Microvascular Thrombosis, Fibrinolysis, Ischemic Injury, and Death After Cerebral Thromboembolism Are Affected by Levels of Circulating α2-Antiplasmin

Guy L. Reed, Aiilyan K. Houng, Dong Wang

Objective—Ischemic stroke is primarily attributable to thrombotic vascular occlusion. Elevated α2-antiplasmin (a2AP) levels correlate with increased stroke risk, but whether a2AP contributes to the pathogenesis of stroke is unknown. We examined how a2AP affects thrombosis, ischemic brain injury, and survival after experimental cerebral thromboembolism.

Approach and Results—We evaluated the effects of a2AP on stroke outcomes in mice with increased, normal, or no circulating a2AP, as well as in mice given an a2AP-inactivating antibody. Higher a2AP levels were correlated with greater ischemic brain injury ($r_s=0.88$, $P<0.001$), brain swelling ($r_s=0.82$, $P<0.001$), and reduced middle cerebral artery thrombus dissolution ($r_s=0.93$, $P<0.001$). In contrast, a2AP deficiency enhanced thrombus dissolution, increased cerebral blood flow, reduced brain infarction, and decreased brain swelling. By comparison to tissue plasminogen activator (TPA), a2AP inactivation hours after thromboembolism still reduced brain infarction ($P<0.001$) and hemorrhage ($P<0.05$). Microvascular thrombosis, a process that enhances brain ischemia, was markedly reduced in a2AP-deficient or a2AP-inactivated mice compared with TPA-treated mice or mice with increased a2AP levels (all $P<0.001$). Matrix metalloproteinase-9 expression, which contributes to acute brain injury, was profoundly decreased in a2AP-deficient or a2AP-inactivated mice versus TPA-treated mice or mice with increased a2AP levels (all $P<0.001$). a2AP inactivation markedly reduced stroke mortality versus TPA ($P<0.0001$).

Conclusions—a2AP has profound, dose-related effects on ischemic brain injury, swelling, hemorrhage, and survival after cerebral thromboembolism. By comparison to TPA, the protective effects of a2AP deficiency or inactivation seem to be mediated through reductions in microvascular thrombosis and matrix metalloproteinase-9 expression. (Arterioscler Thromb Vasc Biol. 2014;34:2586-2593.)

Key Words: brain ischemia ■ fibrinolysis ■ plasminogen activator ■ thromboembolism ■ tissue-type plasminogen activator

Ischemic stroke affects 16.9 million people a year worldwide; it kills >5.4 million and disables millions of others.1 Thrombotic obstruction of a cerebral artery may be triggered by thromboembolism, plaque rupture, atrial fibrillation, and other vascular events.2,3 Thrombotic occlusion initiates a cascade of events that cause neuronal cell death, matrix metalloproteinase (MMP) expression, breakdown of the blood brain barrier with brain swelling, hemorrhage, morbidity, and mortality.4,6 Downstream from a thrombotic occlusion, ischemia may trigger the formation of microvascular thrombi that cause further reductions in blood flow, thereby enhancing blood brain barrier breakdown and ischemic tissue injury.2,57 Clinical and experimental studies have demonstrated that tissue plasminogen activator (TPA), which initiates the conversion of plasminogen to plasmin, dissolves occlusive thrombi to improve outcomes after ischemic stroke.5 However, there is little known in ischemic stroke about the role of molecules that regulate fibrinolysis downstream of TPA.

See accompanying editorial on page 2522

α2-Antiplasmin (a2AP) is a serine protease inhibitor that rapidly inhibits plasmin.8 Most a2AP circulates in the blood, but a portion is crosslinked to fibrin by activated factor XIII during thrombus formation.9 Recent studies have emphasized the role of thrombus-bound a2AP in regulating fibrinolysis or dissolution of pathological thrombi.8,9 Circulating a2AP inhibits circulating plasmin 100- to 1000-fold more efficiently than it inhibits fibrin or thrombus-bound plasmin; this has led some to propose that a primary role of circulating a2AP is to prevent bleeding by preventing the degradation of coagulation factors.10,11 However, recent studies indicate that high levels of circulating a2AP contribute to the failure of TPA therapy to dissolve thrombi and restore blood flow during ischemic stroke.12,14 Moreover, genetic deletion of a2AP protects against ischemic brain injury induced by nonthrombotic permanent surgical ligation of the middle cerebral artery.15,16 Yet, within the neuronal and vascular compartments, a2AP and serpins...
that block TPA-initiated proteolytic pathways, such as the activation of MMP-9, may protect the brain by reducing cell death or neurotoxicity and may prevent bleeding complications.17–20

In this report, we investigated how circulating and thrombus-bound a2AP affect endogenous fibrinolysis, microvascular thrombosis, hemorrhage, brain injury, and other outcomes in an experimental thromboembolic model with translational relevance to human ischemic stroke. We find that thrombus-bound a2AP modulates dissolution of the culprit thromboembolus, whereas circulating a2AP activity also has dynamic, deleterious effects on the development of microvascular thrombosis, MMP-9 expression, brain injury, hemorrhage, disability, and death after cerebral thromboembolism.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

Dose-Related Effects of Circulating a2AP on Cerebral Thromboembolism

If a2AP activity directly contributes to the pathogenesis of stroke after thromboembolism, there should be a dose relationship between circulating levels of a2AP levels and outcomes. Three different experimental groups were examined: mice with increased levels of a2AP (achieved by intravenous supplementation), normal physiological a2AP levels (controls), or no circulating a2AP (a2AP−/− mice).14 Intravenous supplementation increased blood a2AP levels by a median of 87.1 μg/mL (mean 79.3±14.3 μg/mL) in mice measured at the end of the experiment, which approximately doubled the a2AP levels found in normal mice.25 Laser Doppler monitoring showed that middle cerebral artery (MCA) thromboembolism was associated with a marked drop in hemispheric blood flow to ≤20% of the initial baseline in all 3 groups as expected (Figure 1A). During the period of observation, there was a partial restoration of blood flow to a2AP+ mice without circulating a2AP (Figure 1A), whereas hemispheric blood flow remained suppressed in mice with normal or increased levels of a2AP. Pathologically, there was evidence of proximal MCA occlusion (Figure 1B) after thromboembolism, and the extent of brain infarction differed among the experimental groups as shown in TTC (2,3,5-triphenyltetrazolium chloride)-stained representative images of brain slices (Figure 1B).

When compared with mice with normal a2AP levels, mice with increased circulating levels of a2AP had larger areas of brain infarction (Figure 1B and 1C; P<0.001). In contrast, mice without circulating a2AP had significant reductions in brain infarction by comparison to mice with normal (Figure 1B and 1C; P<0.01) or increased levels of a2AP (Figure 1C; P<0.001). There was a significant positive dose-related correlation between a2AP levels and brain infarction (Spearman’s r=0.88; P<0.001).

In these experiments, we monitored the dissolution of 125I-fibrin–labeled MCA thromboemboli (formed with pooled normal a2AP+ plasma) at 6 hours to assess brain injury during a time when thrombus dissolution was still dynamic, as suggested by our pilot studies. By comparison to mice with normal physiological levels of a2AP, mice with increased blood levels of a2AP had markedly decreased dissolution of the MCA thrombus (Figure 1D; P<0.01). In contrast, the dissolution of the MCA thrombus was accelerated in a2AP-deficient versus mice with normal a2AP levels (Figure 1D; P<0.001) or versus mice with increased levels of circulating a2AP (Figure 1D; P<0.001). There was a significant negative correlation between the level of circulating a2AP and dissolution of the MCA thromboembolus (Spearman’s r=−0.93; P<0.001). There was no significant relationship between a2AP levels and hemorrhage (Figure 1E).

