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RESEARCH PAPER

Combined argatroban and anti-oxidative agents prevents increased vascular contractility to thrombin and other ligands after subarachnoid haemorrhage

Katsuharu Kameda[1,2], Yuichiro Kikkawa[1,2], Mayumi Hirano[1], Satoshi Matsuo[2], Tomio Sasaki[2] and Katsuya Hirano[1]

[1]*Division of Molecular Cardiology, Research Institute of Angiocardiology, Graduate School of Medical Sciences, Kyushu University, Maidashi, Higashi-ku, Fukuoka, Japan, and* [2]*Department of Neurosurgery, Graduate School of Medical Sciences, Kyushu University, Maidashi, Higashi-ku, Fukuoka, Japan*

Correspondence
Katsuya Hirano, Division of Molecular Cardiology, Research Institute of Angiocardiology, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan. E-mail; khirano@molcar.med.kyushu-u.ac.jp

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BACKGROUND AND PURPOSE
Increased vascular contractility plays a fundamental role in cerebral vasospasm in subarachnoid haemorrhage (SAH). We investigated the role of thrombin and its receptor, proteinase-activated receptor 1 (PAR1), and other G protein-coupled receptors in the increased contractility, and examined the preventive effects of the thrombin inhibitor, argatroban, and anti-oxidative agents, vitamin C and tempol.

EXPERIMENTAL APPROACH
A rabbit model of SAH was utilized. Contractile responses of the isolated basilar artery and the level of oxidative stress of brain tissues were evaluated.

KEY RESULTS
Contractile responses to thrombin and PAR1-activating peptide (PAR1-AP) were enhanced and prolonged after SAH. The thrombin-induced contraction persisted even after terminating thrombin stimulation. When sequentially stimulated with PAR1-AP, the second response was maintained in SAH, while it was substantially attenuated in the control. Only a combination of argatroban with vitamin C or tempol prevented both the enhancement and prolongation of the contractile response to PAR1-AP and restored the reversibility of the thrombin-induced contraction. The responses to angiotensin II, vasopressin and $PGF_{2\alpha}$ were enhanced and prolonged after SAH to varying degrees, and responded differently to the treatment. The response to vasopressin exhibited a similar phenomenon to that seen with PAR1-AP. Oxidative stress was increased in SAH, and normalized by the treatment with argatroban, vitamin C or their combination.

CONCLUSIONS AND IMPLICATIONS
Increased vascular reactivity to agonists in SAH was attributable to the enhancement and prolongation of the contractile response. A combination of argatroban and anti-oxidative agents was required to prevent both the enhancement and prolongation of the contractile response.

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Abbreviations
p-APMSF, *p*-amidinophenyl methansulphonyl fluoride; PAR1, proteinase-activated receptor 1; PAR1-AP, PAR1-activating peptide; PSS, physiological salt solution; ROS, reactive oxygen species; SAH, subarachnoid haemorrhage; TBARS, thiobarbituric acid reactive substances

Introduction

Cerebral vasospasms determine the prognosis of patients with subarachnoid haemorrhage (SAH) (Kassell *et al.*, 1985). The prevention and treatment of cerebral vasospasms therefore has a crucial role in the management of SAH patients. Elucidating the mechanisms of cerebral vasospasm will greatly facilitate the development of effective therapeutic strategies. It has been suggested that increased production of spasmogens, impaired endothelial vasodilator function and increased vascular contractility are involved in the development of cerebral vasospasms (Kai *et al.*, 2008; Sasaki and Kassell, 1990). However, the precise mechanism of cerebral vasospasm development is still unknown.

Thrombin is suggested to play an important role as a spasmogen (White and Robertson, 1985). Thrombin induces smooth muscle contraction, proliferation and the production of reactive oxygen species (ROS), primarily via proteinase-activated receptor 1 (PAR1) (Coughlin, 2000; Hollenberg, 2005; Hirano, 2007; Alexander *et al.*, 2011). We have recently demonstrated that the expression of PAR1 is up-regulated and the contractile response to thrombin is enhanced in a rabbit double SAH model (Maeda *et al.*, 2007). We have also demonstrated that intrathecal treatment with heparin or a PAR1 antagonist prevents the up-regulation of PAR1 and the enhanced contractile response to thrombin (Kai *et al.*, 2007; Maeda *et al.*, 2007). It is therefore suggested that the activation of PAR1 by heparin-sensitive proteinases leads to the up-regulation of PAR1 and the enhancement of contractile response during SAH. It is conceivable that thrombin is responsible for these events. However, its role still remains to be established. The present study therefore utilized argatroban, a more specific inhibitor of thrombin (Kikumoto *et al.*, 1984), and investigated the involvement of thrombin in the enhancement of contractile responses during SAH.

In addition to the enhancement of the contractile response to thrombin, feedback regulation of the PAR1 activity was found to be impaired during SAH (Kikkawa *et al.*, 2010). As a result, the thrombin-induced contraction was not only enhanced, but also prolonged and irreversibly persisted even after terminating the thrombin stimulation in SAH (Kikkawa *et al.*, 2010). However, the mechanism of the impaired feedback regulation of PAR1 remains to be investigated. In the present study, argatroban was found to prevent the enhancement, but not the prolongation, of the contractile response to PAR1 agonists in a rabbit double SAH model. Some additional treatments therefore appear to be required to prevent both the enhancement and prolongation of the contraction.

Oxidative stress is suggested to contribute to the pathogenesis of cerebral vasospasm (Sasaki *et al.*, 1981). Oxyhaemoglobin liberated from erythrocytes is a major source of ROS such as superoxide anion (Misra and Fridovich, 1972; Kellogg and Fridovich, 1977). The mechanisms by which oxidative stress cause cerebral vasospasm are multifaceted. They include the reduction of NO bioavailability, damaging of perivascular nerves, release of arachidonic acid metabolites and lipid peroxidation (Kolias *et al.*, 2009). A hydroperoxide of arachidonic acid has been shown to induce a sustained contraction in the canine basilar artery *in vivo* (Sasaki *et al.*, 1981). Furthermore, the intrathecal administration of anti-oxidative reagents has been reported to prevent cerebral vasospasm in experimental and human SAH (Asano *et al.*, 1984; Luo *et al.*, 1995). Vitamin C is a water-soluble anti-oxidative agent and scavenges a wide range of reactive oxygen and nitrogen species (Carr *et al.*, 2000). Tempol is a superoxide dismutase mimetic, and it also reduces the formation of hydroxyl radicals by oxidizing Fe^{2+} (Monti *et al.*, 1996). Accordingly, the effect of vitamin C and tempol on the impaired feedback regulation of PAR1 activity was examined in the present study.

Methods

Preparation of SAH model

All animal care and experimental procedures complied with the guidelines of the Ministry of Education, Culture, Sports, Science and Technology, Japan and were approved by the Animal Care and Use Committee, Kyushu University. The double SAH model was established as previously reported (Kai *et al.*, 2007). In brief, adult male Japanese white rabbits (2.5–3.0 kg) were anaesthetized with an i.m. injection of ketamine (40 $mg \cdot kg^{-1}$) and an i.v. injection of sodium pentobarbital (20 $mg \cdot kg^{-1}$). First, 1 mL of CSF was aspirated from the cisterna magna with the use of a 23-gauge butterfly needle percutaneously, and then 2.5 mL of non-heparin treated autologous blood obtained from the middle branch of the ear artery was immediately injected into cisterna magna (day 0). The animal was then kept in a prone position with the head tilting down at 30 degrees for 30 min. The second injection of autologous blood was similarly performed 2 days after the first injection (day 2). The control animals received injections of the same volume of normal physiological salt solution (PSS) instead of autologous blood (control). When the animals were treated with the thrombin inhibitor argatroban (0.1–10 $\mu g \cdot kg^{-1}$ per injection) or vitamin C (0.6 $mg \cdot kg^{-1}$ per injection), these compounds were injected into cisterna magna on day 0 and day 2 together with the autologous blood.

