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# PAR-1 antagonist SCH79797 ameliorates apoptosis following surgical brain injury through inhibition of ASK1-JNK in rats

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ABSTRACT

Neurosurgical procedures inevitably produce intraoperative hemorrhage. The subsequent entry of blood into the brain parenchyma results in the release of large amounts of thrombin, a known contributor to perihematomal edema formation and apoptosis after brain injury. The present study seeks to test 1) the effect of surgically induced brain injury (SBI) on thrombin activity, expression of thrombin's receptor PAR-1, and PAR-1 mediated apoptosis; 2) the effect of thrombin inhibition by argatroban and PAR-1 inhibition by SCH79797 on the development of secondary brain injury in the SBI model on rats.

A total of 88 Sprague–Dawley male rats were randomly divided into sham, vehicle-, argatroban-, or SCH79797-treated groups. SBI involved partial resection of the right frontal lobe under inhalation isoflurane anesthesia. Sham-operated animals received only craniotomy. Thrombin activity, brain water content, and neurological deficits were measured at 24 h following SBI. Involvement of the Ask1/JNK pathway in PAR-1-induced post-SBI apoptosis was characterized by using Ask1 or JNK inhibitors.

We observed that SBI increased thrombin activity, yet failed to demonstrate any effect on PAR-1 expression. Argatroban and SCH79797 reduced SBI-induced brain edema and neurological deficits in a dose-dependent manner. SBI-induced apoptosis seemed mediated by the PAR-1/Ask1/JNK pathways. Administration of SCH79797 ameliorated the apoptosis following SBI.

Our findings indicate that PAR-1 antagonist protects against secondary brain injury after SBI by decreasing both brain edema and apoptosis by inactivating PAR-1/Ask1/JNK pathway. The anti-apoptotic effect of PAR-1 antagonists may provide a promising path for therapy following SBI.



## Introduction

Neurosurgical procedures result in brain injury by various means, including intraoperative hemorrhage (Jadhav et al., 2007b). Thrombin, a serine protease, can be generated in the brain locally from pro-thrombin or may enter the brain parenchyma as a result of blood brain barrier (BBB) disruption (Sokolova and Reiser, 2008). Although low concentrations of thrombin have been demonstrated to be neuroprotective (Striggow et al., 2000), accumulating literature suggests that thrombin in high concentration may result in BBB disruption and development of brain edema (Han et al., 2011; Nagatsuna et al., 2005; Ohyama et al., 2001). Thrombin inhibition has been shown to be neuroprotective in several models of brain injury (Jin et al., 2002; Sugawara et al., 2009).

Thrombin signals are mediated by three types of protease-activated receptors-1 (PAR-1,-3, and -4). Detrimental thrombin signaling is mostly mediated by the PAR-1 receptor, the activation of which results in increased secondary brain injury (Junge et al., 2003; Olson et al., 2004). In this study we examined: 1) if thrombin activity is upregulated after SBI, 2) if SBI affects expression of thrombin's receptor PAR-1, 3) the role of the PAR-1/Ask1/JNK pathway in apoptosis following SBI, and 4) if thrombin or PAR-1 inhibition will result in the amelioration of secondary brain injury after SBI.

## Materials and methods

### *Animals and SBI model*

All procedures were approved by the Loma Linda University Animal Care Committee. Eighty-eight adult male Sprague–Dawley rats (280–350 g, Harlan, Indianapolis, IN) were used. SBI was performed as previously described. All procedures were approved by Loma Linda University Animal Care Committee. Animals were housed in a controlled-

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environment animal housing facility. SBI involved partial resection of the right frontal lobe under inhalation isoflurane anesthesia. SBI induction was conducted as described before (Yamaguchi et al., 2007). Briefly, skin over the dorsum of the skull was shaved. With the aid of a surgical dissecting microscope, skin and connective tissue was dissected by using a No. 11 surgical blade. The right frontal skull was exposed by a periosteal elevator. 5-mm edge square operating area was identified on the frontal skull bone, with the lower left corner of the square lying at the bregma. Using a flat blade, we made two separate incisions leading away from the bregma along the sagittal and coronal planes to sever an area of the brain 2 mm lateral to the sagittal suture and 1 mm proximal to the coronal suture. Sham animals received only craniotomy.

*Drug doses and administration routes*

All drugs were administrated intraperitoneally, 1 h after SBI. The following concentrations were administered: Argatroban (thrombin inhibitor): 0.3 or 0.9 mg/kg; SCH79797 (PAR-1 antagonist): 5 or 25 μg/kg; selenite (Ask1 inhibitor): 0.3 mg/kg; Sp600125 (JNK inhibitor): 30 mg/kg; and Anisomycin (JNK activator): 5 mg/kg.

*Thrombin activity measurement*

Thrombin activity was spectrophotometrically determined by using thrombin-specific chromogenic substrate as described previously (Lee et al., 2005) (See Supplementary materials).

*Brain water content*

Brain water content was calculated as described previously (Jadhav et al., 2007a) (See Supplementary materials).

*Neurological evaluation*

Neurological deficits were evaluated by an investigator blinded to treatment, 24 h after SBI. The modified Garcia, Paw placement and Beam balance tests were used (See Supplementary materials).

*Immunohistochemical studies*

Standard fluorescent immunostaining protocol was adopted (Supplementary materials).