Brain swelling is one of the more serious complications of proximal MCA thrombosis. Brain swelling was significantly greater in mice with high circulating levels of a2AP than with normal a2AP levels (Figure 1F; P<0.01) or a2AP-deficient mice (Figure 1F; P<0.001). There was a significant positive correlation between circulating a2AP levels and brain swelling (Spearman’s r=0.82; P<0.001).

Effects of Thrombus-Bound a2AP on Outcomes in a2AP-Deficient Mice

The previous experiments had examined the effect of different levels of circulating a2AP on outcomes in mice with MCA thromboemboli made from plasma containing physiological levels of a2AP. To examine the potential additional contribution of thrombus-bound a2AP, we compared outcomes in a2AP-deficient mice with MCA thromboemboli made from plasma containing normal a2AP levels versus a2AP-deficient plasma. As expected, thromboemboli made from normal (a2AP+ normal a2AP+/+ plasma) at 6 hours to assess brain injury during a time when thrombus dissolution was still dynamic, as suggested by our pilot studies. By comparison to mice with normal physiological levels of a2AP, mice with increased blood levels of a2AP had markedly decreased dissolution of the MCA thrombus (Figure 1D; P<0.01). In contrast, the dissolution of the MCA thrombus was accelerated in a2AP-deficient versus mice with normal a2AP levels (Figure 1D; P<0.001) or versus mice with increased levels of circulating a2AP (Figure 1D; P<0.001). There was a significant negative correlation between the level of circulating a2AP and dissolution of the MCA thromboembolus (Spearman’s r=−0.93; P<0.001). There was no significant relationship between a2AP levels and hemorrhage (Figure 1E).

Brain swelling is one of the more serious complications of proximal MCA thrombosis. Brain swelling was significantly greater in mice with high circulating levels of a2AP than with normal a2AP levels (Figure 1F; P<0.01) or a2AP-deficient mice (Figure 1F; P<0.001). There was a significant positive correlation between circulating a2AP levels and brain swelling (Spearman’s r=0.82; P<0.001).

Effects of a2AP During Prolonged Ischemia After Cerebral Thromboembolism

Taken together, these studies indicate that at the time of thromboembolism, levels of circulating a2AP have profound effects on stroke outcome. To examine whether a2AP continues to exert dynamic effects on the brain for hours after cerebral thromboembolism, mice were treated with a monoclonal
antibody that inhibits a2AP (a2AP-I) and prevents the neutralization of plasmin. This a2AP-I bound specifically to mouse plasma clots in vitro (Figure 2B). After MCA thromboembolism, laser Doppler monitoring showed a marked drop in hemispheric blood flow to ≤20% of the initial baseline in all 3 groups as expected (Figure I in the online-only Data Supplement). Two and one half hours after cerebral thromboembolism, mice were treated with a standard dose of TPA or with the a2AP-I. Plasma samples from mice receiving the a2AP-I confirmed that it significantly accelerated the dissolution of ex vivo clots (Figure II in the online-only Data Supplement), and blood levels of the a2AP-I were 149±23 μg/mL (mean±standard error). By comparison to TPA, a2AP-I significantly reduced infarct size (Figure 2C; P<0.001). Bederson neurological scores were better (ie, lower) in mice treated with a2AP-I versus TPA (median, 1 versus 3; P<0.02), indicating that a2AP-I treatment was associated with less neurological impairment. However, thrombus dissolution was significantly greater in TPA-treated mice than in those receiving a2AP-I.

Figure 1. The effects of circulating and thrombus-bound α2-antiplasmin (a2AP) on brain infarction, swelling, and dissolution of the culprit middle cerebral artery (MCA) thrombus. A, Changes in relative cerebral blood flow (mean±SE) in the ischemic hemisphere in mice with elevated circulating levels of a2AP (a2AP), normal a2AP levels (control, Ctl), and absent a2AP (a2AP−/−). Representative images of a proximal middle cerebral artery thrombus (arrow) and of TTC (2,3,5-triphenyltetrazolium chloride)-stained brain slices showing viable (red) and infarcted tissue (white) 6 hour after thromboembolism. Percent (C) infarction, (D) dissolution of the MCA thrombus, (E) hemorrhage, and (F) swelling of the ischemic hemisphere 6 hour after placement of an 125I-fibrin-labeled MCA thrombus (containing normal levels of a2AP). Immunoblot analysis of a2AP in thromboemboli formed from a2AP+/+ and a2AP−/− mice. Immunostained bands consistent with thrombus-bound a2AP (fibrin-cross linked a2AP, arrows) in a2AP+/+ but not a2AP−/− clots. Top, Immunostaining with polyclonal anti-a2AP antibody; bottom, immunostaining with pooled monoclonal a2AP antibodies. H–K, Effects of thrombus-bound a2AP in vivo on the percent (H) MCA thrombus dissolution, (I) infarction, (J) hemorrhage, and (K) swelling in brains from a2AP−/− mice with MCA thromboemboli containing normal a2AP (a2AP+/+) or no a2AP (a2AP−/−). n=7 per group; see Materials and Methods for additional details. Data in C–F, H–K represent the means±SE. **P<0.01; ***P<0.001; NS, not significant.

Figure 2. Binding specificity and effects of an α2-antiplasmin (a2AP)-inactivating (a2AP-I) monoclonal antibody and tissue plasminogen activator (TPA) on thrombus dissolution, infarction, swelling, and hemorrhage after thromboembolism. A, The a2AP-I monoclonal antibody binds specifically to mouse a2AP vs BSA in an ELISA by comparison to a control (Ctl; anti-digoxin) monoclonal antibody. See Materials and Methods for additional details. B, The a2AP-I (o) accelerates the dissolution of 125I-fibrin-labeled mouse plasma clots in vitro by comparison to no monoclonal antibody ( ). Clot dissolution was determined as described in Materials and Methods. Effect of a2AP-I (9 mg/kg) or TPA (10 mg/kg) treatment given 2.5 hour after thromboembolism on the percent (C) brain infarction, (D) thrombus dissolution, (E) brain swelling, and (F) hemorrhage. Brains were examined 6 hour after cerebral thromboembolism. Data represent the means±SE. n=7 mice per group (B–E). *P<0.05; ***P<0.001; NS, not significant.
a2AP-I (Figure 2D; P<0.0001). No significant differences were detected in brain swelling (Figure 2E), but there was significantly less brain hemorrhage in mice after a2AP-I administration than in mice treated with TPA (Figure 2F; P<0.05).

**Effects of a2AP on the Development of Microvascular Thrombosis**

Previous studies have shown that brain ischemia triggers the development of downstream microvascular thrombosis that enhances vascular obstruction, aggravates tissue ischemia, and contributes to breakdown of the blood brain barrier. Consistent with previous reports, in situ zymography activity seemed highest in the ischemic cortex of mice with increased a2AP levels, intermediate in mice with normal a2AP levels, and lowest in mice with a2AP-deficiency. (Figure III in the online-only Data Supplement). TPA treatment significantly increased MMP-9 expression by comparison to elevated levels of a2AP (Figure 4, bar graph; P<0.05) or by comparison to control mice with normal levels of a2AP (Figure 4, bar graph; P<0.001). By comparison, a2AP-I (Figure 4, bar graph; P<0.001) or a2AP deficiency (P<0.001) significantly reduced MMP-9 expression by comparison to TPA-treated mice. Overall, there was a positive correlation between the levels of active a2AP and the amount of microvascular thrombosis (Figure 3, line graph; Spearman’s r=0.94; P<0.001).

