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Inhibition of PI3K γ by AS605240 plus low-dose tissue plasminogen activator (tPA) combination improves thrombolytic therapy in a rat model of embolic stroke

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Abstract

Our previous study showed that PI3K γ inhibition with AS605240 plus a standard rat-dose tPA (10 mg/kg) combination attenuates delayed tPA-induced brain hemorrhage and ameliorates acute stroke injury 3 days after ischemic stroke in rats. The purpose of this study was to investigate whether combining AS605240 with tPA can enhance thrombolytic efficacy, so that lower doses of tPA can be applied to improve long-term outcome after ischemic stroke. The results showed that AS605240 plus low-dose tPA (5 mg/kg) combination therapy at 4h after stroke onset significantly reduced infarct volume and neurological deficits at 24h after stroke compared with saline, AS605240 or low-dose tPA alone group. Importantly, the combination therapy significantly reduced the delayed tPA-associated brain hemorrhage. Moreover, the combination therapy significantly decreased the size of the residual embolus within the middle cerebral artery, which was associated with a decrease in plasma plasminogen activator inhibitor-1 (PAI-1) activity compared with saline and tPA alone. Finally, AS605240 plus low-dose tPA combination improved long-term outcome for at least 35 days after stroke compared with the saline-treated group. Taken together, these findings suggest that PI3K γ inhibition with AS605240 might act as an adjunct approach for enhancing tPA thrombolytic efficacy in acute ischemic stroke.

Keywords

PI3-kinase gamma; tPA; PAI-1; combination therapy; thrombolysis; embolic stroke

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Authors' contributions

RJ, WZ, SL, and MW performed experiments and data analysis. RJ and GL designed and supervised the study. RJ wrote the manuscript. GL revised the manuscript.

Disclosures

None

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Introduction

Ischemic stroke accounts for about 87% of all strokes with limited treatment options [1]. To date, recombinant tissue-type plasminogen activator (tPA) remains the only FDA-approved drug for the treatment of patients with acute ischemic stroke [1]. However, only 3% to 8.5% of potentially eligible patients receive tPA in the United States mainly due to narrow time windows for thrombolysis and an increased risk of serious cerebral bleeding after delayed tPA treatment [2]. It is well acknowledged that intravenous tPA treatment beyond 3 hours of stroke onset significantly increases cerebral bleeding, although its treatment time window could be extended to 4.5 hours in carefully selected patients [3]. Moreover, due to its suboptimal efficiency, about 50% of all acute ischemic stroke patients treated intravenously with tPA do not achieve successful reperfusion [4]. Thus, there is an urgent need for developing an adjuvant therapy to extend the therapeutic time window and enhance the efficacy of tPA and thereby more victims of acute ischemic stroke are eligible for tPA treatment.

Phosphoinositide 3-kinase gamma (PI3K γ), the unique member of the class IB, is expressed mainly by leukocytes, platelets, and endothelial cells [5]. Genetic deletion and pharmacological inhibition of PI3K γ exerts anti-inflammatory and protective effects against many neurological diseases, including ischemic brain injury, traumatic brain injury, surgical brain injury, and Alzheimer's disease [6] [7] [8] [9]. Recent studies have shown that inhibition of PI3K γ significantly decreases the expression of plasminogen activator inhibitor-1 (PAI-1) in preclinical models of diseases such as obesity and glucose intolerance [10] [11]. Increasing evidence indicates that plasma PAI-1 activity is critically involved in the regulation of the fibrinolytic system [12] [13]. PAI-1 deficiencies cause accelerated fibrinolysis and bleeding [14], whereas elevated PAI-1 levels are closely associated with vascular thrombosis [15]. Clinically, an increase in plasma PAI-1 activity has been reported in acute ischemic stroke patients, and patients with higher concentrations of PAI-1 in plasma before tPA treatment showed poor recanalization rates [16] [17]. Experimental studies have demonstrated a beneficial effect of PAI-1 as a drug target in mouse models of thrombosis and stroke [18] [19]. As a main endogenous inhibitor of tPA, tPA's thrombolytic activity can be masked by PAI-1 in the circulation. Therefore, decreasing the plasma PAI-1 activity might be a novel approach to improve the thrombolytic efficacy of tPA for stroke treatment. Our recent study has shown that inhibition of PI3K γ with AS605240 significantly decreases the PAI-1 expression in the ischemic brain endothelium and thus reduces delayed tPA-induced brain hemorrhage after embolic stroke in rats [20]. In the present study, we further tested whether PI3K γ inhibition with AS605240 can enhance the thrombolytic efficacy of tPA treatment for acute stroke treatment via decreasing plasma PAI-1 activity in a rat model of embolic stroke.