*Western blot analysis*

For western blot the following primer antibodies were used from Santa Cruz Biotechnology: anti-PAR-1, anti-Ask1, anti-(p-JNK), anti-Caspase3 anti p-Ask1 Ser967; from Cell Signaling: anti-JNK, anti-Bim, anti-Bcl2, anti-Bax (Cell Signaling) (See Supplementary materials for more details).

*Statistical analysis*

The data are expressed as mean ± SEM. Differences between groups were assessed with a one-way analysis of variance (ANOVA) and Holm–Sidak post-hoc test. A value of $p<0.05$ was considered statistically significant.

## Results

*Effect of SBI on the thrombin activity*

Animals were divided into SBI and sham operated animals (N = 6 each group). At 24 h after SBI, we observed a 3.82-fold increase in thrombin enzyme activity in the ipsilateral frontal lobe of SBI animals compared to that of the sham operated animals ($p<0.05$, Fig. 1). SBI also caused a 1.69-fold increase in thrombin activity in the contralateral frontal lobe of SBI animals, compared to that of sham animals ($p<0.05$, Fig. 1). Additionally, we detected that the thrombin activity in the ipsilateral frontal lobe of SBI animals was significant higher compared to that of the contralateral frontal lobe (3.76 fold, $p<0.05$, Fig. 1).

*Both thrombin inhibitor and PAR-1 receptor antagonists decrease SBI-induced brain edema and ameliorate neurological deficits*

Animals were divided into sham-operated, SBI vehicle and SBI argatroban-treated animals (N = 6 each group). At 24 h, brain water content was elevated in the ipsilateral frontal lobe of the vehicle-treated compared to that of the sham-operated animals (82.93 +/− 0.63 vs. 79.61 +/− 0.25, respectively, $p<0.05$, Fig. 2A). While the low dose of argatroban exhibited no effect on SBI-induced increase in brain water content, the high dose significantly reduced the brain water content in argatroban-treated compared to that of vehicle treated-animals (81.43 +/− 0.73 vs. 82.93 +/− 0.63, $p<0.05$, Fig. 2A).

All three neurobehavioral tests used in the study revealed significant neurological deficits in the SBI compared to sham operated animals 24 h after surgery ($p<0.05$, Figs. 2B–C). While the low dose of argatroban proved ineffective, high dose of argatroban ameliorated neurological deficits in the forelimb placement test ($p<0.05$, Fig. 2B) and showed a tendency to improve performance in the modified Garcia and beam balance tests (Figs. 2C–D).

Additionally, we examined the efficacy of PAR-1 antagonist, SCH97979, in treating SBI-induced secondary injury. High dose of SCH7979 (N = 6) significantly decreased the SBI-induced increase of brain water content in the ipsilateral frontal lobe (81.07 +/− 0.21%, compared to the vehicle treated animals 82.93 +/− 0.63, $p<0.05$ Fig. 3A) and improved neurological deficits in all the three neurobehavioral tests used ($p<0.05$, Figs. 3B–D). The low dose of SCH97979 (N = 6) was ineffective (Fig. 3).

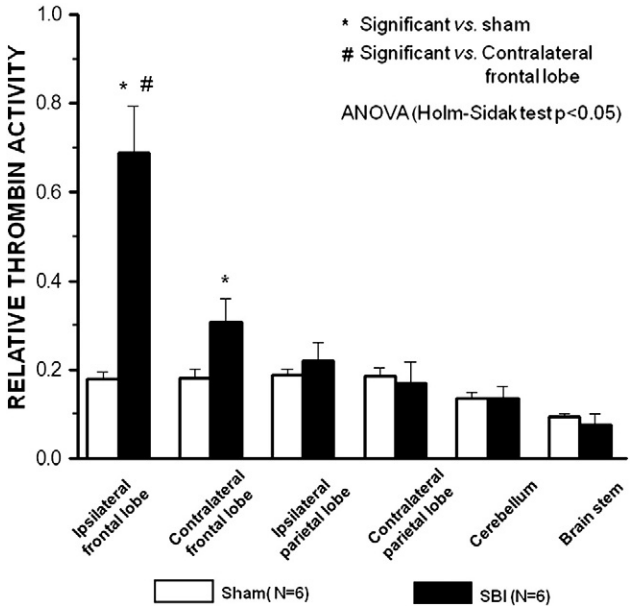


**Fig. 1.** SBI caused a significant increase of thrombin activity at 24 h after operation. SBI increased thrombin activity in the frontal lobe of SBI compared to sham-operated animals. (N = 6 each group, * Significant vs. sham; #Significant vs. Contralateral frontal lobe, $p<0.05$ (ANOVA, Holm–Sidak test)).