**Circulating a2AP and MMP-9 Expression**
The close link between thrombosis and inflammation in ischemic stroke has suggested that ischemic stroke is a thrombo-inflammatory disease. MMP-9 expression is linked to the inflammatory response to ischemic injury and is notably increased after cerebral thromboembolism. MMP-9 has been identified as a key acute mediator of breakdown of the blood brain barrier, hemorrhage, and brain edema in ischemic stroke. In control mice with normal a2AP levels, cerebral thromboembolism was associated with higher levels of expression of MMP-9 versus shams (P<0.001, not shown). When compared with control mice with normal circulating levels of a2AP, mice with high levels of a2AP showed increased MMP-9 expression as assessed by quantitative immunofluorescence (Figure 4, bar graph; P<0.05). MMP-9 expression was significantly lower in a2AP-deficient mice (P<0.001) or a2AP-I treated mice (P<0.01) by comparison to mice with elevated a2AP levels. Consistent with MMP-9 immunofluorescence and previous reports, in situ zymography activity seemed highest in the ischemic cortex of mice with increased a2AP levels, intermediate in mice with normal a2AP levels, and lowest in mice with a2AP-deficiency. (Figure III in the online-only Data Supplement). TPA treatment significantly increased MMP-9 expression by comparison to elevated levels of a2AP (Figure 4, bar graph; P<0.05) or by comparison to control mice with normal levels of a2AP (Figure 4, bar graph; P<0.001). By comparison, a2AP-I (Figure 4, bar graph; P<0.001) or a2AP deficiency (P<0.001) significantly reduced MMP-9 expression by comparison to TPA-treated mice. Overall, there was a positive correlation between the levels of active a2AP and MMP-9 expression (Figure 4, line graph; Spearman’s r=0.94, P<0.0001).

**Dynamic Effects of a2AP Activity Survival After Cerebral Thromboembolism**
Proximal MCA stroke in humans is linked to extensive brain infarction and brain swelling that are associated with significant mortality. Because a2AP affected infarction and brain swelling after experimental thromboembolism, we examined whether a2AP-I after stroke onset may protect against mortality.
by comparison to control, untreated, and TPA-treated mice. Control and untreated mice had a shortened survival after proximal MCA thromboembolism (Figure 5A). Mice treated with TPA also had significant mortality after thromboembolism (Figure 5A). Mice treated with a2AP-I, in the doses used in previous studies above, had significantly greater survival than control mice (P<0.05) or TPA-treated mice (P<0.001). To determine whether the dose or the molecular form of the a2AP-I affected outcomes, we tested high dose a2AP in the form of an immunoglobulin (21.3 mg/kg) and as a Fab (9.5 mg/kg). All mice treated with high-dose a2AP-I in immunoglobulin or Fab form survived (Figure 5A). The survival of mice treated with high-dose a2AP-I was significantly greater than mice treated with low-dose a2AP-I (9.3 mg/kg; P<0.01), TPA treatment (P<0.0001), or control mice (P<0.0001).

Surviving mice were examined to determine how these treatments affected ischemic brain injury. By comparison to control mice, mice treated with all doses of a2AP inactivation had significantly smaller areas of brain infarction than control, untreated mice (Figure 5B; P<0.001), or TPA-treated mice (Figure 5B; P<0.001). In a similar fashion, a2AP-I treated mice had smaller brain hemorrhage than control mice (Figure 5C; P<0.05) or TPA-treated mice (Figure 5C; P<0.001). Brain swelling was markedly reduced in mice treated with a2AP-I compared with control mice (Figure 5D; P<0.001) or with mice treated with TPA (Figure 5D; P<0.001).

Discussion
These data suggest that circulating a2AP has important effects on the dissolution of the culprit MCA thrombus and the development of brain microvascular thrombosis, infarction, blood brain barrier breakdown, hemorrhage, swelling, mortality, and disability after cerebral thromboembolism. Several effects were dose-related because there were significant positive correlations between circulating a2AP levels or activity and the extent of brain infarction, brain swelling, microvascular thrombosis, and MMP-9 expression. Conversely, there was a significant negative correlation between a2AP levels and dissolution of the culprit MCA thrombus. When compared with TPA-treated mice, a2AP-I given late after cerebral thromboembolism had a longer therapeutic window than TPA for protecting against brain injury and hemorrhage.

The finding that increased a2AP levels enhance brain infarction and the resistance of MCA thrombus to dissolution is consistent with clinical observations that a2AP may contribute to stroke in humans. In a case–cohort design, the Atherosclerosis Risk in Communities Study found that elevated a2AP levels were associated with an increased univariate risk of subsequent stroke. Recent studies have noted that genetic deficiency or plasmin-induced deficiency of a2AP contribute to stroke in nonthrombotic models of focal ischemia induced by temporary or permanent surgical ligation of the MCA. The present findings are also consistent with

Figure 4. Matrix metalloproteinase-9 (MMP-9) expression after middle cerebral artery (MCA) thromboembolism. Representative images of MMP-9 immunofluorescence staining (red) in the ischemic hemispheres of mice with increased a2AP (†a2AP), normal a2AP levels (control, Ctl), a2AP deficiency (a2AP−−), and mice treated with a2AP inactivation (a2AP-I) or TPA. MMP-9 expression was assessed by double immunofluorescence staining for MMP-9 (DyLight549, red) and collagen IV (DyLight488, green). Representative micrographs with DAPI (4',6-diamidino-2-phenylindole)-stained nuclei (blue) were taken using a Zeiss LSM 710 confocal microscope at 40× magnification. Bar graph, quantitative digital analysis of the density of MMP-9 immunofluorescence staining (immunofluorescence units) of 10 40× fields from each stroke hemisphere. Data represent means±SE of n=4 to 5 mice per group. Line graph, relation between MMP-9 immunofluorescence staining density (units) and a2AP blood levels in mice supplemented with a2AP, normal, and a2AP-deficient mice. *P<0.05, **P<0.001, and ***P<0.0001. Bar=50 μm.

Figure 5. Effect of α2-antiplasmin (a2AP) inactivation (a2AP-I) after cerebral thromboembolism on survival, brain infarction, hemorrhage, and swelling. A. Survival in mice treated with no agent (control, Ctl), tissue plasminogen activator (TPA; 10 mg/kg), low dose a2AP-I (9 mg/kg), or high dose a2AP-I (as whole antibody [21.3 mg/kg] or Fab [9.3 mg/kg]) 30 minutes after stroke onset. Mice were observed for 7 days post stroke. Effects of treatment on brain infarction (B), brain hemorrhage (C), and brain swelling (D) after thromboembolism in surviving mice. Survival groups n=12 to 16 in each cohort, data represent means±SE. *P<0.05, **P<0.001, and ***P<0.0001 vs control or TPA.
recent reports that increased levels of a2AP may contribute to the failure of TPA treatment in humans and in mice.\textsuperscript{13,14}

In humans and in mice, thromboembolic occlusion of the MCA is associated with significant ischemic brain injury, edema, hemorrhage, and reduced survival.\textsuperscript{31,32} Consistent with these data, our study showed that proximal MCA thromboembolism had a high mortality. Treatment with an a2AP-I caused a marked reduction in mortality by comparison to TPA and to controls. Examination of surviving mice showed that a2AP-I treatment was associated with reduced hemorrhage and brain swelling, 2 key factors that contribute to mortality after MCA stroke.