Evaluation of the clot formation on day 7

On day 7, the rabbits were killed by an overdose of pentobarbital sodium (100 $mg \cdot kg^{-1}$). The whole brain with the basilar artery was excised out *en bloc* and immediately photographed with a CCD camera (PowerShot G1, Canon, Tokyo, Japan). The photograph image was then analysed with ImageJ ver.1.4.3.67 (National Institute of Health, Bethesda, MD, USA). The area of the clot was expressed as a value normalized by the length of the basilar artery in the arbitrary unit (Figure 2A).

Tension measurement in the basilar artery

The basilar artery was excised out from the brain and ring preparations made as described previously (Kai *et al.*, 2007; Maeda *et al.*, 2007). In brief, after removal of the adventitial tissue, the artery was cut into ring preparations measuring 1 mm in width in a circular direction. The endothelium was mechanically removed by rubbing the luminal surface with human hair. The reasons for removing the endothelium were as follows: (1) our previous studies demonstrated that ACh

and thrombin induced an endothelium-dependent relaxation to a similar extent in the basilar artery isolated from both the control and SAH rabbits, thus suggesting that there was no significant change in the vasorelaxing function of the endothelial cells during SAH (Kai *et al.*, 2007; Maeda *et al.*, 2007). (2) There were concerns that the endothelium-dependent relaxant effect of thrombin (Maeda *et al.*, 2007) might affect the accurate evaluation of the contractile response of smooth muscle to thrombin. The arterial rings were mounted onto two tungsten wires in an organ bath containing 2 mL normal PSS (5.9 mM K^+). One of the wires was fixed, while the other was connected to a force transducer (U gauge; Minebea, Nagano, Japan). The preparations were equilibrated in normal PSS at 37°C for at least 60 min. During this equilibration period, the rings were stimulated a couple of times with 118 mM K^+ PSS for 5 min to confirm the reproducibility of contractile responses to 118 mM K^+ depolarization before starting the experimental protocols. The final response was used as a reference response. The level of tension obtained at 5 min during this contraction was assigned a value of 100%, while that obtained in normal PSS was assigned a value of 0% (unless otherwise specified), when expressing the data for tension as a percentage. The responses to thrombin and PAR1-AP were evaluated at the maximal level of the contraction, unless otherwise specified.

Measurement of thiobarbituric acid reactive substances (TBARS) in brain tissue

The lipid peroxidation of the brain tissue was quantified as an index of the oxidative stress in the subarachnoid space. After removal of the basilar artery from the brain, a block of the brain, measuring approximately 6 mm wide, 10 mm long and 2 mm deep, was excised out from the regions beneath the basilar artery. The block was immediately rinsed with PSS containing 10 U·mL^{-1} heparin, snap-frozen in liquid nitrogen, and then maintained at –80°C until use in the assay. The specimens were homogenized with 500 µL lysis buffer (50 mM HEPES, 150 mM NaCl, 0.5% Triton X-100) containing 0.05% of butylated hydroxytoluene to prevent further oxidation of lipid during sample processing and the thiobarbituric acid reaction. The homogenate was then centrifuged at 13 000× *g* for 20 min. The clear supernatant was saved for analysis. The protein concentrations of the samples were determined with a Coomassie protein assay (Thermo Scientific, Rockford, IL, USA), and then the samples were diluted with lysis buffer to adjust the protein concentration to 60 µg in 50 µL. The samples were mixed with 50 µL of 8% SDS and 1250 µL of thiobarbituric acid reagent, which was adjusted to pH 3.5 (Ohkawa *et al.*, 1979), and then the mixture was boiled for 60 min. The samples were then extracted with 100% buthanol. After the centrifugation, the buthanol fractions were subjected to fluorometry with wavelengths of the 535 nm (excitation) and the 550 nm (emission), using a fluorescence spectrophotometer 650-40 (Hitachi, Tokyo, Japan). Malondialdehyde was used in place of the tissue specimens to obtain the standard curve for the fluorescence intensity (Figure 8).

Drugs and solution

The composition of the normal PSS (in mM) was NaCl 123, KCl 4.7, $NaHCO_3$ 15.5, KH_2PO_4 1.2, $MgCl_2$ 1.2, $CaCl_2$ 1.25 and D-glucose 11.5. The 118 mM K^+ PSS was prepared by substituting NaCl for an equimolar KCl. All solutions were aerated with a mixture of 5% CO_2 and 95% O_2 (pH 7.4, 37°C) during the experiment. Thrombin (bovine plasma; 1880 IU·mg^{-1} protein), human angiotensin II, [Arg^8]-vasopressin, $PGF_{2\alpha}$, thiobarbituric acid, 4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl (tempol) and *p*-amidinophenyl methansulphonyl fluoride (*p*-APMSF) were purchased from Sigma (St. Louis, MO, USA). PAR1-AP, TFLLR-NH_2, was purchased from Bachem (Bubendorf, Switzerland). Malondialdehyde was purchased from Cayman Chemical Co, Ltd (Ann Arbor, MI, USA). Argatroban, ((2R,4R)–4–methyl–1-[N^{2-}((RS)–3–methyl-1,2,3,4–tetrahydro–8-quinolinesulphonyl)-L-arginyl]-2-piperidinecarboxylic acid hydrate, $C_{23}H_{36}N_6O_5S \cdot H_2O$; MW = 526.65) was kindly provided by Mitsubishi Tanabe Pharma (Tokyo, Japan).

Data analysis

The data are expressed as the means ± SEM. One strip obtained from one animal was used for each experiment, and, therefore, the number of experiments indicates the number of animals. The StatView®-5.0 software program was used for the statistical data analyses. Student's *t*-test or a Factorial ANOVA with the Tukey–Kramer multiple comparison test or Student–Newman–Keuls test (Figure 6B) as a *post hoc* test were used to determine the statistical significance of the differences. Differences were considered significant at $P < 0.05$.

Results

Enhancement and prolongation of the contractile response to PAR1 agonists in the basilar artery isolated from SAH rabbit

The absolute values of the developed tension induced by 118 mM K^+ depolarization in the isolated basilar artery were not significantly different between the control and SAH (Figure 1B). Thus, the response to 118 mM K^+ depolarization was used as a reference response and the levels of tension induced by PAR1 agonists were expressed as a percentage of this response; those obtained at rest and those obtained with 118 mM K^+ depolarization were assigned to be 0% and 100%, respectively.

Thrombin (1 U·mL^{-1}) induced a small and transient contraction in basilar artery isolated from the control rabbit, while it induced an enhanced and prolonged contractile response in SAH (Figures 1A and 5A). The concentration-dependent effects of thrombin were then evaluated by administering thrombin in a cumulative manner to each preparation (Figure 1C). Thrombin induced only a small amount of contraction even at 3 U·mL^{-1} in the control (Figure 1C). In contrast, thrombin induced an enhanced contraction at lower concentrations in SAH (Figure 1C).