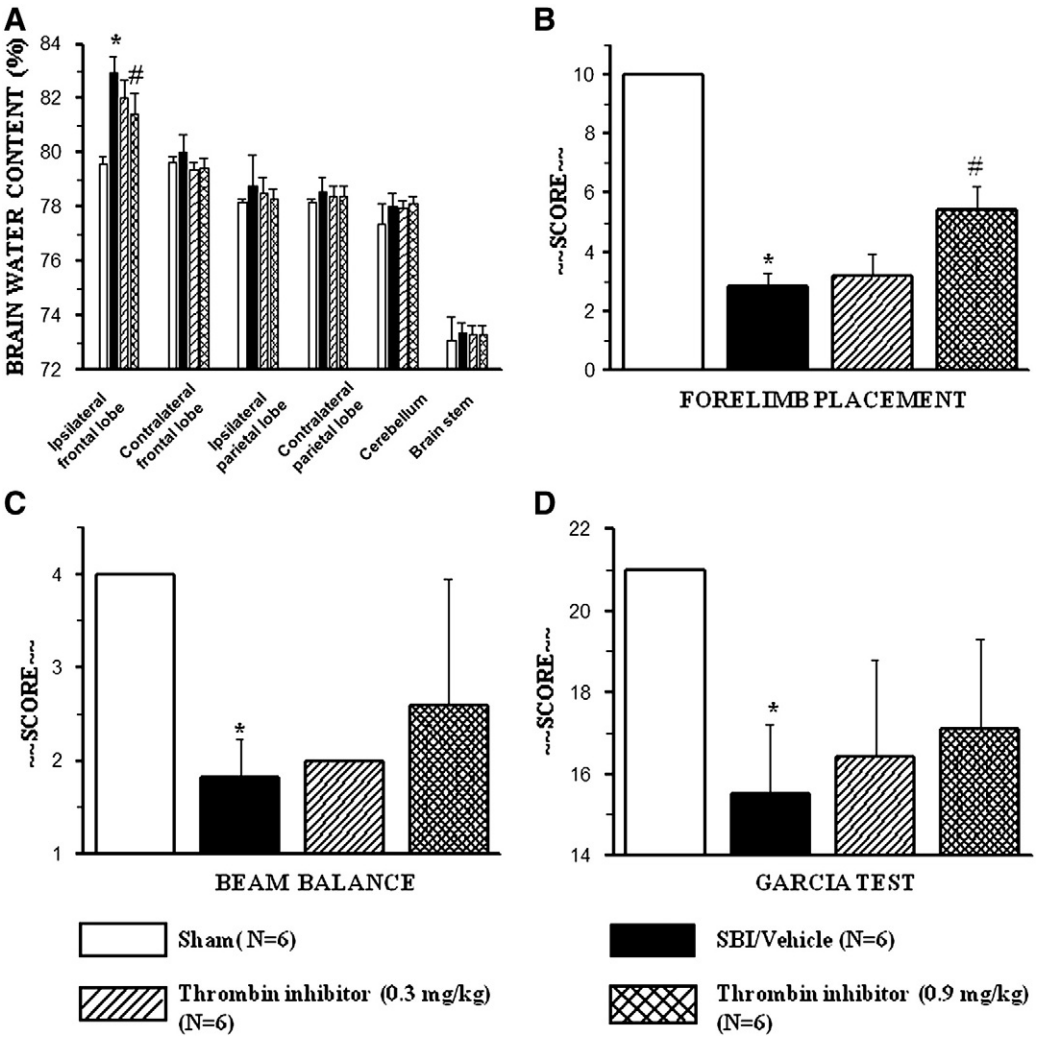


**Fig. 2.** Thrombin inhibitor ameliorates SBI induced increase of brain water content and improves neurological deficits at 24 h after operation. SBI increased brain water content (A) and caused neurological deficits (C–D) in SBI compared to sham operated animals. While low dose of argatroban (thrombin inhibitor) was ineffective, high dose decreased brain water content and ameliorated neurological deficits in treated compared to untreated animals. (N = 6 each group, *Significant vs. sham; #Significant vs. vehicle, $p<0.05$ (ANOVA, Holm–Sidak test)).

*Effects of SBI and PAR-1 antagonists on PAR-1 receptor expression and PAR-1 downstream signaling pathway*

1) SBI activates Ask1/JNK pathway via PAR-1 receptor.
   We investigated whether PAR-1 via Ask1/JNK pathway could participate in the manifestation of secondary brain injury after SBI by performing a triple stain against PAR-1, Ask1 and JNK. The stains showed that these three proteins are expressed in the same cells (Fig. 4A). Based on the features of cell morphology, most of the positive cells are likely neurons and astrocytes.
2) Effects of SBI and PAR-1 antagonist on PAR-1 receptor expression. Neither SBI nor SCH97979 (PAR-1 antagonist) demonstrated any effects on PAR-1 (Fig. 4B).
3) Effect of SBI and PAR1 antagonist on Ask1 and JNK activation.
   Neither SBI nor SCH97979 affected Ask1 expression. However, SBI resulted in the significant increase in the activation of ASK, resulting in the decrease of phosphorylated Ask1 (pAsk1) level and pAsk1/Ask ratio (Fig. 4C). We also examined the effects of selenite (Ask1 inhibitor) and found out that selenite reversed the SBI-induced decrease in pAsk1/Ask1 level (Fig. 4C). Neither SBI nor SCH97979 showed any effect on the expression of JNK. However, SBI resulted in increased pJNK/JNK level compared to that of sham animals ($p<0.05$, Fig. 4D). SCH97979 decreased SBI-induced JNK phosphorylation without affecting JNK expression ($p<0.05$, Fig. 4D). Administration of selenite (Ask1 inhibitor) resulted in decreased levels of pJNK and pJNK/JNK in selenite compared to those of the vehicle-treated animals. Likewise Sp600125 (JNK inhibitor) significantly reduced SBI-induced JNK phosphorylation and resulted in decreased pJNK/JNK level ($p<0.05$, Fig. 4D). Administration of JNK activator, anisomycin, simultaneously with Ask1 inhibition by selenite reversed the effect of Ask1 inactivation and resulted in increased level of pJNK/JNK ($p<0.05$, Fig. 4D).