In studies designed to examine the dynamics of MCA thrombus dissolution in relation to tissue injury, a2AP levels were inversely proportional to thrombus dissolution measured 6 hours after thromboembolism, which is consistent with previous studies showing that circulating a2AP levels or activity regulate the extent of fibrinolysis.\textsuperscript{22,33,34} In addition, combined deficiency of thrombus-bound a2AP and circulating a2AP were associated with even greater dissolution of the MCA thromboembolus, than deficiency of circulating a2AP alone, indicating the important role played by thrombus-associated a2AP in this process.\textsuperscript{35,36} Because of its role in fibrinolysis of the MCA thromboembolus, thrombus-bound a2AP may have been expected to affect tissue ischemia, but there was no detectable additional effect on brain infarction and swelling.

By comparison to controls or TPA-treated mice, the reduction of brain bleeding seen in mice treated with a2AP-I is unanticipated because a2AP is often considered to protect against hemorrhage in fibrinolytic states.\textsuperscript{10} Because TPA and a2AP-I act in the same pathway to enhance plasmin activity, they may be expected to have similar effects on cerebral thromboembolism. It is notable that TPA therapy caused greater dissolution of the MCA thromboembolus than a2AP-I, but it also caused greater ischemic brain injury, swelling, and death. The mechanisms responsible for the different effects of TPA and a2AP-I are currently unknown, but may be attributable to singular effects of these agents on reperfusion injury, MMP-9 expression, acute inflammation, microvascular thrombosis, neuroprotection, and other factors. Several previous studies suggest that during prolonged ischemia, TPA has neurotoxic effects on the ischemic brain, which include cleavage of the NMDA (N-methyl-D-aspartate) receptor, enhancement of excitotoxicity, blood brain barrier breakdown, promotion of apoptosis, and so on.\textsuperscript{30,37}

Microvascular thrombosis plays an important role in ischemic stroke because microvascular thrombosis diminishes plasma flow, which accentuates brain injury and is associated with breakdown of the blood brain barrier, hemorrhage, and other complications.\textsuperscript{38-40} Microvascular thrombosis may be triggered initially by exposure of tissue factor, reflecting activation of endothelial cells, leukocytes, or blood brain barrier breakdown.\textsuperscript{40,42} Inhibition of fibrin formation reduces ischemic injury, arguing that it plays a key pathophysiologic role in ischemic stroke; still the role of fibrinolysis in microvascular thrombosis is poorly understood.\textsuperscript{40,43} These data provide the first evidence that levels of a2AP are positively correlated, in a dose-dependent fashion, with the extent of microvascular thrombosis in the ischemic hemisphere. These differences cannot be explained by changes in blood cell populations as the complete blood counts of a2AP\textsuperscript{+/+} and a2AP\textsuperscript{−−/−} mice were similar (Table I in the online-only Data Supplement). Chopp and colleagues postulated that inhibition of fibrinolysis may accentuate microvascular thrombosis in stroke, and studies have shown that microvascular thrombosis induced by endotoxemia is enhanced when fibrinolysis is inhibited.\textsuperscript{24,44-46} Consistent with that hypothesis, our data shows that differences in a2AP levels, which affect dissolution of the MCA thrombus, also modulate the development of microvascular thrombosis. Still, it is important to note that, despite their common effects on promoting thrombus dissolution through plasmin, TPA accentuated, whereas a2AP-I diminished, microvascular thrombosis. The cause of this difference is unknown, though pharmacological TPA has been shown to have prothrombotic or procoagulant effects after prolonged ischemia.\textsuperscript{47}

Cerebrovascular ischemia triggers the release of endogenous TPA, which generates plasmin, and plasmin increases brain MMP-9 activity.\textsuperscript{20} MMP-9 expression and activity in ischemic regions is important in the mechanism of brain injury, as MMP-9 expression follows a similar pattern, and previous studies have shown that MMP-9 expression and activity in ischemic stroke are closely related.\textsuperscript{48} Although the exact mechanism remains to be determined, a2AP may have a role in modulating cellular injury and repair beyond its role in fibrinolysis.\textsuperscript{15,21,33,54,55} In this translational model, it is noteworthy that delayed a2AP inactivation after stroke onset reversed the deleterious effects of a2AP to reduce brain injury, bleeding, swelling, disability, and death. By comparison to TPA, the protective effects of a2AP deficiency or inactivation seem to be mediated in part through reductions in microvascular thrombosis and MMP-9 expression. It will be important to better define the fibrinolytic
and nonfibrinolytic mechanisms responsible for a2AP’s effects and to further assess the extent to which therapeutic modulation of a2AP may protect ischemic tissue after thrombotic occlusion.

Sources of Funding
This research was supported in part by National Institute of Health grants to G.L. Reed (HL58496, HL92750, and NS73147).

Disclosures
G.L. Reed is a founder of Translational Sciences. The other authors have no disclosures.

References


Clinical observations suggest that α2-antiplasmin (a2AP) may increase the risk of ischemic stroke. We found that levels of a2AP were directly related to the severity of ischemic brain injury after cerebral thromboembolism. High levels of circulating a2AP increased brain infarction and swelling and interfered with the dissolution of cerebral thromboemboli. Conversely, low levels of a2AP were neuroprotective. In a similar fashion, high levels of circulating a2AP worsened microvascular thrombosis and matrix metalloproteinase expression, two factors that contribute to ischemic brain injury. When compared with tissue plasminogen activator therapy, a2AP inactivation was less potent at dissolving thromboemboli. However, a2AP inactivation was significantly more effective than tissue plasminogen activator at reducing brain infarction, swelling, hemorrhage, and mortality. Further studies are necessary to elucidate the mechanisms through which a2AP enhances microvascular thrombosis and ischemic injury during stroke, as well as to determine the potential protective value of a2AP inactivation.
Microvascular Thrombosis, Fibrinolysis, Ischemic Injury, and Death After Cerebral Thromboembolism Are Affected by Levels of Circulating α2-Antiplasmin
Guy L. Reed, Aiilyan K. Houng and Dong Wang

Arterioscler Thromb Vasc Biol. 2014;34:2586-2593; originally published online September 25, 2014;
doi: 10.1161/ATVBAHA.114.304530

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2014 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/34/12/2586

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2014/09/25/ATVBAHA.114.304530.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
Materials and Methods

Thromboembolic stroke. Experiments were approved by the Institutional Animal Care and Use Committee. Adult male C57Black/6 (Jackson Labs, Bar Harbor, ME), a2AP+/+ mice or a2AP-deficient (C57Black/6; a2AP−/−) mice (UC Davis, KOMP) weighing 29 -35 g were anesthetized with isoflurane. Autologous thromboemboli were made with pooled fresh frozen mouse plasma from a2AP+/+ and a2AP−/− mice as described. MCA thromboembolism was performed as described with monitoring by a laser Doppler flow meter (see below, ML-191, ADInstruments, Oxford Optonix, UK). Appropriate embolization was confirmed by a decline in hemispheric blood flow by 80%. One experimental group received purified human a2AP (4.2 mg/kg, Athens Research & Technology, Inc. Athens, GA) intravenously via the contralateral jugular vein immediately after thromboembolism. Other experimental groups received a purified monoclonal antibody inhibitor of a2AP (9.3 or 21.3 mg/kg, 4h9 whole IgG or 9.5 mg/kg 4h9 Fab, Innovative Research, Novi, MI) or TPA (10 mg/kg, 20% bolus, 80% as infusion over 1 h, Genentech, South San Francisco, CA) at the indicated times after onset of ischemia. After anesthesia, mice were supported by infusions of 250 µL of saline intraperitoneally and free access to food and water. Mice were euthanized 6 h or 7 days after thromboembolism or if there were signs of being in extremis or moribund in compliance with IACUC rules. Bederson neurologic scores were assessed in a2AP-I and TPA-treated mice 6 hours after thromboembolism as described. In six hour studies, 1 mouse supplemented with a2AP and 1 TPA-treated mouse died; no control mice, a2AP+/− mice with a2AP+/− clots, a2AP−/− mice with a2AP−/− clots or a2AP-I treated mice died. After euthanasia, blood was collected by cardiac puncture and saline tissue perfusion was performed as described.