Material and methods

Embolic Middle Cerebral Artery Occlusion Model

All animal experiments were approved by the Institutional Animal Care and Use Committee at Penn State University College of Medicine. Adult male Sprague-Dawley rats (weighing 280 to 340g, purchased from Charles River Laboratories, Wilmington, MA) were subjected

to embolic middle cerebral artery occlusion (MCAO) according to the standard operating procedure in our laboratory [21]. Rats were anesthetized with isoflurane (3% for induction and 1-2% for maintenance). Body temperature was maintained steadily at $37.0 \pm 0.5^{\circ}\text{C}$ by use a feedback-regulated heating pad system throughout the surgical procedures. Laser doppler flowmetry (MSP300XP; ADInstruments Inc) was used to monitor regional cerebral blood flow (rCBF) in the MCA territory (2 mm posterior and 5 mm lateral to the bregma on the right parietal skull) as we described previously [22].

Experimental Protocols

To test the dose-response thrombolysis of tPA, ischemic rats were randomly assigned into the following 5 groups: normal saline (0.9% sodium chloride) and varied doses of tPA including human dose 0.9 mg/kg, low doses (2.5, 5.0 mg/kg), and the standard rat dose (10 mg/kg). tPA (Alteplase, Genentech, Inc., San Francisco, CA) or equal volume of saline (10% as a bolus and 90% as a 30-minutes infusion) was administrated intravenously at 2 hours after ischemia onset using a syringe infusion pump as we described previously [22]. Animals were killed at 4 or 24 hours after stroke for the evaluation of effectiveness of thrombolytic therapy.

Based on the dose-response assessment, a low dose of tPA (5 mg/kg) was used in this study. Ischemic rats were randomly assigned to the following 5 groups: saline at 4h, tPA at 2h or 4h, AS605240 (30 mg/kg) at 4h, and combination treatment with AS605240 and tPA at 4h after the onset of ischemia. The dose (30 mg/kg) for AS605240 was chosen based on the dose-response assessment (5-50 mg/kg/day) in the same rat model of embolic stroke in our previous study [20]. For the measurement of outcome in acute phase of stroke, animals were killed at 24h after stroke. For the measurement of long-term outcome of stroke, AS605240 was initiated at 4 hours and repeated once daily for 4 days, and all surviving animals were followed up to 35 days after stroke. Brain atrophy was determined by measuring cortical width index (CWI) as we described previously [22]. At the end of each experiment, animals were euthanized immediately by exposure to carbon dioxide (CO₂) in an enclosed container.

Behavioral Tests.

Behavioral tests, including modified Bederson score, modified neurological severity scores (mNSS), and foot fault test, were performed by a blinded investigator before and at the indicated time points after stroke, as we previously described [20][22].

Infarct Volume, Brain Edema and Intracerebral Hemorrhage

At 24 hours after stroke, infarct volume and brain swelling were measured in TTC-stained coronal sections. Then, the TTC-stained sections were homogenized and the hemoglobin levels were assessed by a spectrophotometric assay using Drabkin reagent (Sigma-Aldrich), as we previously described [20][22].

Embolus Measurement

At 4 hours after embolization, rats were euthanized by transcardial perfusion with saline containing 2 units/ml heparin. The ventral surface of the brain was photographed with a

digital camera. The area of embolus (mm²) was measured by tracing the outline of the embolus from the image with the NIH Image program, as previously described [23].