*Effect of PAR1 antagonist on apoptosis after SBI*

The effects of SBI and SCH97979 (PAR-1 antagonist) on the expression of pro- and anti-apoptotic proteins were investigated by western blot and TUNEL staining at 24 h after SBI. Compared to sham animals, SBI animals demonstrated significantly increased production of pro-apoptotic BIM and shifted BAX/Bcl2 levels toward pro-apoptotic BAX. Both SCH97979 and inhibitors of Ask1 or JNK

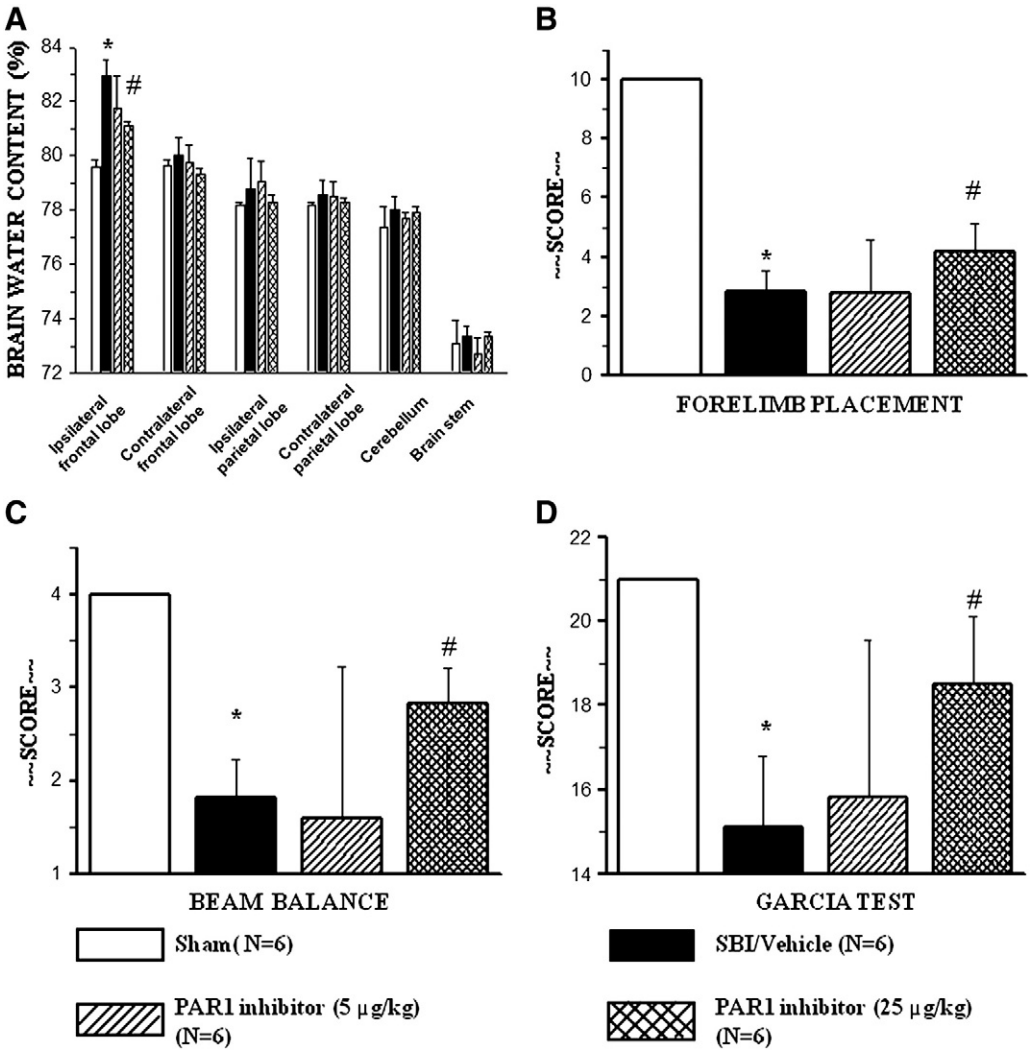


**Fig. 3.** PAR-1 inhibitor ameliorates SBI induced increase of brain water content and improves neurological deficits at 24 h after operation. SBI increased brain water content (A) and caused neurological deficits (C–D) in SBI compared to sham operated animals. While low concentration of SCH97979 (PAR1 antagonist) was ineffective, high concentration of SCH97979 decreased brain water content and ameliorated neurological deficits in treated compared to untreated animals. (N = 6 each group, *Significant vs. sham; #Significant vs. vehicle, $p<0.05$ (ANOVA, Holm–Sidak test)).

shifted the balance of pro-apoptotic proteins toward anti-apoptotic Bcl2 ($p<0.05$, Figs. 5A–B) compared to their vehicle-treated counterparts. Co-administration of JNK activator and Ask1 inhibitor reversed the anti-apoptotic effect of Ask1 inhibition and increased BIM and BAX/Bcl2 level compared to that of animals treated with Ask1 inhibitor only ($p<0.05$, Figs. 5A–B).