PAR1-AP (100 µM) also induced a transient contraction in the control artery, while it induced enhanced and prolonged contraction in SAH (Figure 1A). Some differences in the initial response between thrombin and PAR1-AP were noted. The differences may be related to the activation of the different sets of G proteins and downstream signalling pathways or the

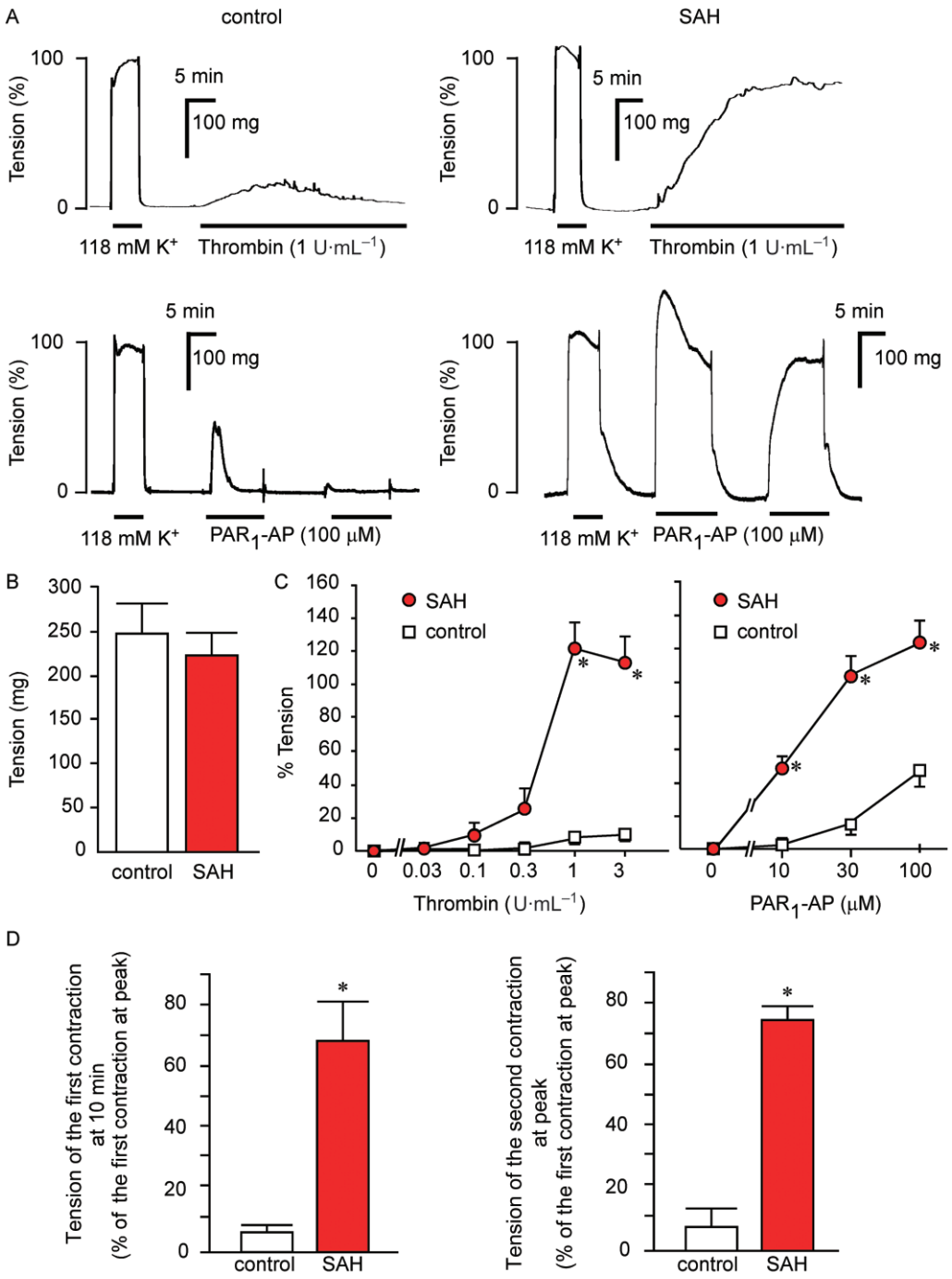

Figure 1

Enhancement and prolongation of the contractile response to thrombin and PAR1-AP in the basilar artery after SAH. (A) Representative recordings of the contractile responses to 118 mM K^+ depolarization, 1 U·mL^{-1} thrombin and 100 μM PAR1-AP in the basilar artery obtained from the control or SAH rabbits. The levels of tension obtained at rest and during 118 mM K^+ depolarization were assigned values of 0% and 100% respectively. (B) Summary (n = 8–9) of the absolute values of developed tension induced by 118 mM K^+ depolarization. (C) A summary of the concentration–response curves for the contractions induced by cumulative applications of thrombin (n = 4 for control, n = 7 for SAH) and PAR1-AP (n = 4 for control, n = 6 for SAH) in the basilar artery obtained from the control and SAH animals. (D) Summaries of the levels of tension induced by the first stimulation with 100 μM PAR1-AP at 10 min and the level of the peak tension development induced by the second stimulation with PAR1-AP in the basilar artery obtained from the control (n = 6) and SAH animals (n = 6). The data were expressed as a percentage of the peak level of the first response. The experimental protocol is shown in (A). The data are expressed as the mean ± SEM. *P < 0.05 versus control.

differences in the mode of activation or the kinetics of drug diffusion (Blackhart *et al.*, 2000; McLaughlin *et al.*, 2005). The concentration–response curves for the contractile effect of PAR1-AP were also obtained by cumulative applications of PAR1-AP and the contractile response to PAR1-AP was enhanced in SAH (Figure 1C). The conversion of the transient contraction in the control to a sustained contraction in SAH was depicted by the quantitative evaluation of the level of tension 10 min after the stimulation in relation to the peak level (Figure 1D). In the control, the level of tension at 10 min decreased to 7.15 ± 0.77% (n = 6) of the peak level (Figure 1D). In SAH, the level of tension at 10 min stayed at 68.8 ± 12.2% (n = 6) of the peak level (Figure 1D). Thereafter, the arterial preparations were challenged with a second stimulation. In this protocol, the arteries were consecutively stimulated with 100 μM PAR1-AP for 10 min each at 10 min intervals (Figure 1A). In the control, the second stimulation induced 8.37 ± 3.91% (n = 6) of the first contraction (Figure 1D). This phenomenon is consistent with tachyphylaxis. In SAH, the second stimulation induced a contraction to the level of 74.4 ± 4.3% (n = 6) of that obtained with the first stimulation (Figure 1D). This conversion of the transient contraction to a prolonged contraction, and the impaired tachyphylactic attenuation of the contractile response suggests the negative feedback regulation of the contractile response is impaired in SAH.

Effect of intrathecal treatment with argatroban and vitamin C on clot formation in the rabbit SAH model

Argatroban is an inhibitor of the proteolytic activity of thrombin. It may affect the clot formation in SAH model. The clot formation over the basilar artery was therefore examined at the time of death. The area covered by the clot, recognized as a high density area by an image analysis programme ImageJ (Figure 2A, converted image), was determined as an indication of the amount of clot, as described in Methods. A significant amount of clot was observed in SAH in comparison to the control (Figure 2A and B). The intrathecal treatment with argatroban had no significant effect on the clot formation in SAH, up to a dose of 1 μg·kg^{-1} per injection (Figure 2B). However, the treatment with 10 μg·kg^{-1} argatroban per injection substantially suppressed the clot formation (Figure 2B). Vitamin C (0.6 mg·kg^{-1} per injection), either alone or in combination with argatroban (1 μg·kg^{-1} per injection), had no significant effect on clot formation (Figure 2B). Hence, the dosage mainly used in the following experiments, that is, 1 μg·kg^{-1} argatroban per injection and 0.6 mg·kg^{-1} vitamin C per injection, had no effect on clot formation.