PAI-1 Activity Assays

At the indicated time points, blood samples were collected into 3.8% sodium citrate (dilution, 1:9) and centrifuged immediately at 3000 X g for 15 minutes. It is important to ensure the preparation of platelet-free plasma since platelets can release PAI-1 [24]. The levels of PAI-1 activity in plasma was assayed with a rat tPA activity assay kit (Molecular Innovation Inc, Catalog No. RPAIKT), according to the manufacturer's introduction. The latent or complexed PAI-1 did not bind to the plate and was not detected by this assay kit. All samples were run in triplicate.

Statistical Analysis

Data is expressed as mean $\pm$ SD (standard deviation). The GraphPad Prism 8 software package was used for statistical analysis. The normality of data was assessed with the D'Agostino-Pearson omnibus test. For normally distributed variables, one-way analysis of variance (ANOVA) followed by the Bonferroni post hoc test were used to assess differences between groups. If only 2 groups were compared, an unpaired, 2-tailed Student *t* test, was applied. The Kruskal–Wallis and Mann–Whitney U tests were used to explore differences between groups in non-normally distributed variables. Nonparametric functional outcome scores were compared by the Kruskal–Wallis test with post hoc Dunn corrections. For a comparison of survival data, the log-rank test was used. *p* < 0.05 was considered statistically significant.

Results

Dose-response thrombolysis of tPA in the rat embolic MCAO model

The data showed that all varied doses of tPA (0.9 mg/kg to 10 mg/kg) initiated at 2 hours after stroke did not cause intracerebral hemorrhage compared with saline-treated group (Figure 1A). Treatment with 5.0 and 10 mg/kg of tPA, but not 0.9 and 2.5 mg/kg of tPA, significantly reduced infarct volume (Figure 1B) and brain swelling (Figure 1C) compared to the saline-treated group. tPA therapy reduced neurological deficits in a dose-dependent manner (Figure 1D).

To evaluate the successful recanalization, the residual emboli within the origin of the MCA and regional cerebral blood flow (rCBF) were measured. In saline- and 0.9 mg/kg tPA-treated groups, the intact emboli were found at the origin of MCA and no recanalization occurred (0/6) in all animals (Figure 2A and 2B). In addition, the 2.5 mg/kg of tPA had no significant thrombolytic effect (recanalization: 1/6). However, the size of the residual emboli was significantly decreased in the 5.0 and 10 mg/kg of tPA (recanalization: 4/6 and 6/6, respectively) treated groups compared to saline-treated group. rCBF was restored to 80% of the baseline within half hour after 5.0 and 10 mg/kg tPA treatment (Figure 2C). In contrast, 2.5 mg/kg tPA only achieved partial reperfusion. 0.9 mg/kg of tPA and saline had no effect on rCBF values (Figure 2C).

AS605240 plus tPA combination reduces delayed tPA-associated brain hemorrhage and ameliorates acute brain injury

Unlike the early (at 2 hours) tPA (5.0 mg/kg) treatment, tPA alone at 4 hours had no effect on infarct size (Figure 3B), but promoted brain hemorrhage (Figure 3A) and brain swelling (Figure 3C). Treatment with AS605240 alone at 4 hours had no detectable effect on hemorrhage (Figure 3A) and infarct size (Figure 3B), but it decreased the brain edema formation (Figure 3B) compared to the saline-treated group. However, the combination treatment at 4 hours not only blocked the delayed tPA-associated brain hemorrhage (Figure 3A), but also significantly attenuated infarct volume (Figure 3B), brain edema (Figure 3C), and neurological deficits (Figure 3D).

Inhibition of PI3K γ attenuates plasma PAI-1 activity levels after stroke

PAI-1 activity levels in plasma were significantly increased at 4 hours and further elevated at 6 hours after stroke, but the levels were significantly decreased in both the AS605240-treated group and the tPA-treated group (neutralized by exogenous tPA) at 6 hours after stroke (Figure 4A). Intriguingly, the combined AS605240 (30 mg/kg) plus tPA (5 mg/kg) treatment at 4 hours almost completely abolished the stroke-associated increase of plasma PAI-1 activity levels (Figure 4A). Moreover, we checked the residual embolus within the origin of MCA at 6 hours after the injection of the blood clots. The blood clots were clearly present within the origin of MCA in the ischemic rats treated with saline, tPA (5 mg/kg), or AS605240 (40 mg/kg) at 4 hours alone, but they were almost completely dissolved in the ischemic rats co-treated with AS605240 plus tPA at 4 hours. Together, these data indicate that the combination treatment with AS605240 effectively enhances the thrombolytic efficacy of delayed tPA compared to tPA alone (Figure 4B).