Consistent with these findings, SBI significantly increased the cleavage of caspase-3, a key regulator of apoptosis in SBI versus sham-operated animals ($p<0.05$, Fig. 6A). Both SCH97979 and the inhibitors of Ask1/JNK signaling pathway reduced cleavage of caspase-3. Co-administration of JNK activator and Ask1 inhibitor reversed this beneficial effect of Ask-1 inhibitor and resulted in increased expression of cleaved caspase-3 compared to the animals treated with JNK activator only ($p<0.05$, Fig. 6A).

TUNEL staining revealed that SBI increased the number of TUNEL positive cells in ipsilateral frontal lobe. Blockage of PAR-1 receptor decreased the amount of TUNEL positive cells in treated compared with untreated animals. Double staining NeuN/TUNEL revealed reduced TUNEL positive neurons in SCH97979-treated, compared to vehicle-treated SBI animals (Fig. 6B).

## Discussion

In this study, we demonstrated for the first time that SBI increased thrombin activity in the frontal lobe of operated animals. We also demonstrated that thrombin, via its PAR-1 receptor, contributed to the development of secondary brain injury after SBI. We showed that thrombin inhibition and PAR-1 antagonism reduced the SBI-induced brain edema and neurological deficits. Additionally, we showed that a PAR-1 receptor antagonist reduced SBI-induced apoptosis suppressing SBI-induced activation of PAR-1/Ask1/JNK pathway.

Thrombin, a key enzyme of the blood coagulation system, accumulates in the brain either by increased production by the injured brain or by increased permeability to thrombin induced by the breakdown of the BBB (Chen et al., 2010; Gong et al., 2008; Thevenet et al., 2009). Increased thrombin activity has previously been documented in animal models of brain injury (Gong et al., 2008; Hua et al., 2003; VanLandingham et al., 2008); however the effect of SBI on thrombin activity had not yet been evaluated. We measured thrombin activity in different sections of the brain in SBI animals as previously described by others (Hua et al., 2003). We observed a significant increase in