Effects of intrathecal treatment with argatroban and vitamin C on the enhanced contractile response to PAR1 agonists in the isolated basilar artery of SAH

Intrathecal treatment with argatroban or vitamin C, either alone or in combination, had no significant effect on the tension developed in response to 118 mM K^+ at any dosage (Figure 3A). However, intrathecal treatment with argatroban dose-dependently inhibited the contractile response to thrombin (Figure 3B). The inhibitory effect of argatroban

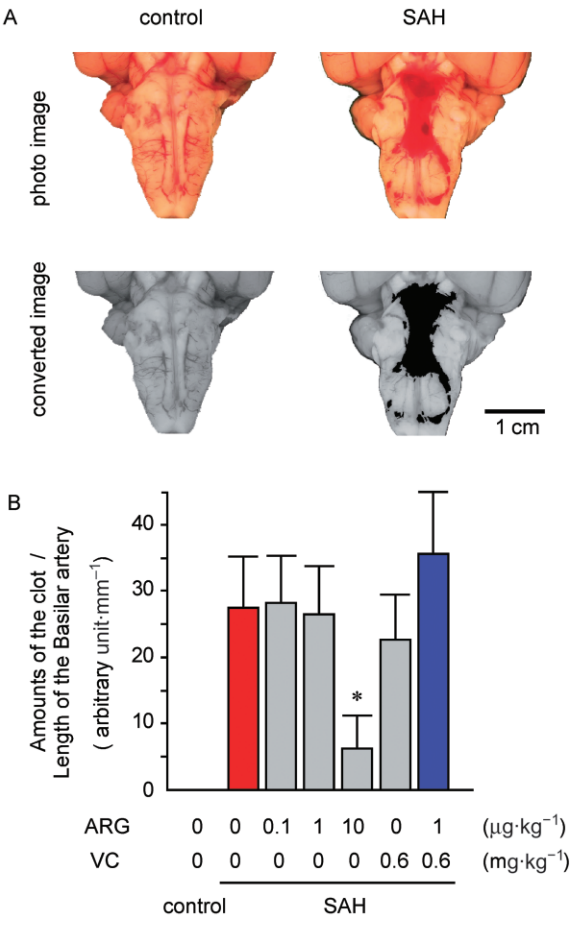

Figure 2

Effect of intrathecal treatment with argatroban and vitamin C on clot formation. (A) Representative photo images of the ventral surface of the brain excised on day 7, and the converted images showing the clot-covered area as a dark region. (B) Summary of the estimated amount of clot in the control and SAH with or without treatment with either argatroban (ARG) or vitamin C (VC) at the indicated dosages per injection. The data are expressed as the mean ± SEM (n = 5–8). *P < 0.05 versus SAH with no treatment.

reached a maximum with the dosage of 1 μg·kg^{-1} per injection (Figure 3B). Further increasing the dosage of argatroban to 10 μg·kg^{-1} per injection caused no additional inhibition (Figure 3B), although this dosage significantly suppressed clot formation (Figure 2B). Moreover, the addition of vitamin C 0.6 mg·kg^{-1} per injection also caused no additional effect to that obtained with 1 μg·kg^{-1} argatroban per injection alone (Figure 3B). Vitamin C (0.6 mg·kg^{-1} per injection) alone had no significant effect on the contractile response to thrombin (Figure 3B).

The contractile response to PAR1-AP was also significantly inhibited by the treatment with argatroban 1 μg·kg^{-1} per injection, while the maximal response to 100 μM PAR1-AP remained unaffected (Figure 3C). In the case of PAR1-AP, addition of vitamin C 0.6 mg·kg^{-1} per injection to this dosage of argatroban caused further inhibition of the contractile response to PAR1-AP (Figure 3C). However, the response to 100 μM PAR1-AP was resistant to this combination of arga-

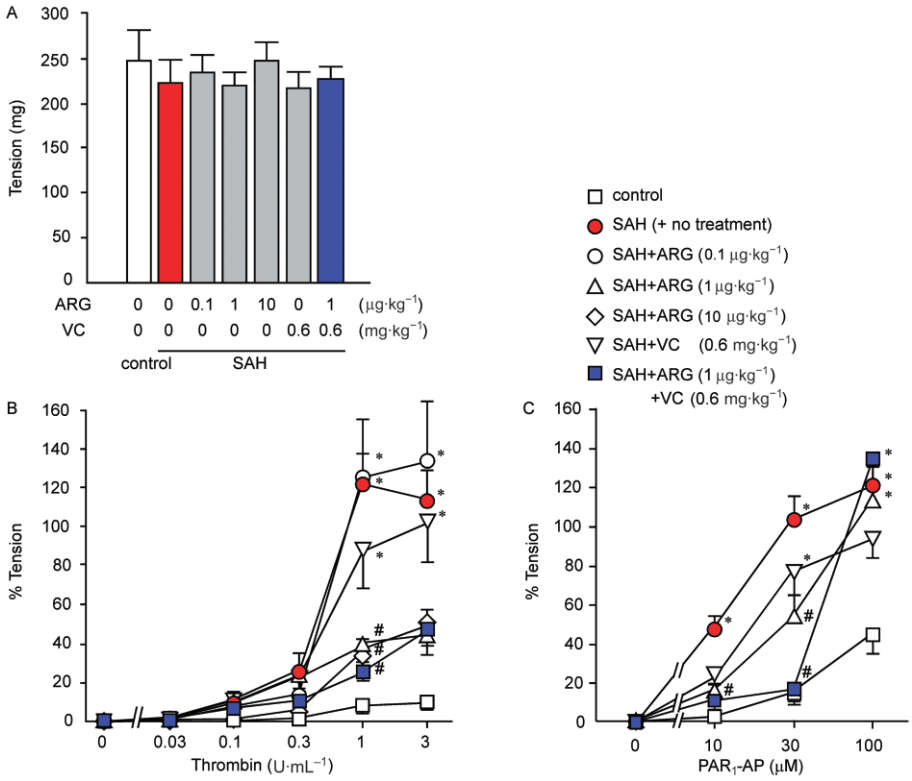

Figure 3

The effects of intrathecal treatment with argatroban and vitamin C on the contractions induced by 118 mM K^+ depolarization and PAR1 agonists. A summary (n = 8–9) of the developed tension induced by 118 mM K^+ depolarization (A) and the concentration–response curves for the contractions induced by the cumulative applications of thrombin (B; n = 4–7) and PAR1-AP (C; n = 4–6) in the basilar arteries obtained from the control and SAH animals with or without treatment with argatroban (ARG), vitamin C (VC) or the combination of ARG and VC (ARG + VC) at the indicated dosages per injection. The data for the control and SAH in (A) are the same as in Figure 1B. The data for the control and SAH in (B) and (C) are the same as in Figure 1C. The data are expressed as the mean ± SEM. *P < 0.05 versus control; #P < 0.05 versus SAH.

troban and vitamin C (Figure 3C). Vitamin C alone at 0.6 mg·kg^{-1} per injection had no significant effect on the contractile response to PAR1-AP (Figure 3C). As a result, 1 μg·kg^{-1} argatroban per injection was used as an optimal dosage to obtain the maximal effect in the present study. The optimal dosage of vitamin C was determined in the following experiments shown in Figure 4.

