., 2006). To explore the role of A2bR in protection of the neurovascular network after cerebral ischemia, we performed immunofluorescence using cell-specific markers NeuN for neurons, GFAP for astrocytes, and vWF for endothelium to explore which cell type expressed A2bR. We found that A2bR-positive cells were co-labeled with NeuN, GFAP, and vWF in the peri-infarct area (Fig. 1D).

3.2. tPA reduces A2bR expression in ischemic brain microvessels

A2bR activation was shown to be vasoprotective in a femoral artery injury model (Chen et al., 2011). In our study, increased A2bR activation in neurons, astrocytes, and endothelium after tMCAO might indicate initiation of the endogenous protective mechanism. We investigated the effects of tPA on A2bR expression in the peri-infarct brain of rats subjected to 2-h tMCAO plus 24 h reperfusion. tMCAO raised A2bR levels in the peri-infarct tissue, but tPA treatment significantly reduced A2bR expression compared with that in the vehicle-treated tMCAO group (Fig. 2A). We also investigated the effect of tPA on endothelial cells using Western blotting and immunostaining. We measured the A2bR protein level in homogenates of microvessels isolated from the whole brain hemispheres. tPA treatment inhibited the ischemia-reperfusion-induced increase in A2bR expression in microvessels (Fig. 2B). Immunofluorescence studies also demonstrated that A2bR and vWF co-staining signals were markedly less in tPA-treated tMCAO rats than in vehicle-treated tMCAO rats (Fig. 2C), consistent with the Western blot results. These findings indicate that tPA reduces A2bR expression in ischemic brain microvessels.

3.3. Physiologic data and fibrinolytic activity

No significant differences in MABP, blood gases, body temperature, or blood glucose were observed among the four groups (Supplementary Table 1). However, plasma levels of D-D dimer and FDP, molecular markers of fibrinolysis, were significantly higher in tPA-treated rats than in vehicle-treated rats at 6 h after tMCAO. As expected, BAY 60-6583 administration did not affect the fibrinolytic activity of tPA (Supplementary Fig. 1).

3.4. Effects of BAY 60-6583 on infarct volume and behavioral deficits after tMCAO with or without tPA

A schematic diagram of the research design and experimental groups is shown in Fig. 3A. Administration of BAY 60-6583 to tMCAO rats reduced infarct volume compared with that in vehicle-treated rats ($p < 0.01$; Fig. 3B). Rats treated with tPA showed a trend toward enlarged infarct, but the volume did not differ significantly from that of vehicle-treated rats ($p = 0.207$). Combination treatment with BAY 60-6583 and tPA reduced infarct volume compared with that in rats treated with tPA alone ($p < 0.05$; Fig. 3B). Rats that underwent tMCAO also exhibited significant neurologic deficit, based on their performance in the neurologic deficit score, rotarod, and corner tests. tPA administration did not worsen the performance on these tests. Rats treated with BAY 60-6583 alone exhibited significant improvement on the rotarod test and corner test at 24 h post-ischemia ($p < 0.01$; Fig. 3C), and those administered BAY 60-6583 and tPA in combination performed better on all three tests than did rats treated with tPA alone (Fig. 3C).

3.5. Effects of BAY 60-6583 on brain swelling and HT after tMCAO with tPA

By gross inspection, we detected mild hemorrhage in the vehicle-treated group, but substantially more hemorrhage in the tPA-treated group (Fig. 4A). In addition, brain swelling was significantly greater in the tPA group than in the vehicle group but was reduced by BAY 60-6583 (Fig. 4B). Next, we measured the hemoglobin concentration in homogenates of the ipsilateral hemisphere. The increase in tPA-induced bleeding was significantly suppressed by BAY 60-6583 administration (Fig. 4C). These data indicate that BAY 60-6583 prevented the exacerbation of HT by tPA in tMCAO rats.