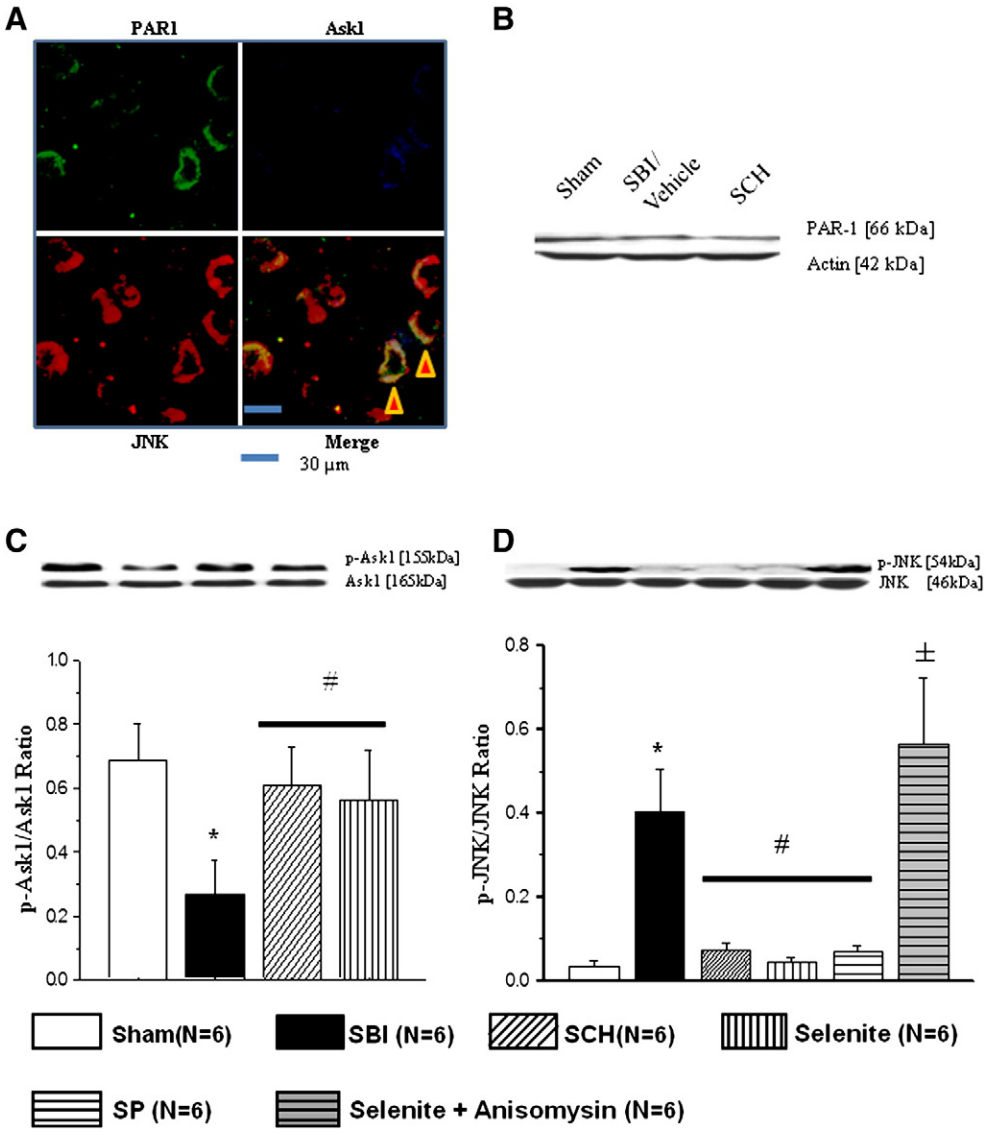


**Fig. 4.** SBI had no effect on PAR-1 expression, however it induced activation of PAR-1/Ask1/JNK pathway. (A) Triple staining against PAR-1/Ask1/JNK revealed that all three molecules are expressed in the same cell after SBI ( ). (B) Neither SBI nor SCH97979 (PAR-1 antagonist) showed effects on PAR-1 expression compared to sham animals. (C) While SBI increased Ask1 activation via decreased Ask phosphorylation, SCH97979 ameliorated SBI-induced Ask1 activation. Similarly selenite (Ask1 inhibitor) resulted in decreased Ask1 activation. (D) Concomitant with Ask1 activation SBI induced JNK activation evaluated by increased JNK phosphorylation. PAR-1, Ask1 or JNK inhibitor decreased SBI-induced JNK activation. Anisomysin (JNK activator), administrated simultaneously with selenite (Ask inhibitor), reversed the effect of Ask inhibition. (Western blot study N = 6 each group, *Significant vs. sham; #Significant vs. vehicle, ± Significant vs. selenite $p<0.05$ (ANOVA, Holm–Sidak test)).

thrombin activity in the frontal lobe of SBI animals evaluated at 24 h after SBI induction. We also detected this increase in thrombin activity correlated with the development of brain edema in SBI animals, suggesting that there was a relation between these two parameters.

While low concentrations of thrombin are protective (Wang et al., 2007b), high concentrations aggravate secondary brain damage (Hua et al., 2009; Lee et al., 1997; Ma et al., 2011; Shirakawa et al., 2010; Zhang et al., 2010). It has been shown that systemic administration of thrombin inhibitors ameliorates secondary brain injury and improves neurological outcomes in experimental models of brain injury (Jin et al., 2002; Morris et al., 2001; Ohyama et al., 2001). We tested the hypothesis in the current study that thrombin inhibition decreases secondary brain injury after SBI by using thrombin inhibitor, argatroban, a drug previously used in animal models of hemorrhagic stroke (Nagatsuna et al., 2005; Sugawara et al., 2009). In agreement with previous literatures, we found that thrombin inhibition significantly reduced SBI-induced brain edema and ameliorated neurological deficits.

Thrombin's effects may involve signaling through three receptors, PAR-1, -3 and -4, but are mainly mediated by PAR-1. Pathological conditions may change PAR-1 expression in the brain (Wu et al., 2010; Zhang et al., 2011; Zhou et al., 2011). Our study evaluated PAR-1 expression in brain ipsilateral frontal lobe of SBI animals; no change in PAR-1 expression was observed. Effect of PAR-1 activation on the secondary brain injury is a controversy discussed in the literature. It has been demonstrated that PAR-1 activation via systemic anti-coagulant and anti-inflammatory factor APC (activated protein C) is protective (Cheng et al., 2003, 2006; Guo et al., 2004; Liu et al., 2004). Yet, PAR-1 activation contributes to secondary brain injury (Junge et al., 2003; Olson et al., 2004). PAR-1 deficiency and pharmacological inactivation of PAR-1 have been shown to decrease brain injury and neurological deficits (Hamill et al., 2007; Junge et al., 2003; Olson et al.,

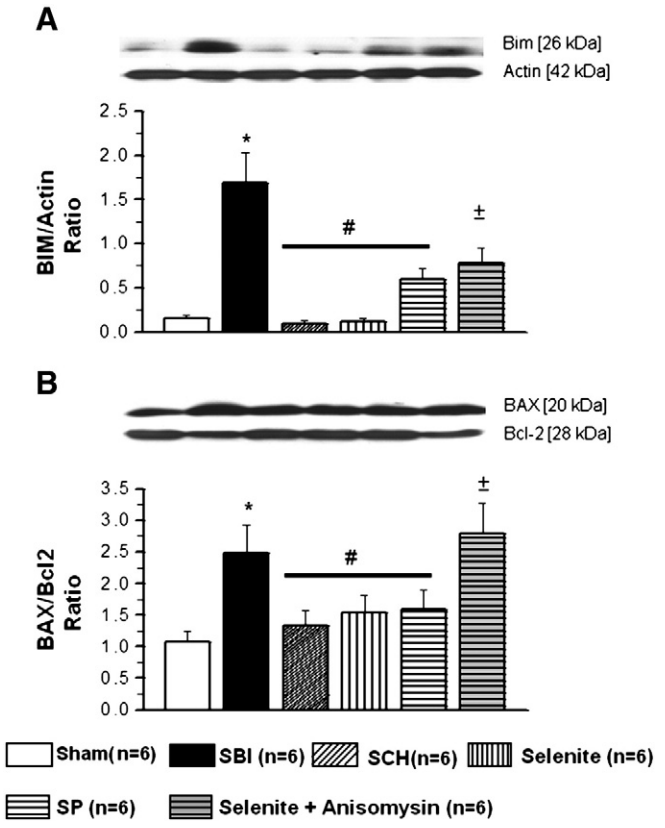


**Fig. 5.** Inhibition of PAR-1/Ask1/JNK pathway decreased production of apoptotic markers after SBI. SBI increased production of pro-apoptotic BAX (A) and ratio pro-(BAX) to anti-apoptotic (Bcl2) (B) proteins. PAR-1, Ask1 or JNK inhibitor decreased SBI induced apoptosis. Anisomysin (JNK activator) administrated simultaneously with selenite (Ask1 inhibitor) reversed the anti-apoptotic effects of Ask1 inhibition. (N = 6 each group, *Significant vs. sham; #Significant vs. vehicle, ±Significant vs. selenite $p<0.05$ (ANOVA, Holm–Sidak test)).