Effects of argatroban, vitamin C and tempol on the contractile responses to repetitive stimulations with PAR1-AP in the isolated basilar artery

Intrathecal treatment with argatroban 1 μg·kg^{-1} per injection had no significant effect on either the prolonged duration of the PAR1-AP-induced contraction (Figure 4A and B) or the maintained response to the second stimulation (Figure 4A and C). This dosage of argatroban was optimal for preventing the enhancement of the contractile response to PAR1 agonists (Figure 3). Accordingly, some intervention in addition to the thrombin inhibitor was required to normalize the vascular reactivity. Therefore, the effect of the addition of various dosages of vitamin C (3, 0.6 and 0.12 mg·kg^{-1}) to this dosage of argatroban was investigated. As a result, 0.6 mg·kg^{-1} vitamin C per injection in combination with 1 μg·kg^{-1} argatroban per injection induced a near complete inhibition of both the prolongation of the PAR1-AP-induced contraction and the maintenance of the responsiveness to the second stimulation in SAH (Figure 4A–C). Treatment with vitamin C 0.6 mg·kg^{-1} per injection alone significantly but partly reduced the sustained phase of the PAR1-AP-induced contraction (Figure 4B), while it had no significant effect on the maintained response to the second stimulation (Figure 4C).

Furthermore, the combination of 1 μg·kg^{-1} argatroban and 120 μg·kg^{-1} tempol per injection caused a near complete inhibition of both the prolongation of the PAR1-AP-induced contraction and the maintenance of responsiveness to the second stimulation (Figure 4). However, the combination of argatroban and tempol had no significant effect on the maximal response to 100 μM PAR1-AP (data not shown). These observations were similar to those seen with the combination of argatroban and vitamin C (Figures 3C, 4B and 4C).

The administration of argatroban and vitamin C only on day 2 also prevented the prolongation of the PAR1-AP-induced contraction and the maintenance of the responsiveness to the second stimulation in SAH (Figure 4). The delayed treatment also prevented the enhancement of the contractile

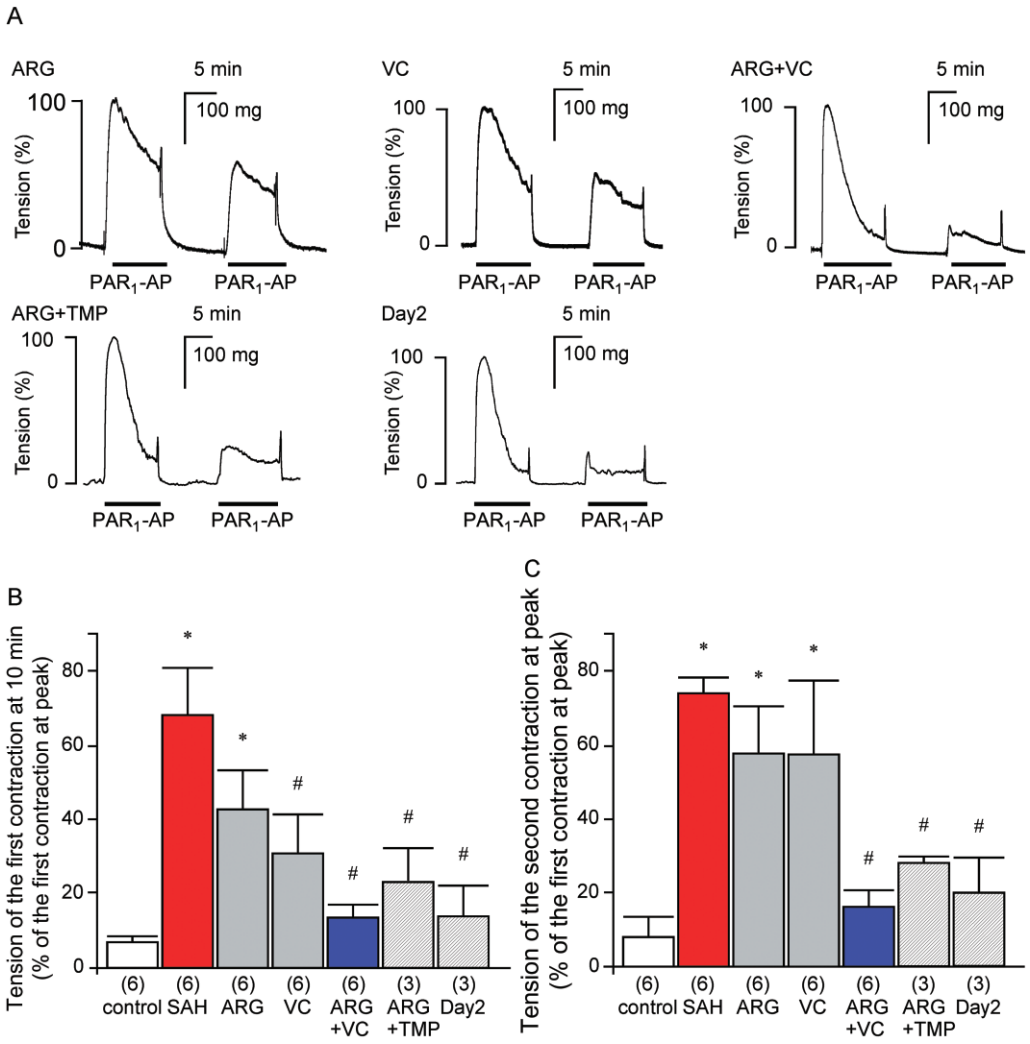

Figure 4

The effects of intrathecal treatment with argatroban and vitamin C on the contractile responses during repetitive stimulations with PAR1-AP. (A) A representative recording of the contractile responses induced by repetitive stimulation with 100 μM PAR1-AP in the basilar artery obtained from SAH animals treated with 1 μg argatroban·kg^{-1} per injection (ARG), 0.6 mg vitamin C·kg^{-1} per injection (VC), the combination of ARG and VC (ARG + VC), the combination of ARG and 120 μg tempol·kg^{-1} per injection (ARG + TMP) and the combination of ARG and VC only on day 2 (Day2). The strips were stimulated twice with PAR1-AP for 10 min at 10 min intervals. Refer to Figure 1A for the representative traces for the control and SAH. (B and C) Summaries of the effects of ARG, VC, TMP and the delayed treatment (Day2) on the level of the first response at 10 min (B) and the level of the peak response to the second stimulation (C), expressed as a percentage of the peak level of the first response. The data for the control and SAH were the same as shown in Figure 1D. The data are expressed as the mean ± SEM of the experimental numbers indicated in parentheses. *P < 0.05 versus control; #P < 0.05 versus SAH.

response to thrombin (data not shown). These preventive effects were comparable to those seen after the treatment on both day 0 and day 2.