PI3K γ inhibition in combination with delayed tPA improves long-term stroke outcome

Finally, we investigated whether the combination treatment with AS605240 can improve long-term stroke outcome. The rats subjected to MCAO showed significant neurological deficits compared to sham animals, as determined by the modified neurological severity score and foot-fault test up to 35 days after stroke (Figure 5A and B). The combined AS605240 plus delayed tPA treatment at 4 hours significantly improved neurological function, while AS605240 alone was less effective (Figure 5A and B). The cortical width index (CWI) is a commonly used method to measure brain atrophy (Figure 5C). The AS605240 plus tPA combination significantly increased CWI compared to vehicle-treated group (Figure 5D). However, treatment with AS605240 or tPA alone showed no significant effect on CWI (figure 5D). Although the 35-day survival rate was higher in the combination treatment group than other stroke groups, it did not reach statistical significance because of the small sample size (Figure 5E).

Discussion

The present study, for the first time, demonstrates that therapeutic inhibition of PI3K γ with AS605240 combined with low-dose tPA improves thrombolytic therapy in a rat model of focal embolic stroke. Mechanistically, this beneficial effect conferred by AS605240 is likely attributed to reduced levels of plasma PAI-1 activity. Our findings suggest that PI3K γ

inhibition with AS605240 may become a new approach for improving the efficacy of tPA intravenous thrombolysis for treating acute ischemic stroke.

Among various stroke models currently available, the embolic MCAO model closely mimics human ischemic stroke and is most suitable for preclinical investigation of thrombolytic therapy [25]. The therapeutic window of tPA is 2 to 3 hours after embolic MCAO in rats. Thus, tPA was given at 2h to test the dose-response thrombolysis of tPA. It has been shown that the standard rodent dose of tPA (10 mg/kg) lost its effectiveness beyond 4 hours after embolic MCAO in rats and instead increased infarct volume, as well as hemorrhagic transformation [26]. Thus, the combination therapy with tPA plus AS605240 was initiated at 4h after stroke onset. The present study demonstrated that, unlike the early (at 2 hours) tPA (5.0 mg/kg) treatment, tPA alone at 4 hours had no effect on infarct size, but promoted brain hemorrhage and brain swelling. Treatment with AS605240 alone at 4 hours had no detectable effect on hemorrhage and infarct size, but it decreased the brain edema formation compared to the saline-treated group. However, the combination treatment at 4 hours not only blocked the delayed tPA-associated brain hemorrhage, but also significantly attenuated infarct volume, brain edema, and neurological deficits. Encouragingly, we showed that the combination treatment enhanced long-term survival rate and functional outcome up to 35 days after stroke.

Translational stroke research studying thrombolytic improvement requires a “human equivalent” dose of tPA that reflects the clinical recanalization rates. The high recanalization rates (67-100% in various stroke models [27] [28][29][30]) with the standard “rat” dose tPA (10 mg/kg) are not reflective of clinical recanalization. Although the thrombolytic efficacy of tPA has been well established clinically, its efficacy is suboptimal. In large-scale clinical trials encompassing 2066 patients, the reported recanalization rates were: intravenous tPA (46.2%), intra-arterial tPA (63.2%), combined intravenous-intra-arterial tPA (67.5%) [4]. Similarly, other clinical study reported that complete recanalization was achieved in 56.3% (36/64) stroke patients after tPA treatment [31]. In the present study, we evaluated the thrombolytic efficacy of varied doses of tPA (from 0.9 to 10 mg/kg) treated at 2 hours after ischemia onset in a well-established rat embolic stroke model. Our data show that 10 mg/kg tPA was too effective (100% recanalization), and 0.9 mg/kg tPA (0% recanalization) and 2.5 mg/kg tPA (16.7% recanalization) were ineffective for thrombolysis in situ and thus neither dose mimics clinical tPA response, whereas 5.0 mg/kg tPA (66.7% recanalization) is closer to the clinical response. Our results are similar to the previous report [32]. Although only two studies showed that the human dose (0.9 mg/kg) tPA produced beneficial effects with early (at 1h) treatment in a rat embolic stroke model [33] and in a mouse model of MCA occlusion by injection of thrombin into left MCA [27], other studies demonstrated that neither 5 mg/kg nor 2.5 mg/kg doses of tPA was effective in the rat embolic stroke model [34][35]. It is likely that tPA doses required to mimic clinical recanalization rates will differ with regards to the model choice, incorporating clot type, species, and co-morbidities. Therefore, to study improved thrombolytic therapies over tPA alone, it is necessary to determine the “human equivalent” dose that generates recanalization rates that correlate with the clinical conditions being tested.