3.6. Effects of BAY 60-6583 on BBB disruption after tMCAO with tPA

Increased BBB disruption is commonly characterized by the extravasation of blood-pool agents, which, under normal physiologic conditions, do not cross the BBB to enter brain parenchyma. In this study, to assess BBB integrity, we measured extravasation of gadoteridol (the gadolinium MRI contrast reagent, MW 558 Da) on MRI and leakage of albumin (MW 68 kDa) by histochemistry and Western blotting at 24 h post-MCAO. Contrast-enhanced T1-weighted images were used to estimate BBB leakage volume (PBV), which indicates the area of BBB disruption (Fig. 5A). Thus, we used overall extravasated gadoteridol volume ($\text{CER} \times \text{PBV}$) to express the degree of BBB disruption. PBV in the ipsilateral hemisphere was $68.0 \pm 5.8 \text{ mm}^3$ in the vehicle group and $87.5 \pm 7.7 \text{ mm}^3$ in the tPA group ($p = 0.088$; Fig. 5B). Strikingly, the ipsilateral $\text{CER} \times \text{PBV}$ value was significantly higher in the tPA group (35.0 ± 9.1) than in the vehicle group (13.4 ± 2.2 ; $p < 0.01$; Fig. 5C). However, BAY 60-6583 significantly attenuated gadoteridol extravasation in tPA-treated ischemic rats (Fig. 5B and C). Immunostaining results revealed albumin in the extracellular space surrounding the MCA perfusion territory at 24 h post-MCAO; administration of BAY 60-6583 attenuated albumin extravasation in tPA-treated ischemic rats (Fig. 5D and E). MRI results demonstrated extravasation of gadoteridol in an area adjacent to the infarct area, but this extravasation in the cortex was mild, possibly because of the severe cortical damage that might result in extremely low or no blood flow into that region. Importantly, both contrast-enhanced MRI and albumin immunostaining showed that tPA exacerbated tMCAO-induced

BBB disruption and that Bay 60-6583 reversed this effect. We also explored the effect of tPA treatment on degradation of tight junction protein zonula occludens-1 (ZO-1). Western blotting showed that band density of ZO-1 was reduced in the ischemic hemisphere (Fig. 5F). tPA treatment exacerbated the ZO-1 degradation after tMCAO, whereas BAY 60-6583 treatment significantly prevented the tPA-induced ZO-1 degradation.

3.7. Effects of BAY 60-6583 on MMP-9/TIMP-1 activity after tMCAO with tPA

Over-activation of MMP-2 and MMP-9 leads to increased cerebrovascular permeability after ischemia-reperfusion injury (Mishiro et al., 2012). TIMP-1 can inhibit activation of MMP-9 (Souvenir et al., 2011). To elucidate the underlying mechanisms of tPA-induced BBB disruption after cerebral ischemia, we first determined the expression levels of MMP-9 and TIMP-1 in the reperfused brain tissue. MMP-9 and TIMP-1 protein levels were significantly greater in ischemic brain tissue 24 h after tMCAO than they were in the sham group. tPA reduced TIMP-1 expression and increased MMP-9 expression, but BAY 60-6583 reversed this MMP-9 activation. Strikingly, TIMP-1 protein levels were significantly higher in the BAY 60-6583 group than in both the tPA group and the vehicle group (Fig. 6A–C). Next we used zymography to examine the effect of BAY 60-6583 on MMP-9 activity. Densitometric analysis showed that tPA caused a significant increase in MMP-9 activity at 24 h after tMCAO that was blocked by BAY 60-6583 (Fig. 6D).

4. Discussion

In this study, we examined BBB permeability and HT after tMCAO and evaluated the effects of tPA, BAY 60-6583, and tPA plus BAY 60-6583. We found that A2bR activation with BAY 60-6583 provided marked neurovascular protection after tMCAO with or without tPA. It reduced neurologic deficits and infarct volume and prevented tPA-associated BBB damage and HT. Moreover, the neurovascular protection afforded by BAY 60-6583 appears to derive from stimulation of TIMP-1 production, inhibition of tPA-induced MMP activation, and prevention of tight junction protein degradation. Our findings indicate that A2bR activation during ischemia and reperfusion provides neurovascular protection that may offer an important therapeutic strategy to reduce HT.

Previous studies have revealed neuromodulatory effects of adenosine in the central nervous system (Eltzschig, 2009). In view of the involvement of glutamate-associated excitotoxicity in the etiology of ischemic brain injury, interest in the potential role of adenosine receptors is growing (Latini and Pedata, 2001; Melani et al., 2014). Controlling excitatory transmission by adenosine receptors might constitute a therapeutic strategy to treat brain disorders. Prior studies focused on A1R and A2aR in the control of ischemic brain damage. Early activation of A1R was shown to be protective; however, chronic manipulation of A1R function had the opposite effect (Cunha, 2005). Studies of the A2aR activity-induced regulation of inflammatory responses and brain damage have yielded some complex and seemingly paradoxical results (Chen et al., 2007). Because A2bR has a low affinity for adenosine, it appears that the higher adenosine concentrations required for activation of A2bR are mainly achieved during ischemic conditions. Under such conditions, elevated endogenous adenosine not only activates the high-affinity A1R and A2aR but also low-

affinity A2bR. A significant increase in A2bR expression under ischemic conditions might be caused by transcriptional regulation, and the robust increase in extracellular adenosine is suggestive of an endogenous neuroprotective response.