Effects of intrathecal treatment with argatroban, vitamin C and tempol on the thrombin-induced irreversible contraction in the isolated basilar artery in SAH

Thrombin activation of PAR1 is an irreversible process because it depends on the proteolytic cleavage of the extracellular region (Coughlin, 2000). The negative feedback regulation therefore plays an important role in terminating the signalling activity of the proteolytically activated PAR1 (Trejo, 2003). Otherwise, thrombin elicits persistent signalling (Booden *et al.*, 2004). In fact, in SAH, the thrombin-induced contraction irreversibly persisted even after removing thrombin from the bathing solution and also adding a protease inhibitor, *p*-APMSF, at 10 μM (Figure 5A). Treatment with 1 μg kg^{-1}argatroban reduced the maximal level of the thrombin-induced contraction (Figure 5B), which was consistent with the observation shown in Figure 3B. Terminating the thrombin stimulation during the sustained phase of the contraction induced an approximately 50%

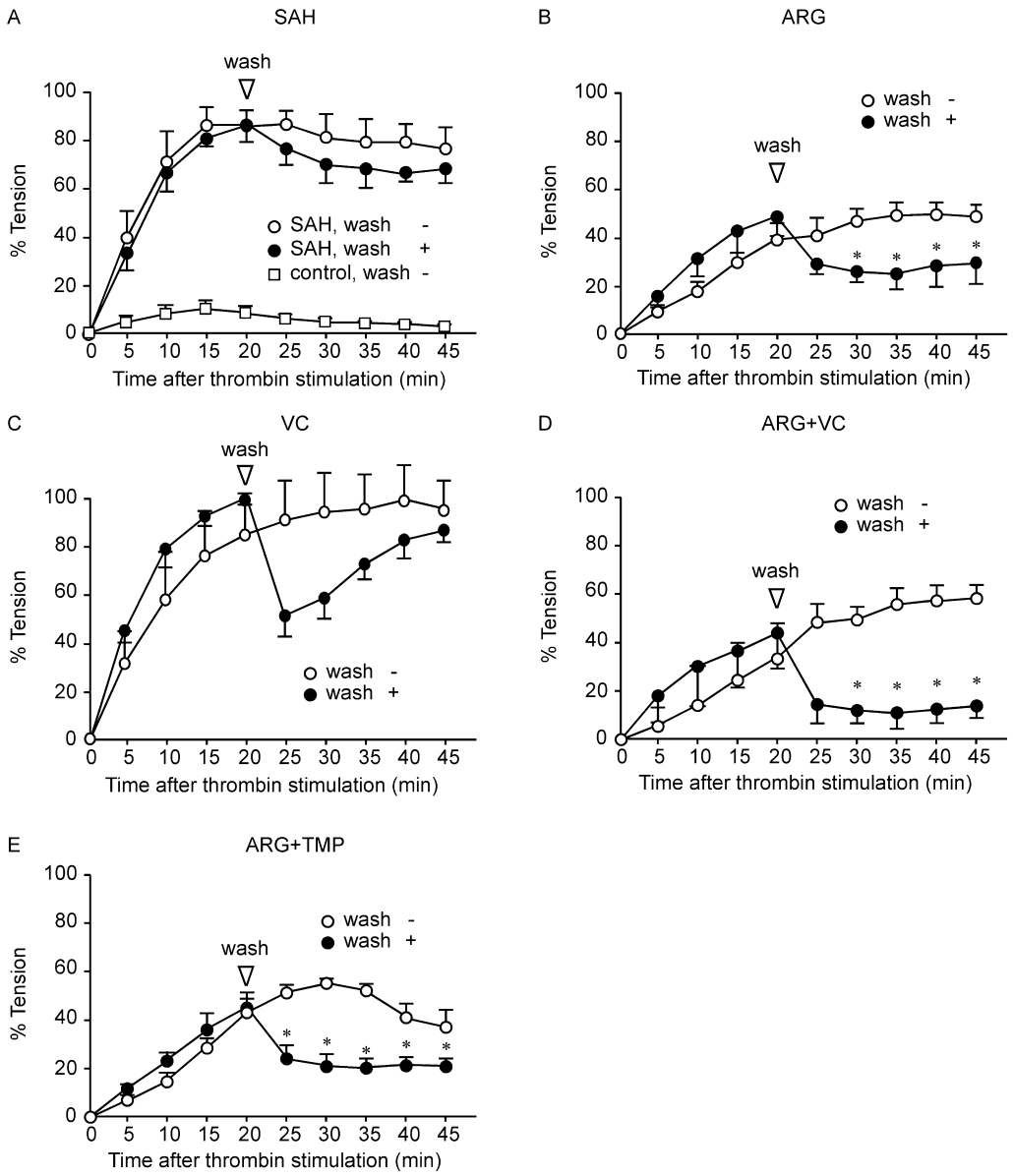

Figure 5

Effect of intrathecal treatment with argatroban and vitamin C on the time course of the contractile response to thrombin. Summaries of the time courses of the contractile responses initiated by 1 U·mL^{-1} thrombin, either with (wash+) or without (wash–) subsequent thrombin wash-out at 20 min, in the basilar artery obtained from SAH animals without (SAH; n = 4 for wash+, n = 5 for wash–) or with treatment with 1 μg argatroban·kg^{-1} per injection (ARG; n = 4 for wash+, n = 6 for wash–), 0.6 mg vitamin C·kg^{-1} per injection (VC; n = 6 for wash+, n = 7 for wash–), the combination of ARG and VC (ARG + VC; n = 5 for wash+, n = 7 for wash–) and the combination of ARG with 120 μg tempol·kg^{-1} per injection (ARG + TMP; n = 3). When thrombin was removed by changing the bathing buffer, the proteinase inhibitor p-APMSF was added to the bathing buffer at the final concentration of 10 μM to ensure the termination of the thrombin stimulation. Panel (A) also shows the time course of the thrombin-induced contraction without subsequent thrombin wash-out in the control basilar artery. The data are expressed as the mean ± SEM. *P < 0.05 versus wash–.

reduction of the tension (Figure 5B). However, the level of tension persisted at this reduced level, which was significantly higher than at the resting level (Figure 5B). Treatment with vitamin C had no effect on the maximal level of the thrombin-induced contraction (Figure 5C), which is consistent with the observation shown in Figure 3B. Termination of thrombin stimulation induced 50% reduction of tension (Figure 5C). However, the tension eventually increased to the level similar to that seen without termination of thrombin stimulation (Figure 5C). The combination of argatroban and vitamin C reduced the maximal level of the thrombin-induced contraction to a similar extent to that seen with argatroban alone (Figure 5B vs. D). However, the termination of thrombin stimulation induced a significantly greater

reduction of tension than that seen with either argatroban or vitamin C alone (Figure 5D vs. B and C). The level of tension decreased to the level closer to the resting level (Figure 5D). The combination of argatroban and tempol also reduced the maximal level of the thrombin-induced contraction to a similar extent as that seen with either argatroban alone or in the combination with vitamin C (Figure 5E). The termination of the thrombin also induced a significant reduction of tension. However, this reduction was slightly smaller than that seen with the combination of argatroban and vitamin C.

The effects of intrathecal treatment with argatroban and vitamin C on the contractile responses to angiotensin II, vasopressin and $PGF_{2\alpha}$

In order to determine whether or not the enhancement and prolongation of the contractile responses and the preventive effects of the treatment were unique to PAR1, the contractile responses to three different agonists, angiotensin II, vasopressin and $PGF_{2\alpha}$, were examined. The contractile effects of these agonists exhibited three different patterns with regard to their alteration during SAH and their response to the treatment (Figure 6).