Cerebral Blood Flow. Cerebral blood flow in the MCA territory was monitored by a blood flow meter (ML-191, ADInstruments, Oxford Optonix, UK) using a laser Doppler probe (MSF 100XP, ADInstruments) through a fiberoptic filament attached by use of a tissue adhesive to the intact skull 2 mm caudal to bregma and 6 mm lateral to midline of the affected hemisphere. The blood flow was recorded using a Power Lab 2/26 data acquisition system (ADInstruments) and successful MCA occlusion was confirmed by ~80% drop in relative blood flow. Changes in cerebral blood flow after MCA occlusion were expressed as percentage (mean ± standard error) of the baseline value prior to thromboembolism.

Measurement of Brain Infarction, Hemorrhage and Swelling. Analyses were performed as previously described. Following euthanasia and perfusion, brains were sliced coronally into 2 mm sections in a rostral-caudal orientation. Brain slices were rapidly digitally photographed on both sides through a microscope. Then brain slices were immediately incubated in triphenyl tetrazolium chloride (TTC, 2%) to assess cellular viability followed by digital photography as above. Digital microscopic images were analyzed by a blinded observer using Image Pro Plus 6.2 software to measure areas of brain hemorrhage, TTC staining and hemisphere swelling. To determine the percent hemisphere infarction, the TTC-stained areas of the ischemic and non-ischemic hemispheres were measured on both faces of each brain slice. The percent infarction was calculated for each brain by the formula: infarct percentage = 100 × (Vc−Vi)/Vc, where Vc = TTC-stained area in the control hemisphere × slice thickness, Vi = TTC-stained area in the infarct hemisphere × slice thickness. The percent brain hemorrhage in the infarct hemisphere was determined by measuring the area of hemorrhage in digital microscopic images on both sides of each brain slice for the ischemic and contralateral, unaffected control hemisphere (in which there was no hemorrhage). The percent hemorrhage = 100 × (volume of hemorrhage in the infarcted hemisphere/volume of the control hemisphere). The swelling in the ischemic hemisphere was determined by comparing the volume of the ischemic hemisphere and the contralateral hemisphere for
both faces of each brain slice. The percent swelling was determined for each brain by the formula:
swelling percentage = 100 × (volume of the infarcted hemisphere – volume of the control hemisphere)/
volume of the control hemisphere).

**Thrombus formation, dissolution and immunoblotting.** *In vivo studies.* A fibrin-rich thrombus was
formed with citrated pooled fresh frozen mouse plasma from a2AP+/+ and a2AP−/− mice using calcium and
thrombin, with and without trace amounts of 125I-fibrinogen as described.1 Pooled fresh frozen mouse
plasma (5 ul) and 125I-fibrinogen (~12,500 cpm) were mixed with 1ul of (premixed thrombin 0.25 U and
CaCl2 100 mM) on ice. Immediately 2 ul (5,000 cpm) of plasma mix was drawn into 40 cm PE-10 tubing
and clotted overnight at 4°C. The PE-10 tubing containing the clot was cut into a 40 cm length and
attached to two syringes filled with sterile PBS with 1% BSA. The clot was washed by back and forth
movement in the tubing by alternate syringe aspiration for 5 minutes. The clot was expelled and stained
in 0.5% Evans blue in PBS for 10 minutes for visualization. After washing twice with 1% BSA in PBS, the
clot was cut into 6 pieces to facilitate loading back into the tubing and then gently compressed and
washed with sterile saline. The clot was counted in gamma counter (COBRA II, Packard) and pulled into a
30 cm segment of modified PE-08 tubing filled with saline for insertion to MCA. At the conclusion of the
study, the brain was gamma counted and the amount of thrombus dissolution was determined by the
formula, thrombus dissolution % = 100 × (cpm of the original clot – residual brain thrombus cpm)/ cpm
of the original clot).1

*In vitro studies of clot dissolution.* Clots were formed at 37 °C for 1 hour in 5 ml tubes (Sarstedt
Inc. Newton, NC.) from pooled mouse plasma or from individual mouse plasmas by mixing 30 ul mouse
plasma labeled with trace amounts of 125I-fibrinogen, a2AP-I or buffer (20 ul) and thrombin and calcium
(10 ul, 0.05 u thrombin, 8.3 uM calcium, final). Then 90 ul of Tris-buffered saline was added together
with TPA (10ul, 1 mM final). Test tubes were placed at 37 °C and 10 ul samples of the supernatant were
sampled hourly and subjected to gamma scintillation counting (COBRA II, Packard) to determine the rate
of clot dissolution according to the thrombus dissolution formula above. A standard curve was formed
that related the concentration of a2AP-I to the amount clot dissolution. Plasma clots made from mice
receiving a2AP-I were compared to a standard curve which, by linear regression, related the effect on
fibrinolysis of different concentrations of a2AP-I added to pooled mouse plasma (r² = 0.98).

**Immunoblotting studies.** For immunoblotting, a2AP+/+ and a2AP−/− thrombi were made as above,
washed and subjected to SDS-PAGE under reducing conditions. After electrophoretic transfer to
polyvinylidene fluoride membranes and blocking with 1% BSA, membranes were incubated with pooled
monoclonal antibodies specific for mouse a2AP (monoclonal antibodies 173, 766 as well as 4h9, 27c9: 5
ug/ml; Molecular Innovations, Inc. MI.) or rabbit polyclonal antibody (1:100) directed to the NH2 and C-
terminal peptides of a2AP as described.6 Bound antibodies were detected with a secondary goat anti-
mouse antibody IRDye 800CW or anti-rabbit labeled with IRDye-680 (Li-COR Biosciences, Lincoln, NE)
and imaged with an infrared imager (LI-COR ODYSSEY).

**Measurement of a2AP levels.** Levels of human a2AP were measured in the plasma of mice given
supplemental a2AP by intravenous infusion. At the conclusion of experiments, six hours after a2AP
administration, citrated blood samples were obtained anesthetized mice undergoing euthanasia. Plasma
was obtained by differential centrifugation at 4 °C (1800 rpm for 20 minutes followed by repeat plasma
centrifugation at 8000 rpm for 5 minutes). Wells of a microtiter plate were coated with a human
chimeric monoclonal antibody specific for human a2AP (10 ug/ml) for 1 hour at 37°C. Then wells were
washed and blocked with 1% goat serum for 1 hour. Wells were washed four times with 0.1% Tween
and three times with PBS. Then mouse plasma containing various amounts of human a2AP was diluted
1:100 in PBS containing 1% goat serum was added to wells in duplicate in seven, two-fold dilutions or
no mouse plasma, to create a standard curve. For comparison samples from mice administered a2AP
were added in various dilutions to mouse plasma (1:100) in PBS for 1 hour. After washing, a mouse antibody to human a2AP was added for an hour. Then wells were washed again and goat antimouse-horse radish peroxidase (1:5000 diluted in 1% BSA; Santa Cruz Biotechnology, Dallas, TX) was added for an hour. After washing four times with 0.1% Tween and three times with PBS, wells were developed with 3,3',5,5'-tetramethylbenzidine (TMB) peroxidase substrate (Thermo Scientific, Rockford, IL) and monitoring in a microtiter plate reader at A370 nm. The level of human a2AP in mice given intravenous human a2AP was determined by an ELISA. The standard curve related human a2AP levels spiked into mouse plasma with the ELISA signal ($r^2 = 0.95$).