2004). To examine whether PAR-1 activation contributes to brain injury after SBI, we applied a selective PAR-1 antagonist, SCH 79797 (Strande et al., 2007). Similar to thrombin inhibition, PAR-1 inhibition resulted in the reduction of SBI-induced brain edema and neurological deficits.

To examine the mechanism of PAR-1 inhibition in the SBI model, we evaluated whether PAR-1 inhibition acted on SBI-induced apoptosis, a secondary brain injury process that characterizes the SBI model as previously shown (Bravo et al., 2008; Matchett et al., 2006). We tested whether SBI leads to the activation of PAR-1/Ask1/JNK pathway followed by cleaving caspase, a key mediator of apoptosis (Thornberry, 1998) (Supplemental Fig. 1). Ask1, a member of the mitogen-activated protein kinase family, is activated in the initial phase of apoptosis by various stress signals including thrombin via PAR-1 (Yu et al., 2009). Ask1 activity is regulated by the regulatory protein, 14-3-3 protein. Phosphorylation of Ask1 on at Ser967 is critical for Ask1 association with 14-3-3 protein. 14-3-3 antagonizes the pro-apoptotic activity of Ask1, suppressing cell death (Zhang et al., 1999). We demonstrated that SBI significantly decreased the ratio of phosphorylated to non-phosphorylated Ask1 (pAks1/Ask1), thus inducing apoptosis, which is consistent with previous publications (Bravo et al., 2008; Matchett et al., 2006). We also demonstrated that PAR-1 inhibition abolished SBI-induced activation of Ask1. Selenite, an Ask1 inhibitor known to inactivate Ask1 by phosphorylation, has been previously demonstrated to have anti-apoptotic properties (Wang et al., 2007a; Whanger and Butler, 1988; Yeo and Kang, 2007). In accordance with the previous literature, we observed that selenite increased the pAsk1/Ask1 ratio, indicating that selenite may have an anti-apoptotic effect in SBI model as well.

JNK, a member of the MAPK family, is activated by phosphorylation following stress stimuli. Ask1 activation causes JNK activation following brain injury (Kim et al., 2012; Xu et al., 2008). In our study we found that JNK activation follows SBI-induced Ask-1 activation. We were able to demonstrate that PAR-1 inhibition significantly decreased SBI-induced JNK activation. In addition to western blot investigation, we performed a triple immune-staining against PAR-1/Ask1/JNK. We have shown that PAR-1, Ask1 and JNK can be co-expressed in the same cells, suggesting that PAR-1/Ask1/JNK pathway is important for apoptotic reaction after SBI.

To strengthen our hypotheses that SBI induces JNK activation via Ask1 pathway, we tested the effect of Ask1 inhibition (selenite) on JNK phosphorylation. Ask1 inhibition significantly decreased SBI-induced JNK phosphorylation. Moreover, JNK antagonist Sp600125 (Liu et al., 2011) significantly reduced SBI-induced JNK phosphorylation. Finally, we examined the effects of simultaneously inhibiting Ask1 and activating JNK by using JNK activator anisomycin as previously described (Hong et al., 2007). We demonstrated that an anisomycin reversed selenite-induced inactivation of JNK.

After establishing that SBI activates PAR-1/Ask1/JNK pathway, we investigated whether SBI shifts production of anti- and pro-apoptotic proteins toward a pro-apoptotic balance, leading to cleavage and subsequent activation of caspase-3. Upon phosphorylation, JNK, in turn, phosphorylates a number of transcription factors and regulates production of pro and anti-apoptotic factors such as BIM, Bcl-2 and BAX (Gao et al., 2005; Wang and Youle, 2009). A pro-apoptotic protein Bim, which is a BH3-only Bcl-2 family member, functions upstream of BAX (Putcha et al., 2001). BAX and Bcl-2 are essential regulatory factors, acting as positive and negative regulatory factors, respectively. Their ratio can predict an apoptotic cell death (Cory and Adams, 2002). Upon activation, BAX penetrates the mitochondrial outer membrane and elicits a release of cytochrome c from the intermembrane space to the cytosol, activating caspase-9, which in turn activates caspase-3, a key factor of apoptosis (Niquet and Wasterlain, 2004; Troy et al., 2011). In our study we found that SBI caused significant upregulation of BIM production and shifted the ratio of pro-apoptotic (BAX)/anti-apoptotic (Bcl-2) proteins towards pro-apoptotic, which resulted in increased cleavage of caspase-3. We confirmed this finding by TUNEL staining. There are more TUNEL positive cells in the frontal lobe of SBI compared to the sham-operated animals. We found that PAR-1 inhibition reversed SBI-induced upregulation of pro-apoptotic proteins and decreased caspase-3 cleavage resulting in a decreased amount of TUNEL positive cells in the frontal lobe of treated compared to that of un-treated animals. In addition, inhibition of PAR-1 downstream molecule such as Ask1 and JNK led to the amelioration of SBI-induced apoptosis evaluated by caspase-3 cleavage. Finally, we found that the simultaneous inhibition of Ask1 and activation of JNK reversed the anti-apoptotic effect of Ask1 inhibition by upregulation of pro- vs. anti-apoptotic protein production and increased cleavage of caspase-3.