Angiotensin II (1 μM) induced a small transient contraction in the control artery and the level of tension at 10 min was therefore 0% of the peak (Figure 6A and B). The second stimulation induced a residual contraction (Figure 6A and B). The contractile response was significantly enhanced after SAH (Figure 6A and C) and was slightly prolonged (Figure 6A and B). The response to the second stimulation was also slightly augmented (Figure 6C). Treatment with argatroban and vitamin C prevented the enhancement of the contractile response to angiotensin II and maintained it at the control level (Figure 6C). However, the treatment had no significant effect on the prolongation of the contraction and the maintenance of the responsiveness to the second stimulation (Figure 6B).

Vasopressin (10 nM) induced an initial transient contraction followed by a slightly sustained contraction in the control (Figure 6A and B). The second stimulation induced a contraction that was approximately 50% of the first contraction (Figure 6B). In SAH, the contractile response to vasopressin was enhanced (Figure 6C) and prolonged (Figure 6B). The responsiveness to the second stimulation was also increased (Figure 6B). Treatment with argatroban and vitamin C partially inhibited the enhancement of the contractile response to vasopressin (Figure 6C), the prolongation of the vasopressin-induced contraction and the maintenance of the responsiveness to the second stimulation (Figure 6B).

$PGF_{2\alpha}$ (1 μM) exerted no apparent contractile effect in the control artery (Figure 6A and C). In the SAH rabbits, a significant contractile response to $PGF_{2\alpha}$ was observed (Figure 6C). $PGF_{2\alpha}$ induced a sustained contraction, which reached a maximum at around 10 min (Figure 6A). The responsiveness to the second stimulation was also well maintained (Figure 6B). The treatment with argatroban and vitamin C had little effect on the contractile response to $PGF_{2\alpha}$ (Figure 6C), the sustained phase of the contraction or the maintenance of the responsiveness to the second stimulation (Figure 6B).

Ex vivo *effect of argatroban on thrombin-induced contraction in the basilar artery of SAH*

The effect of *ex vivo* treatment with argatroban on the thrombin-induced contraction was examined to evaluate the inhibitory effect of argatroban on the enzymatic activity of thrombin to activate PAR1 in basilar artery (Figure 7A and B). Thrombin was pretreated with argatroban, and then applied to the arteries. Argatroban concentration-dependently inhibited the contractile effect of thrombin, with an IC_{50} of 53 μM (Figure 7B). A substantial inhibition was observed with 300 μM argatroban (Figure 7B vs. Figure 1A). In contrast, 300 μM argatroban had no apparent effect on the contractile response to 118 mM K^+ PSS (Figure 7A).

Effect of intrathecal treatment with argatroban and vitamin C on the oxidative stress in the brain tissues

Oxidative stress plays a critical role in the development of vasospasm (Sano *et al.*, 1980; Sasaki *et al.*, 1981; Asano *et al.*, 1984). The level of oxidative stress in the brain tissues just beneath the basilar artery was evaluated with a TBARS assay. A linear relationship between the concentrations of malondialdehyde and the fluorescence intensity was obtained with 0–1 μM malondialdehyde (Figure 8A and B). An evaluation of the brain tissues was performed within this linear range. The level of oxidative stress in SAH was three times higher than that of the control brain (Figure 8C). Intrathecal treatment with argatroban, vitamin C and their combination significantly prevented the production of oxidative stress in SAH (Figure 8C).

Discussion

Increased vascular contractility plays a fundamental role in the pathogenesis of cerebral vasospasm in SAH (Kai *et al.*, 2008; Hirano and Hirano, 2010). The present study demonstrated that the contractile response to PAR1 agonists was not only enhanced but also prolonged in SAH. Furthermore, the tachyphylactic attenuation of the PAR1-AP-induced contraction was impaired and the thrombin-induced contraction irreversibly persisted in SAH. These observations are consistent with impaired receptor desensitization, as previously reported (Kikkawa *et al.*, 2010). As PAR1 activation by thrombin is an irreversible process, the activity of PAR1 and the thrombin-induced contraction therefore persisted when the receptor desensitization was impaired in SAH. However, the mechanism underlying this impairment of receptor desensitization still remains to be elucidated. The novel finding of the present study is that argatroban alone was sufficient to prevent the enhancement of the contractile response to PAR1 agonists, while a combination of argatroban and vitamin C or tempol was required to prevent both the enhancement of the contractile response and the impaired receptor desensitization, thereby normalizing the vascular reactivity during SAH. Oxidative stress appears to be responsible for the impairment of receptor desensitization, because two chemically different anti-oxidative agents, vitamin C and tempol, exerted a similar effect.

