Thrombin Inhibition by Argatroban Ameliorates Early Brain Injury and Improves Neurological Outcomes After Experimental Subarachnoid Hemorrhage in Rats

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Background and Purpose—We investigated the role of thrombin in early brain injury after subarachnoid hemorrhage (SAH). Methods—The standard intravascular perforation model was used to produce experimental SAH in Sprague Dawley rats. Low-dose (0.3 mg/h) and high-dose (0.9 mg/h) argatroban, a direct thrombin inhibitor, were evaluated for effects on brain edema, blood–brain barrier (BBB) disruption, apoptotic cell death, inflammatory marker, and neurological outcomes after SAH.

Results—Both doses of argatroban attenuated BBB disruption; however, only high-dose was effective in lowering edema in all brain regions, reducing cell death, and inflammatory marker expression, and improving neurological outcomes.

Conclusions—Thrombin inhibition by argatroban improves neurological outcomes and provides neuroprotection against acute events after SAH such as BBB disruption, brain edema, and cell death. (Stroke. 2009;40:1530-1532.)

Key Words: argatroban ▪ brain edema ▪ early brain injury ▪ subarachnoid hemorrhage ▪ thrombin

Early brain injury comprising blood–brain barrier (BBB) disruption, brain edema, and global ischemia is important in the pathophysiology of subarachnoid hemorrhage (SAH); however, the mechanisms are not clearly understood.1 Thrombin, a serine protease coagulation protein, has been implicated in BBB disruption and brain edema after cerebral ischemia and intracerebral hemorrhage.2–4 We investigated the role of thrombin by using argatroban, a direct inhibitor,2–4 in the standard rat intravascular perforation model for SAH.5–6

Materials and Methods

All procedures were approved by Loma Linda University animal care committee. The intravascular perforation SAH model was used as previously described in adult male Sprague Dawley rats (Harlan; Indianapolis, Ind).5–6 One hundred forty-three animals were divided into 4 groups: sham (n = 24), vehicle (n = 39; saline with hydrochloric acid pH 1.4 to 1.6), low-dose (n = 35, 0.3 mg/h), and high-dose (n = 45, 0.9 mg/h) argatroban delivered intraperitoneally 15 minutes after SAH using osmotic minipumps (ALZET; Alza Corp).5 Animals having mild SAH (3 from vehicle-treated, 2 from low-dose, and 2 from high-dose argatroban groups) were excluded from the study per the SAH grading system criteria reported previously; animals obtaining grade ≤5/18 on blinded evaluation were classified as having mild SAH and were excluded.6 The animals were not randomly allocated; however, the neurological status of the animals was evaluated by a blinded observer using an 18-point scoring scale and right forelimb placing test before euthanization.7 Brain water content of the cerebral hemispheres, cerebellum, and brain stem was examined to assess brain edema and spectrophotometric quantitation of Evans blue dye extravasation into the cerebral hemispheres provided a measure of BBB disruption at 24 hours as described previously.5–6 Cell Death Detection ELISA kit (Roche Applied Science) was used to quantify cell death in left hemisphere at 24 hours and 72 hours.8 Standard Western blotting protocol9 using the following antibodies, rabbit polyclonal zona occludens-1 antibody from Invitrogen, mouse monoclonal IL-1β antibody, and goat polyclonal actin antibody from Santa Cruz Biotechnology, was performed on brain tissue from left hemisphere (ipsilateral to perforation) at 24 hours. All molecular studies were performed by blinded researcher. The data are expressed as mean ± SEM and differences between groups were assessed with a 1-way analysis of variance with Holm-Sidak posthoc analysis, with P<0.05 considered statistically significant.

Results

Physiological parameters were not significantly different among groups. Mortality rates (calculated using χ2 test) were not significantly different among the vehicle and treatment groups at 24 hours (vehicle, 22%; low-dose argatroban, 45%; and high-dose argatroban, 40%) and 72 hours (vehicle, 54%; high-dose argatroban, 54%). Sham animals had zero mortality. All animals subjected to experimental SAH had comparable SAH grades.6 Neurological and forelimb placement scores were significantly worse in vehicle group compared to sham over 24 to 72 hours. Neurological deficits were not improved by either dose of argatroban at 24 hours; however, high-dose argatroban

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showed significant improvement at 48 and 72 hours after SAH (Figure 1A–D).