## Conclusion

In this study we demonstrated that SBI increased thrombin activity. Thrombin may activate the PAR-1 receptor and induce apoptosis via the Ask1/JNK pathway. Argatroban (thrombin inhibitor) and SCH79797 (PAR-1 inhibitor) protected against secondary brain injury after SBI, by decreasing brain edema and apoptosis mediated by inhibition of PAR-1/Ask1/JNK pathway.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.nbd.2012.09.004.

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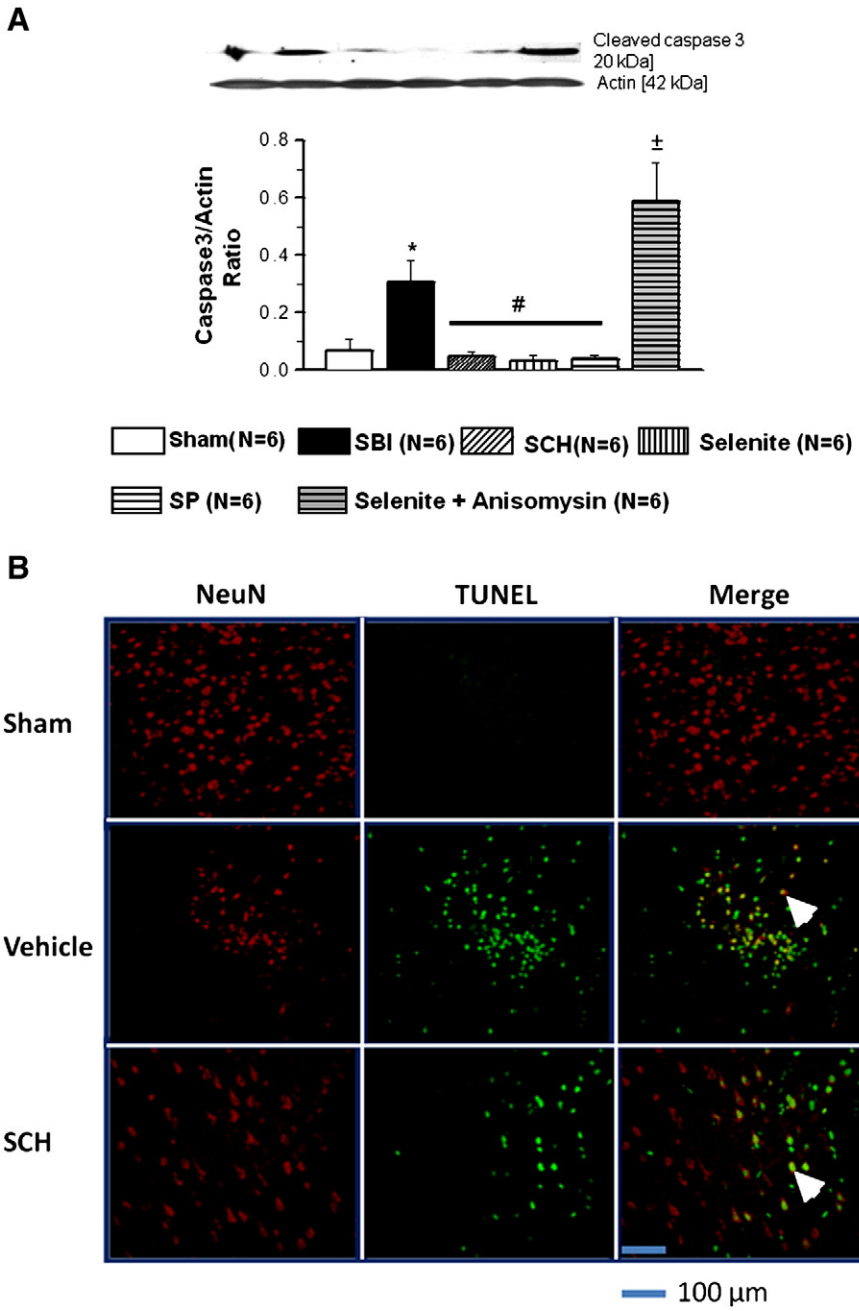


**Fig. 6.** Inhibition of PAR-1/Ask1/JNK pathway decreased cleavage of caspase 3 and amount of TUNEL positive cells after SBI. SBI increased cleavage of caspase-3 (A) and amount of TUNEL positive cells (B). SCH79797 (PAR-1 inhibitor) decreased SBI-induced cleavage of caspase-3 (A) and diminished the number of TUNEL positive cells (B). In addition inhibition of Ask1 or JNK decreased SBI-induced caspase-3 cleavage. Anisomysin (JNK activator), administrated simultaneously with selenite (Ask1 inhibitor) reversed the effect of Ask1 inhibition on caspase-3 cleavage. (Western blot study N = 6 each group, *Significant vs. sham; #Significant vs. vehicle, ± Significant vs. selenite $p<0.05$ (ANOVA, Holm–Sidak test)).

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