PI3K γ is a multifaceted protein, expressed in many different types of immune and non-immune cells [5]. Increased expression of PI3K γ in platelets, leukocytes, and endothelial cells has been implicated in inflammation and cardiovascular disease [5]. Genetic deletion and pharmacological inhibition of PI3K γ has yielded encouraging therapeutic benefits in a number of animal models of human diseases, such as drug-induced liver injury, traumatic brain injury and stroke [6] [7] [36]. We have recently reported that inhibition of PI3K γ with AS605240 (30 mg/kg) extends the therapeutic window for tPA (10 mg/kg) to 6 h after ischemia onset, and combination therapy exerts significant neuroprotection against embolic stroke in rats [20]. In an effort to enhance the efficacy and safety of thrombolytic therapy with tPA, the neuroprotective effect of the combined AS605240 plus low-dose of tPA (5 mg/kg) treatment was investigated in this study. Our findings that the combination therapy significantly improved the efficacy of thrombolysis and reduced the risk of intracerebral hemorrhage suggest that inhibition of PI3K γ with AS605240 might enhance the thrombolytic efficacy of tPA treatment. The underlying mechanisms may be partially related to a decrease in plasma PAI-1 activity by inhibition of PI3K γ after stroke. As a main endogenous inhibitor of tPA, the levels of PAI-1 activity in the circulation is closely related to the reperfusion efficacy and intracerebral hemorrhagic risk of tPA thrombolytic therapy [16][17]. Although the PAI-1 activity in thrombi can be several thousand-fold higher than normal plasma [37], the functionally active PAI-1 in the circulation must be first overcome before the tPA can exert thrombolysis. Elevated plasma PAI-1 activity creates a prothrombotic state and has been shown to be inversely related to clinical outcomes in patients receiving thrombolytic therapy, as high PAI-1 reduces thrombolytic efficacy [17] [38]. Our recent study has demonstrated that the combination treatment with AS605240 and tPA (10 mg/kg) significantly decreases PAI-1 expression in the ischemic brain endothelium after stroke [20]. In the present study, we further demonstrated that the combination treatment with AS505240 and low-dose of tPA (5 mg/kg) significantly inhibited stroke-associated upregulation of plasma PAI-1 activity, thereby improving the tPA's thrombolytic efficacy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlight:

- Therapeutic inhibition of PI3K γ with its selective inhibitor AS605240 effectively enhances the thrombolytic efficacy of tPA and combining AS605240 plus lower-dose tPA therapy significantly reduces acute brain damage and reduces the delayed tPA-caused brain hemorrhage.
- The combination therapy significantly decreases the size of the residual embolus within the MCA, which is partially associated with a decrease in plasma plasminogen activator inhibitor-1 (PAI-1) activity.
- The combination therapy can confer substantial long-term functional and histologic protection.

Perspectives

Our results show that inhibition of PI3K γ with AS605240 improves the thrombolytic efficacy of tPA possibly via inhibition of plasma PAI-1 activity. The combined AS605240 plus low-dose tPA (5 mg/kg) not only reduces delayed tPA-induced brain hemorrhage and ameliorates acute stroke injury but also exerts long-term beneficial effects after embolic stroke in rats. However, there is a limitation in this study. In the circulation, the platelet α granules is the largest pool of PAI-1. In addition to platelets, liver, endothelial cells, macrophages, and adipocytes may all contribute to plasma PAI-1. Further investigation is needed to examine which source of PAI-1 is primarily involved with reduced plasma PAI-1 activity conferred by inhibition of PI3K γ with AS605240. Moreover, Stroke Therapy Academic Industry Roundtable recommends that after initial evaluations in young, healthy male animals, further studies should be performed in females, aged animals, and animals with comorbid conditions [39].