**Brain tissue analyses.** For immunohistochemistry and histochemical analyses, brains were fixed in 4% paraformaldehyde and paraffin-embedded. Sections were deparaffinized and hydrated in serial dilutions of ethanol and water. Antigen was retrieved with sodium citrate buffer (pH 6.0) using heat induction at 98°C (20 min) followed by room temperature (20 min). Sections were washed in PBS and blocked with 10% normal donkey serum in PBS at room temperature (45 min). MMP-9 expression (anti-MMP-9 antibody, AF909, R&D systems) was localized relative to blood vessels by double immunofluorescence staining for collagen IV (rabbit anti-mouse type IV collagen, Karlan Research Products Corporation). Sections were incubated with primary antibody diluted in 2% normal donkey serum in PBS overnight at 4°C. After washing with PBS, secondary antibodies, DyLight488-conjugated donkey anti-goat and DyLight488-conjugated donkey anti-Rabbit IgG (Jackson ImmunoResearch Laboratories, PA) were applied for 45 minutes at room temperature. Total nuclei were counterstained with DAPI using Vectashield mounting media (Vector Laboratories, Burlingame, CA). Slides were examined by fluorescence, confocal or scanning microscopy (Aperio ScanScope, Vista, CA) and digital image analyses were performed using Image-Pro Plus 6.2 software (Media Cybernetics, Bethesda, MD). For quantitative analysis of MMP-9 immunostaining, MMP-9 staining density on red channel images of ten fields (40x) from each stroke hemisphere were analyzed. The MMP-9 staining density in each group represents the average of individual brains and was expressed in arbitrary units by Image-Pro Plus software. Microvascular thrombus formation was identified by the Martius-Scarlet-Blue stain (Atom Scientific) and quantitated by measurement of fibrin positive area (fresh red area) in 12-15 random 40x fields from each individual mice brain stroke hemisphere using Image-Pro Plus software.

In situ zymography assays were performed to evaluate MMP activity in freshly frozen brain slices isolated 6 hours after cerebral thromboembolism. Quenched fluorescein-labeled gelatin (Invitrogen) was used as a substrate to identify gelatinase activity by the development of fluorescence. For in situ zymography, 6 μm brain cryosections were first incubated with reaction buffer (0.05M Tris-HCl, 0.15 M NaCl, 5 mM CaCl2, and 0.2 mM Na3P, pH 7.6) for 10 min and then mixed with the reaction buffer containing 40 μg/ml DQ gelatin at 37°C in a humidified chamber for 5 hours. After incubation, sections were rinsed 3 times for 5 min. intervals in PBS followed by fixation with 4% paraformaldehyde for 10 min at room temperature. Slides were then washed twice for 5 min. intervals in PBS and mounted with Vectashield mounting media with DAPI (Vector Laboratories). Negative controls were obtained by pre-fixation of the sections in 4 % paraformaldehyde solution. Whole brain images were obtained by Zeiss 710 Confocal scanning at 10x magnification.

**Analysis of complete blood counts.** The complete blood counts of a2AP+/+ and a2AP−/− male mice, three mice per group, were analyzed by the UT Endocrinology Clinical Laboratory (Memphis, TN) on a Beckman-Coulter AcT diff Hematology Analyzer (Miami, FL). Blood was collected from anesthetized mice into EDTA containing tubes during euthanasia (BD Vacutainer, K2 EDTA 7.2mg, REF 367681, LOT 6341632).
**Antibody binding.** The binding of the a2AP-I to mouse a2AP or BSA was compared to a control monoclonal antibody (anti-digoxin 40-160) in an ELISA. Wells of a microtiter plate were coated with purified recombinant mouse a2AP (25 ul, 5 ug/ml for 1 hour) or nothing. After washing, non-specific binding sites were blocked with 250 ul of 1% BSA for 30 min. After rinsing, purified a2AP-I (5 ug/ml, 25 ul), control monoclonal antibody (anti-digoxin 40-160, 5 ug/ml, 25 ul) or no MAb (25 ul) were added to the wells for 1 hour in duplicate. After washing, goat anti mouse- horse radish peroxidase (1/2000 in 1% BSA, 25 ul Santa Cruz Biotechnology Inc. Dallas, Texas.) was added for 30 min. at RT. Wells were washed and developed with 3,3',5,5'-tetramethylbenzidine (TMB) peroxidase substrate (Thermo Scientific. Rockford, IL) with detection by a microtiter plate reader at A370 nm.

**Statistics.** Normally distributed data were analyzed by an unpaired Student’s t-test or a one way ANOVA using the Neuman-Keuls correction for multiple statistical inference. Non-Gaussian data were analyzed by a Mann-Whitney test, Spearman’s rank correlation coefficient ($r_s$) or a one way Kruskal Wallis analysis using Dunn’s correction. Survival data were analyzed by the Mantel Cox log rank test. Data are expressed as the mean ± standard error. A two-tailed $p \leq 0.05$ was considered statistically significant.

**References**

Supplemental Materials

Microvascular Thrombosis, Fibrinolysis, Ischemic Injury and Death after Cerebral Thromboembolism Are Affected by Levels of Circulating α2-Antiplasmin

Guy L. Reed MD*, Aiilyan K. Houng BS*, Dong Wang PhD*
Department of Medicine*
University of Tennessee Health Sciences Center, Memphis, TN, 38163, USA
Fig. I. Relative cerebral blood flow following cerebral thromboembolism (indicated by \( \uparrow \)) in control, a2AP-I and TPA-treated mice. Cerebral blood flow in the MCA territory was monitored by a blood flow meter using a laser Doppler probe as described in Materials and Methods.
Fig. II. Enhanced dissolution of clots formed ex vivo in plasma samples from a2AP-I treated vs. control or sham mice. Plasma samples were obtained six hours after the completion of ischemic stroke studies (a2AP-I treated, n= 6, control, n=2) or from sham mice (n=2). Fresh frozen plasma was clotted with trace amounts of $^{125}$I-fibrin, thrombin and calcium. Clots were treated with TPA (1 nM) and the amount of dissolution of the clot was determined by the release of soluble fibrin degradation products into the supernatant after 1 h incubation at 37° C as described in Materials and Methods. ***p<0.001 a2AP-I vs control or sham mice.
### Table I. Complete Blood Counts a2AP<sup>+/+</sup> and a2AP<sup>-/-</sup> mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>a2AP&lt;sup&gt;+/+&lt;/sup&gt;</th>
<th>a2AP&lt;sup&gt;-/-&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Test</strong></td>
<td><strong>Mean</strong></td>
<td><strong>SD</strong></td>
</tr>
<tr>
<td>WBC (x 1000/uL)</td>
<td>11.3</td>
<td>2.2</td>
</tr>
<tr>
<td>Lymphocytes %</td>
<td>89.1</td>
<td>1.2</td>
</tr>
<tr>
<td>Monocytes %</td>
<td>7.2</td>
<td>0.7</td>
</tr>
<tr>
<td>Granulocytes %</td>
<td>3.7</td>
<td>0.5</td>
</tr>
<tr>
<td>RBC (x 10&lt;sup&gt;6&lt;/sup&gt;/uL)</td>
<td>8.7</td>
<td>0.9</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>14.0</td>
<td>1.4</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>29.9</td>
<td>0.8</td>
</tr>
<tr>
<td>RDW (%)</td>
<td>31.1</td>
<td>9.6</td>
</tr>
<tr>
<td>Platelets (x 1000/uL)</td>
<td>1023.0</td>
<td>264.0</td>
</tr>
</tbody>
</table>