Brain water content was significantly increased in all brain areas in vehicle group compared to sham. High-dose argatroban significantly lowered the brain water content in hemispheres, cerebellum, and brain stem; however, low-dose argatroban only reduced brain water content in left hemisphere (ipsilateral to perforation) and cerebellum (Figure 2A).

Evans blue dye extravasation was significantly increased in hemispheres in vehicle group as compared to sham and attenuated by both doses of argatroban (Figure 2B). Expression of zona occludens-1, a tight junction protein, was significantly decreased in vehicle group compared to sham but reversed by high-dose but not low-dose argatroban (Figure 2C).

Significantly higher cell death was detected at 24 hours but not at 72 hours in the vehicle group compared to sham; this was attenuated by high-dose but not low-dose argatroban (Figure 3A). Increased expression of inflammatory marker IL-1β in the vehicle group (compared to sham) at 24 hours was attenuated by high-dose but not low-dose argatroban (Figure 3B).

**Discussion**

The present study showed for the first time to our knowledge that thrombin plays a role in early brain injury after SAH. Brain edema after BBB disruption is a key event in early brain injury after SAH.1,4,10 High-dose of argatroban, a direct thrombin inhibitor, prevented the BBB disruption and reduced the brain edema after SAH with subsequent improvement in neurological status. High-dose argatroban also attenuated cell death and expression of inflammatory marker after SAH.
Cell death and inflammation are critical in BBB disruption and brain edema.14–10–12 The early brain injury peaks at 24 hours after SAH as indicated by cell death observed at 24 hours but not at 72 hours, similar to previous studies; this temporal profile was also seen with brain edema and BBB disruption.11 It remains to be determined whether the anticell death effect of argatroban is cell-specific or pan-cellular,13 however, our BBB studies suggested that argatroban may protect the endothelial cells. Moreover, the expression of zona occludens-1, a tight junction protein was preserved by high-dose argatroban after SAH indicating a protective effect on the BBB.

The lack of argatroban effect in improving neurological deficits at an early time point of 24 hours could be dose-related14 or attributable to incomplete recovery of animals from the SAH postictal state.11 Furthermore, the global ischemia caused by SAH may warrant further higher doses, which will be examined in future studies. Argatroban has been used clinically in Japan and Korea for thromboembolic disruption.11 It remains to be determined whether the anticell death effect of argatroban is cell-specific or pan-cellular,13 however, our BBB studies suggested that argatroban may protect the endothelial cells. Moreover, the expression of zona occludens-1, a tight junction protein was preserved by high-dose argatroban after SAH indicating a protective effect on the BBB.

The lack of argatroban effect in improving neurological deficits at an early time point of 24 hours could be dose-related14 or attributable to incomplete recovery of animals from the SAH postictal state.11 Furthermore, the global ischemia caused by SAH may warrant further higher doses, which will be examined in future studies. Argatroban has been used clinically in Japan and Korea for thromboembolic disorders and more recently for ischemic stroke.15 However, in the United States and Canada it has been approved only for prophylaxis and treatment of thrombosis in patients with heparin-induced thrombocytopenia.15 There are reported side effects such as increased hemorrhagic episodes and gastrointestinal bleeding and hepatic dysfunction. In this study, we did not encounter any hemorrhagic events (brain and gastric) with both low-dose and high-dose argatroban. This evaluation was performed by gross examination after euthanization. Other studies using the same dosage in rats have reported not encountering any intracraniatal or systemic bleeding side effects.2 Macroscopically, the livers also looked normal in all treatment groups. Favorable pharmacokinetics and clinical outcomes in other cerebrovascular disorders make argatroban a promising therapeutic modality to be evaluated in clinical trials for SAH.15

Conclusions
Argatroban, a direct thrombin inhibitor, ameliorated BBB disruption and brain edema with improvement in neurological outcomes and exhibited anticell death and antiinflammatory effects coincident with early brain injury after SAH.

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Disclosures
None.

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