Figure 1. Therapeutic effects of varied doses of tPA in the rat embolic MCAO model. **A** and **B**, Representative images of unstained coronal sections (**A**, top) and TTC-stained coronal sections (white color: infarct tissue) (**B**, top) in the indicated groups 24 h after the onset of ischemia. Quantitative analysis of hemorrhage volume (**A**, bottom) and infarct volume (**B**, bottom). **C** and **D**, Brain edema and Bederson score of the indicated groups 24 h after the onset of ischemia. Brain edema is expressed as percentage of hemispheric enlargement. Drug treatment was initiated at 2 hours after the onset of ischemia. $n=7$ rats per group, * $p < 0.05$ vs. saline control.

Figure 2. Thrombolytic efficacy of varied doses of tPA in the rat embolic MCAO model.

A, Representative images of ventral view of brains. Animals were treated with varied doses of tPA or saline at 2 h after embolization and killed 2 h after tPA treatment. Red color: the injected blood clot located at the origin of MCA. **B**, Quantitative measurement of sizes of residual emboli in the indicated groups. $n=6$ per group. $*p < 0.05$ vs. saline control. **C**, Regional cerebral blood flow (rCBF). Regional cerebral blood flow was monitored by using a Laser Doppler flowmeter. rCBF rapidly dropped below 20% of the preischemic baseline after clot injection. 5 mg/kg and 10 mg/kg tPA treatment at 2 h restored rCBF to 80% within 30 min. 2.5 mg/kg tPA achieved partial reperfusion. 0.9 mg/kg tPA and saline had no effects on rCBF restoration.

Figure 3. AS605240 plus low-dose tPA combination treatment reduces the risk of brain hemorrhage and ameliorates acute stroke injury.

A and B, Representative images of unstained coronal sections (red color: intracerebral hemorrhage) (**A**, top) and TTC-stained coronal sections (white color: infarct tissue) (**B**, top) and quantitative analysis of hemorrhage volume (**A**, bottom), infarct volume (**B**, bottom) in the indicated groups 24 h after embolic stroke. **C and D**, Brain edema and Bederson score of the indicated groups 24 h after embolic stroke. Brain edema is expressed as percentage of hemispheric enlargement. Animals were treated with AS605240 (AS, 30 mg/kg) and tPA (5 mg/kg) alone or in combination at 4h after the onset of ischemia. n=7 to 9 rats per group.

*P<0.05, vs saline; #P<0.05 vs tPA alone.

Figure 4. Inhibition of PI3K γ with AS605240 attenuates plasma PAI-1 activity and enhances thrombolytic efficacy of delayed tPA treatment in the rat embolic MCAO model.

A, Plasma PAI-1 activity in the indicated groups before and at the indicated time points after the onset of ischemia. * $p < 0.05$. $n = 6$ per group. **B**, Representative images of ventral view of brains showing residual emboli at the origin of MCA in the indicated groups. Brain samples were collected at 6 h after embolization (immediately after blood collection).

Figure 5. AS605240 plus low-dose tPA combination treatment improves long-term outcome
A and B, The modified neurological severity score (mNSS) test (**A**) and foot-fault test (**B**) measured before (day 0) and up to 35 days after stroke. * $p < 0.05$ vs. Saline; # $p < 0.05$ vs. tPA alone. Animals were treated with AS605240 (AS, 30 mg/kg) and tPA (5 mg/kg) alone or in combination at 4 h after the onset of ischemia. AS605240 treatment was repeated once daily for 4 days. **C**, Representative images showing whole brain atrophy in the indicated groups. * $p < 0.05$. **D**, quantitative analysis of the cortical width index, $n = 4-8$ rats per group, **E**, Survival curves of animals in the indicated groups up to 35 days after stroke.