Recent evidence supports a pivotal role of A2bR in the regulation of immunity, inflammation, and vascular protection (Feoktistov and Biaggioni, 2011; Hoegl et al., 2015; Ponnoth and Jamal Mustafa, 2011). In our study, mRNA and protein expression levels of A2bR increased to a greater extent than did those of the other three adenosine receptors after tMCAO, and A2bR primarily localized to endothelial cells, neurons, and astrocytes of the ischemic brain. However, tPA reduced A2bR expression in endothelial cells. Additionally, a previous study showed that ambient hypoxia increased BBB permeability in A2bR knockout mice compared to that in A1R, A2aR, or A3R knockout mice (Eckle et al., 2008). Together, these data indicate that A2bR might be a desirable target for protecting the BBB against HT caused by cerebral ischemia and exacerbated by tPA.

BAY 60-6583 is a highly selective ligand for A2bR and has low affinity for the other three adenosine receptor subtypes (Eckle et al., 2007). Activation of A2bR could explain the efficacy of BAY 60-6583 in our study. Previous studies showed that tPA alters the expression of tight junction proteins, causing disruption of the BBB and leading to hemorrhagic complications (Jickling et al., 2014; Jickling and Manolescu, 2012). We found that tPA treatment induced a significant increase in degradation of tight junction protein ZO-1, consistent with previous reports (Li et al., 2011; Won et al., 2014). Notably, BAY 60-6583 prevented ZO-1 degradation in tPA-treated ischemic rats. Furthermore, the reduction of tPA-induced degradation of ZO-1 by BAY 60-6583 was associated with a marked decrease in tPA-aggravated HT. These results supply strong evidence that an A2bR agonist could benefit the entire neurovascular network. One limitation of the study is that we tested BAY 60-6583 at only one dose and one time point. Future studies are needed to determine the dose-response and therapeutic window of Bay 60-6583 in the tMACO model with and without tPA.

The mechanisms that underlie the protective effect of A2bR activation against tPA-aggravated HT are unknown. Previous studies revealed that activation of MMP and the subsequent degradation of endothelial basal lamina and tight junction proteins disrupt cerebrovascular integrity in experimental models of stroke (Kelly et al., 2006; Lakhani et al., 2013; Mishiro et al., 2012). A broad-spectrum MMP inhibitor prevented tPA-aggravated HT and tight junction loss in mice (Mishiro et al., 2012). Also, BAY 60-6583 was shown to reduce MMP-9 expression in peripheral macrophages, whereas A2bR gene deletion increased MMP-9 activity in response to femoral artery intima damage (Chen et al., 2011). In addition to effects on macrophages, BAY 60-6583 stabilizes vascular permeability and exerts anti-inflammatory effects (Bot et al., 2012; Hart et al., 2009). In line with these reports, we found that BAY 60-6583 treatment protected the vasculature in post-ischemic brain by attenuating tPA-induced MMP-9 activity. In our study, A2bR agonist inhibited MMP-9 activation, likely through elevation of TIMP-1, as TIMP-1 inhibits MMP-9 activity (Souvenir et al., 2011; Wu et al., 2015b). Indeed, A2bR activation has previously been shown to increase TIMP-1 activity (Csoka et al., 2012; Koscsó et al., 2013). Because A2bR agonist BAY 60-6583 protects the BBB, it may inhibit the infiltration of leukocytes, a major

source of MMPs. This potential effect needs to be investigated in our future studies. Currently, no therapies specifically target MMP-9 because regulation of MMP activity is complex and MMP inhibitors lack specificity and pose potential adverse effects. Our results provide evidence that A2bR agonists could offer a novel strategy to inhibit HT induced by tPA-MMP-9 activation.

In conclusion, our study showed that the A2bR agonist BAY 60-6583 protects ischemic brain and counteracts the post-ischemic BBB damage, HT, and functional deficits that are exacerbated by tPA in a tMCAO model. Our results suggest that BAY 60-6583 provides this protection by increasing the expression of TIMP-1, which inhibits the tPA-induced increases in MMP-9 activity and tight junction protein degradation. These findings have a high translational relevance and could potentially be developed into novel therapies, especially with the increasing clinical use of thrombolytic or mechanical reperfusion therapies in stroke patients. Our findings suggest that administration of A2bR agonist at the time of reperfusion could be a novel strategy to diminish cerebrovascular complications of reperfusion injury, ultimately resulting in less neurovascular injury and improved stroke outcomes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Characteristics of A2b receptor (A2bR) expression after cerebral ischemia. (A) mRNA and (B) protein expression levels of the four adenosine receptor subtypes during cerebral ischemia-reperfusion (I/R; $n = 6$ rats/group). (C) Immunoblot analysis of A2bR expression in cortex (Ctx), striatum (Str), and hippocampus (Hip) after cerebral ischemia ($n = 6$ rats/group). C, contralateral; I, ipsilateral; AU, arbitrary units. (D) Representative photographs show immunofluorescent co-staining of A2bR (red) with NeuN (green), GFAP (green), and von Willebrand factor (vWF; green) in the ischemic cortex ($n = 4$ rats/group). Data are shown as mean $\pm$ SD. Scale bar = 10 μ m.