Table I. Comparison of complete blood cell counts in a2AP<sup>+/+</sup> and a2AP<sup>-/-</sup> mice. Blood (350 ul) was collected from anesthetized mice in tubes containing EDTA (BD Vacutainer, K2 EDTA 7.2mg, REF 367681, LOT 6341632) and analyzed by the UT Endocrinology Clinical Laboratory (Memphis, TN) on a Beckman-Coulter AcT diff Hematology Analyzer (Miami, FL).
Fig. III. Relationship between circulating a2AP levels and MMP gelatinase activity following cerebral thromboembolism. Brains were isolated 6 h following thromboembolism in mice with increased a2AP levels (↑ a2AP) due to a2AP infusions, normal a2AP levels (Ctl) or a2AP-deficiency (a2AP−/−). Representative images from the ischemic cortex are shown. Collagenase activity is indicated by green fluorescence, nuclei are stained blue with DAPI. See Methods for additional details.
Materials and Methods

Thromboembolic stroke. Experiments were approved by the Institutional Animal Care and Use Committee. Adult male C57Black/6 (Jackson Labs, Bar Harbor, ME), a2AP+/+ mice or a2AP-deficient (C57Black/6, a2AP−/−) mice (UC Davis, KOMP) weighing 29 -35 g were anesthetized with isoflurane. Autologous thromboemboli were made with pooled fresh frozen mouse plasma from a2AP+/+ and a2AP−/− mice as described. MCA thromboembolism was performed as described with monitoring by a laser Doppler flow meter (see below, ML-191, ADInstruments, Oxford Optonix, UK). Appropriate embolization was confirmed by a decline in hemispheric blood flow by 80%. One experimental group received purified human a2AP (4.2 mg/kg, Athens Research & Technology, Inc. Athens, GA) intravenously via the contralateral jugular vein immediately after thromboembolism. Other experimental groups received a purified monoclonal antibody inhibitor of a2AP (9.3 or 21.3 mg/kg, 4h9 whole IgG or 9.5 mg/kg 4h9 Fab, Innovative Research, Novi, MI) or TPA (10 mg/kg, 20% bolus, 80% as infusion over 1 h, Genentech, South San Francisco, CA) at the indicated times after onset of ischemia. After anesthesia, mice were supported by infusions of 250 µL of saline intraperitoneally and free access to food and water. Mice were euthanized 6 h or 7 days after thromboembolism or if there were signs of being in extremis or moribund in compliance with IACUC rules. Bederson neurologic scores were assessed in a2AP-I and TPA-treated mice 6 hours after thromboembolism as described. In six hour studies, 1 mouse supplemented with a2AP and 1 TPA- treated mouse died; no control mice, a2AP−/− mice with a2AP+/+ clots, a2AP−/− mice with a2AP−/− clots or a2AP-I treated mice died. After euthanasia, blood was collected by cardiac puncture and saline tissue perfusion was performed as described.

Cerebral Blood Flow. Cerebral blood flow in the MCA territory was monitored by a blood flow meter (ML-191, ADInstruments, Oxford Optonix, UK) using a laser Doppler probe (MSF 100XP, ADInstruments) through a fiberoptic filament attached by use of a tissue adhesive to the intact skull 2 mm caudal to bregma and 6 mm lateral to midline of the affected hemisphere. The blood flow was recorded using a Power Lab 2/26 data acquisition system (ADInstruments) and successful MCA occlusion was confirmed by ~80% drop in relative blood flow. Changes in cerebral blood flow after MCA occlusion were expressed as percentage (mean ± standard error) of the baseline value prior to thromboembolism.

Measurement of Brain Infarction, Hemorrhage and Swelling. Analyses were performed as previously described. Following euthanasia and perfusion, brains were sliced coronally into 2 mm sections in a rostral-caudal orientation. Brain slices were rapidly digitally photographed on both sides through a microscope. Then brain slices were immediately incubated in triphenyl tetrazolium chloride (TTC, 2%) to assess cellular viability followed by digital photography as above. Digital microscopic images were analyzed by a blinded observer using Image Pro Plus 6.2 software to measure areas of brain hemorrhage, TTC staining and hemisphere swelling. To determine the percent hemisphere infarction, the TTC-stained areas of the ischemic and non-ischemic hemispheres were measured on both faces of each brain slice. The percent infarction was calculated for each brain by the formula: infarct percentage = 100 × (Vc-Vi / Vc), where Vc = TTC-stained area in the control hemisphere × slice thickness, Vi = TTC-stained area in the infarct hemisphere × slice thickness. The percent brain hemorrhage in the infarct hemisphere was determined by measuring the area of hemorrhage in digital microscopic images on both sides of each brain slice for the ischemic and contralateral, unaffected control hemisphere (in which there was no hemorrhage). The percent hemorrhage = 100 × (volume of hemorrhage in the infarcted hemisphere/ volume of the control hemisphere). The swelling in the ischemic hemisphere was determined by comparing the volume of the ischemic hemisphere and the contralateral hemisphere for
both faces of each brain slice. The percent swelling was determined for each brain by the formula:
swelling percentage = \(100 \times \frac{\text{volume of the infarcted hemisphere} - \text{volume of the control hemisphere}}{\text{volume of the control hemisphere}}\).

**Thrombus formation, dissolution and immunoblotting.** *In vivo studies.* A fibrin-rich thrombus was formed with citrated pooled fresh frozen mouse plasma from a2AP\(^{+/+}\) and a2AP\(^{+/−}\) mice using calcium and thrombin, with and without trace amounts of \(^{125}\)I-fibrinogen as described.\(^1\) Pooled fresh frozen mouse plasma (5 ul) and \(^{125}\)I-fibrinogen (~12,500 cpm) were mixed with 1ul of (premixed thrombin 0.25 U and CaCl\(_2\) 100 mM) on ice. Immediately 2 ul (5,000 cpm) of plasma mix were drawn into 40 cm PE-10 tubing and clotted overnight at 4°C. The PE-10 tubing containing the clot was cut into a 40 cm length and attached to two syringes filled with sterile PBS with 1% BSA. The clot was washed by back and forth movement in the tubing by alternate syringe aspiration for 5 minutes. The clot was expelled and stained in 0.5% Evans blue in PBS for 10 minutes for visualization. After washing twice with 1% BSA in PBS, the clot was cut into 6 pieces to facilitate loading back into the tubing and then gently compressed and washed with sterile saline. The clot was counted in gamma counter (COBRA II, Packard) and pulled into a 30 cm segment of modified PE-08 tubing filled with saline for insertion to MCA. At the conclusion of the study, the brain was gamma counted and the amount of thrombus dissolution was determined by the formula, thrombus dissolution % = \(100 \times \frac{\text{cpm of the original clot} - \text{residual brain thrombus cpm}}{\text{cpm of the original clot}}\).\(^1\)

*In vitro studies of clot dissolution.* Clots were formed at 37 °C for 1 hour in 5 ml tubes (Sarstedt Inc. Newton, NC.) from pooled mouse plasma or from individual mouse plasmas by mixing 30 ul mouse plasma labeled with trace amounts of \(^{125}\)I-fibrinogen, a2AP-I or buffer (20 ul) and thrombin and calcium (10 ul, 0.05 u thrombin, 8.3 uM calcium, final). Then 90 ul of Tris-buffered saline was added together with TPA (10ul, 1 nM final). Test tubes were placed at 37 °C and 10 ul samples of the supernatant were sampled hourly and subjected to gamma scintillation counting (COBRA II, Packard) to determine the rate of clot dissolution according to the thrombus dissolution formula above. A standard curve was formed that related the concentration of a2AP-I to the amount clot dissolution. Plasma clots made from mice receiving a2AP-I were compared to a standard curve which, by linear regression, related the effect on fibrinolysis of different concentrations of a2AP-I added to pooled mouse plasma \(r^2 = 0.98\).