Fig. 2. tPA reduces expression of A2b receptor (A2bR) in ischemic brain microvessels. (A) Immunoblot images and quantitative analysis show that tPA reduced expression of A2bR in ischemic brain tissue (A; $n = 6$ rats/group) and in isolated microvessels (B; $n = 6$ rats/group). (C) Immunohistochemical localization of A2b receptor in brain microvessels (stained with the endothelial marker vWF) at 24 h after tMCAO. A2bR (red), vWF (green). The inset images represent higher magnification of the boxed area in the corresponding merged images. Immunohistochemistry suggests that A2bR expression was markedly reduced in brain microvessels from the tPA-treated rats ($n = 4$ rats/group). Data are shown as mean $\pm$ SD. ** $p < 0.01$ vs. sham group. Scale bars = 50 μ m in merged images and 20 μ m in insets.

Fig. 3. A2b receptor agonist reduces infarct volume and neurologic deficit after cerebral ischemia. (A) Schematic diagram of the experimental design. Experimental groups were treated with vehicle, tPA, BAY 60-6583, or tPA + BAY 60-6583. Rats received treatments at the start of reperfusion (short black arrow) and were sacrificed at 24 h after tMCAO (long black arrow). (B) Sections stained with 2,3,5-triphenyltetrazolium chloride at 24 h post-tMCAO show that BAY 60-6583 (BAY) decreased infarct area (unstained regions) in the absence or presence of tPA. Bar graphs show quantification of infarct volumes. $n = 12$ rats/group. (C) Bar graphs show rat performance on the rotarod test, neurologic deficit scores, and corner test scores at 24 h after tMCAO ($n = 16$ –18 rats/group). Data are shown as mean $\pm$ SD (infarct volume, rotarod test, corner test) and medians (neurologic deficit scores). ** $p < 0.01$ vs. sham group; ## $p < 0.01$ vs. vehicle group.

Fig. 4.

A2b receptor agonist inhibits tPA-induced brain swelling and hemorrhagic transformation after cerebral ischemia. (A) Representative 2,3,5-triphenyltetrazolium chloride-stained coronal sections show signs of hemorrhage and brain swelling at 24 h after ischemia-reperfusion. The extent of brain swelling = (volume of ipsilateral hemisphere – volume of contralateral hemisphere)/volume of contralateral hemisphere. The dashed line is drawn from mirroring the contralateral hemisphere. The area between the solid line and dashed line on each brain section indicates the extent of brain swelling. The black arrows indicate small hematomas and microbleeds. Scale bar = 5 mm. (B) Quantification of brain swelling at 24 h after ischemia-reperfusion ($n = 12$ rats/group). (C) Quantification of hemorrhage volume as assessed by hemoglobin spectrophotometric assay ($n = 6$ rats/group). Data are shown as mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$ vs. sham group; # $p < 0.05$ vs. vehicle group.

Fig. 5.

A2b receptor agonist inhibits tPA-induced blood-brain barrier (BBB) damage after cerebral ischemia. (A) Representative images of contrast-enhanced T1-weighted images (CE-T1WI) for BBB permeability at 24 h after ischemia-reperfusion. (B–C) Bar graphs show quantitative gadoteridol-permeable BBB volume (PBV) and overall extravasated gadoteridol amounts (contrast-enhanced ratio [CER] $\times$ PBV) after cerebral ischemia ($n = 6$ rats/group). $^{\#}p < 0.05$, $^{##}p < 0.01$ vs. vehicle group. (D–E) Representative images of albumin immunofluorescent staining (red) and immunoblot analyses of albumin extravasation ($n = 6$ rats/group). (F) Immunoblot images and quantitative data show that A2b receptor agonist

inhibited tPA-induced degradation of the tight junction protein zonula occludens-1 (ZO-1) after cerebral ischemia ($n = 6$ rats/group). Data are shown as mean $\pm$ SD. $*p < 0.05$, $**p < 0.01$ vs. sham group. Scale bar = 50 μm .

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Fig. 6. A2b receptor agonist increases tissue inhibitor of metalloproteinases-1 (TIMP-1) expression and inhibits tPA-induced increases in matrix metalloproteinase-9 (MMP-9) activity after cerebral ischemia. (A–C) Immunoblot images and quantitative data of TIMP-1 and MMP-9 protein level ($n = 6$ rats/group). (D) Representative gel zymograms and densitometric analyses of MMP-9 and MMP-2 activity ($n = 6$ rats/group). Data are shown as mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$ vs. sham group.