**Immunoblotting studies.** For immunoblotting, a2AP\(^{+/+}\) and a2AP\(^{+/−}\) thrombi were made as above, washed and subjected to SDS-PAGE under reducing conditions. After electrophoretic transfer to polyvinylidene fluoride membranes and blocking with 1% BSA, membranes were incubated with pooled monoclonal antibodies specific for mouse a2AP (monoclonal antibodies 173, 766 as well as 4h9, 27c9; 5 ug/ml; Molecular Innovations, Inc. MI.) or rabbit polyclonal antibody (1:100) directed to the NH\(_2\) and C-terminal peptides of a2AP as described.\(^6\) Bound antibodies were detected with a secondary goat anti-mouse antibody IRDye 800CW or anti-rabbit labeled with IRDye-680 (Li-COR Biosciences, Lincoln, NE) and imaged with an infrared imager (LI-COR ODYSSEY).

**Measurement of a2AP levels.** Levels of human a2AP were measured in the plasma of mice given supplemental a2AP by intravenous infusion. At the conclusion of experiments, six hours after a2AP administration, citrated blood samples were obtained anesthetized mice undergoing euthanasia. Plasma was obtained by differential centrifugation at 4 °C (1800 rpm for 20 minutes followed by repeat plasma centrifugation at 8000 rpm for 5 minutes). Wells of a microtiter plate were coated with a human chimeric monoclonal antibody specific for human a2AP (10 ug/ml) for 1 hour at 37°C. Then wells were washed and blocked with 1% goat serum for 1 hour. Wells were washed four times with 0.1% Tween and three times with PBS. Then mouse plasma containing various amounts of human a2AP was diluted 1:100 in PBS containing 1% goat serum was added to wells in duplicate in seven, two-fold dilutions or no mouse plasma, to create a standard curve. For comparison samples from mice administered a2AP
were added in various dilutions to mouse plasma (1:100) in PBS for 1 hour. After washing, a mouse antibody to human a2AP was added for an hour. Then wells were washed again and goat antimus- 
horse radish peroxidase (1:5000 diluted in 1% BSA; Santa Cruz Biotechnology, Dallas, TX) was added for an hour. After washing four times with 0.1% Tween and three times with PBS, wells were developed 
developed with 3,3',5,5'-tetramethylbenzidine (TMB) peroxidase substrate (Thermo Scientific, Rockford, 
and monitoring in a microtiter plate reader at A370 nm. The level of human a2AP in mice given 
intravenous human a2AP was determined by an ELISA. The standard curve related human a2AP levels 
spiked into mouse plasma with the ELISA signal (r² = 0.95).

Brain tissue analyses. For immunohistochemistry and histochemical analyses, brains were fixed in 4% 
parafomaldehyde and paraffin-embedded. Sections were deparaffinized and hydrated in serial dilutions 
of ethanol and water. Antigen was retrieved with sodium citrate buffer (pH 6.0) using heat induction at 
98°C (20 min) followed by room temperature (20 min). Sections were washed in PBS and blocked with 
10% normal donkey serum in PBS at room temperature (45 min). MMP-9 expression (anti-MMP-9 
antibody, AF909, R&D systems) was localized relative to blood vessels by double immunofluorescence 
staining for collagen IV (rabbit anti-mouse type IV collagen, Karlan Research Products Corporation). 
Sections were incubated with primary antibody diluted in 2% normal donkey serum in PBS overnight at 
4°C. After washing with PBS, secondary antibodies, DyLight549–conjugated donkey anti-goat and 
DyLight488-conjugated donkey anti-Rabbit IgG (Jackson ImmunoResearch Laboratories, PA) were 
applied for 45 minutes at room temperature. Total nuclei were counterstained with DAPI using 
 Vectashield mounting media (Vector Laboratories, Burlingame, CA). Slides were examined by 
fluorescence, confocal or scanning microscopy (Aperio ScanScope, Vista, CA) and digital image analyses 
were performed using Image-Pro Plus 6.2 software (Media Cybernetics, Bethesda, MD). For quantitative 
analysis of MMP-9 immunostaining, MMP-9 staining density on red channel images of ten fields (40x) 
from each stroke hemisphere were analyzed. The MMP-9 staining density in each group represents the 
average of individual brains and was expressed in arbitrary units by Image-Pro Plus software. 
Microvascular thrombus formation was identified by the Martius-Scarlet-Blue stain (Atom Scientific) and 
quantitated by measurement of fibrin positive area (fresh red area) in 12-15 random 40x fields from 
each individual mice brain stroke hemisphere using Image-Pro Plus software.

In situ zymography assays were performed to evaluate MMP activity in freshly frozen brain 
slices isolated 6 hours after cerebral thromboembolism. Quenched fluorescein-labeled gelatin 
(Invitrogen) was used as a substrate to identify gelatinase activity by the development of fluorescence. 
For in situ zymography, 6 μm brain cryosections were first incubated with reaction buffer (0.05M Tris- 
HCl, 0.15 M NaCl, 5 mM CaCl₂, and 0.2 mMNaN3, pH 7.6) for 10 min and then mixed with the reaction 
buffer containing 40 μg/ml DQ gelatin at 37°C in a humidified chamber for 5 hours. After incubation, 
sections were rinsed 3 times for 5 min. intervals in PBS followed by fixation with 4% paraformaldehyde 
for 10 min at room temperature. Slides were then washed twice for 5 min. intervals in PBS and mounted 
with Vectashield mounting media with DAPI (Vector Laboratories). Negative controls were obtained by 
pre-fixation of the sections in 4 % paraformaldehyde solution. Whole brain images were obtained by 
Zeiss 710 Confocal scanning at 10x magnification.

Analysis of complete blood counts. The complete blood counts of a2AP+/+ and a2AP−/− male mice, three 
mice per group, were analyzed by the UT Endocrinology Clinical Laboratory (Memphis, TN) on a 
Beckman-Coulter AcT diff Hematology Analyzer (Miami, FL). Blood was collected from anesthetized mice 
into EDTA containing tubes during euthanasia (BD Vacutainer, K2 EDTA 7.2mg, REF 367681, LOT 
6341632).
**Antibody binding.** The binding of the a2AP-I to mouse a2AP or BSA was compared to a control monoclonal antibody (anti-digoxin 40-160) in an ELISA. Wells of a microtiter plate were coated with purified recombinant mouse a2AP (25 ul, 5 ug/ml for 1 hour) or nothing. After washing, non-specific binding sites were blocked with 250 ul of 1% BSA for 30 min. After rinsing, purified a2AP-I (5 ug/ml, 25 ul), control monoclonal antibody (anti-digoxin 40-160, 5 ug/ml, 25 ul) or no MAb (25 ul) were added to the wells for 1 hour in duplicate. After washing, goat anti mouse- horse radish peroxidase (1/2000 in 1% BSA, 25 ul Santa Cruz Biotechnology Inc. Dallas, Texas.) was added for 30 min. at RT. Wells were washed and developed with 3,3',5,5'-tetramethylbenzidine (TMB) peroxidase substrate (Thermo Scientific. Rockford, IL) with detection by a microtiter plate reader at A370 nm.

**Statistics.** Normally distributed data were analyzed by an unpaired Student’s t-test or a one way ANOVA using the Neuman-Keuls correction for multiple statistical inference. Non-Gaussian data were analyzed by a Mann-Whitney test, Spearman’s rank correlation coefficient (r_s) or a one way Kruskal Wallis analysis using Dunn’s correction. Survival data were analyzed by the Mantel Cox log rank test. Data are expressed as the mean ± standard error. A two-tailed p ≤0.05 was considered statistically significant.

**References**