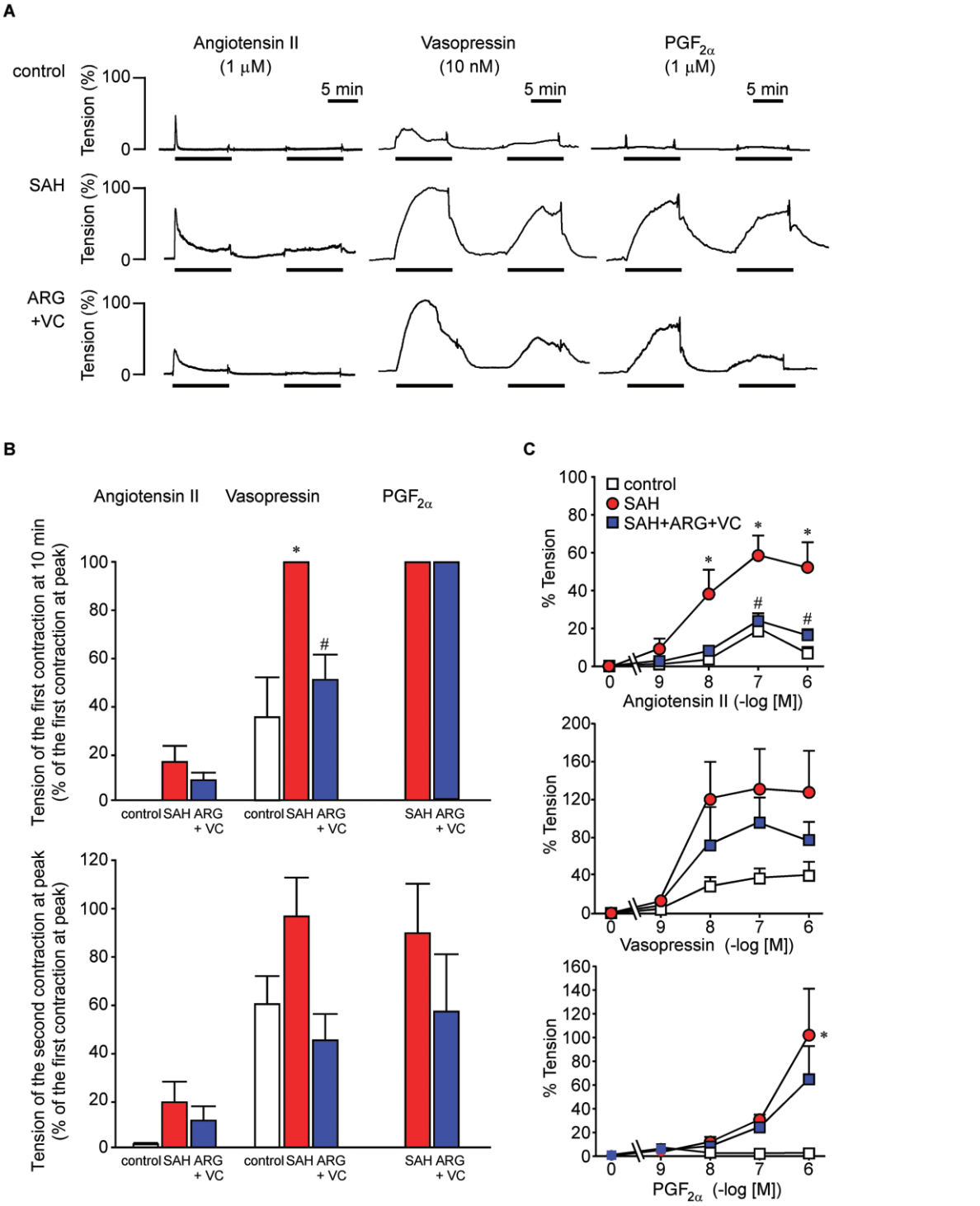

Figure 6

The effects of intrathecal treatment with argatroban and vitamin C on the contractile responses to angiotensin II, vasopressin and $PGF_{2\alpha}$. (A) A representative recording of the contractile responses induced by repetitive stimulation with 1 μM angiotensin II, 10 nM vasopressin and 1 μM $PGF_{2\alpha}$ in the basilar artery obtained from the control, SAH rabbits and SAH rabbits treated with 1 μg argatroban·kg^{-1} per injection and 0.6 mg vitamin C·kg^{-1} per injection (ARG + VC) on day 0 and day 2. The strips were stimulated twice with each agonist for 10 min at 10 min intervals. (B) Summaries of the effects of the treatment with ARG and VC on the level of the first response at 10 min and the level of the peak response to the second stimulation, as expressed as a percentage of the peak level of the first response. (C) A summary of the concentration–response curves for the contractions induced by cumulative applications of each agonist in the basilar artery obtained from the control, SAH rabbits and SAH rabbits treated with argatroban and vitamin C (ARG + VC). The data are expressed as the mean ± SEM ($n = 3$ in B and C). *$P < 0.05$ versus control; #$P < 0.05$ versus SAH.

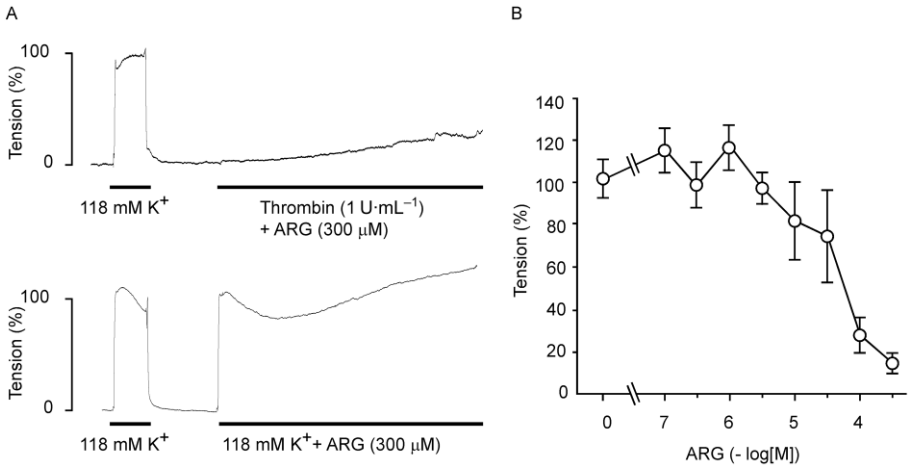

Figure 7

Effect of *ex vivo* treatment with argatroban on the contractile response to thrombin in SAH. (A) Representative traces showing the contractile response to 1 U·mL^{-1} thrombin and 118 mM K^+ depolarization in the presence of 300 μM argatroban. Refer to Figure 1A for the representative traces of the contractile response to 1 U·mL^{-1} thrombin without argatroban in SAH. Thrombin and argatroban were pre-incubated for 10 min in 100 μL normal PSS, and the mixture was applied to the organ bath to obtain the indicated final concentrations of thrombin and argatroban. (B) The concentration-dependent inhibitory effect of argatroban on the thrombin-induced contractions in the rabbit basilar artery of SAH (n = 4–5). The data are expressed as the mean ± SEM.

Argatroban has been shown to prevent cerebral vasospasm by suppressing the expression of platelet-derived growth factor-BB in rabbit SAH (Zhang *et al.*, 2001) and to ameliorate early brain injury by preventing the disruption of the blood brain barrier and the resulting brain oedema in rat SAH (Sugawara *et al.*, 2009). In the present study, argatroban prevented the enhancement of the contractile response to PAR1 agonists. This observation suggests that thrombin is responsible for the enhancement of the contractile response. Notably, this effect of argatroban was not associated with the inhibition of the clot formation. Our previous studies have demonstrated that the enhancement of the contractile response to thrombin can be prevented by the addition of heparin to the autologous blood before injection or by the intrathecal administration of a PAR1 antagonist (Kai *et al.*, 2007; Maeda *et al.*, 2007). As a result, the observations of the present and previous studies collectively suggest that thrombin-mediated activation of PAR1 is responsible for the enhancement of the contractile response to thrombin itself. However, despite the treatment with argatroban, PAR1-AP induced a prolonged contraction, the response to the second simulation with PAR1-AP was maintained, and the thrombin-induced contraction still persisted after thrombin withdrawal. It is therefore suggested that inhibition of thrombin alone was insufficient to prevent the impairment of receptor desensitization during SAH.

Vitamin C has been shown to prevent cerebral vasospasm in SAH patients and experimental SAH models (Sato, 1987; Kawakami *et al.*, 1991; Luo *et al.*, 1995; Kodama *et al.*, 2000). The findings of the present study suggest a new mechanism for the therapeutic effect of vitamin C on cerebral vasospasm. Vitamin C, in combination with argatroban, restored the impaired receptor desensitization and prevented the increased response to PAR1 agonists in SAH, thereby normalizing the increased vascular contractility in SAH. However, vitamin C alone was insufficient to completely restore the impaired receptor desensitization in SAH. A combination of argatroban and vitamin C are required to obtain the complete restoration of the receptor desensitization.

Tempol, a superoxide dismutase mimetic, scavenges super oxide and also reduces the formation of hydroxyl radical by oxidizing Fe^{2+} (Monti *et al.*, 1996). This chemically distinct anti-oxidative agent exerted a similar effect to that seen with vitamin C, while there were some quantitative differences in their effects. This difference may be related to the optimal dosage of these two different agents. Nevertheless, the observations with vitamin C and tempol support the involvement of oxidative stress in the impairment of receptor desensitization. The involvement of other pharmacological actions related to these reagents is thus less likely given their distinct structures.

The TBARS assay demonstrated that the oxidative stress in the brain tissues just beneath the basilar artery significantly increased in SAH. Either argatroban or vitamin C alone or their combination equally decreased the level of oxidative stress to that seen in the controls. These observations therefore suggest that the inhibition of oxidative stress is linked to the partial restoration of the impaired feedback regulation seen with argatroban or vitamin C alone. It appears that the complete restoration seen with this combination is not associated with a further reduction of oxidative stress. However, the mechanism for the inhibition of oxidative stress may differ between argatroban and vitamin C. Thrombin induces ROS production mainly via NADPH oxidase in various cell types, including microglial cells and vascular smooth muscle cells (Patterson *et al.*, 1999; Gorlach *et al.*, 2001; Choi *et al.*, 2005). Argatroban is therefore considered to decrease the oxidative stress by inhibiting the ROS production induced by thrombin. In contrast, vitamin C is thought to decrease oxidative stress by scavenging ROS. This difference appears to

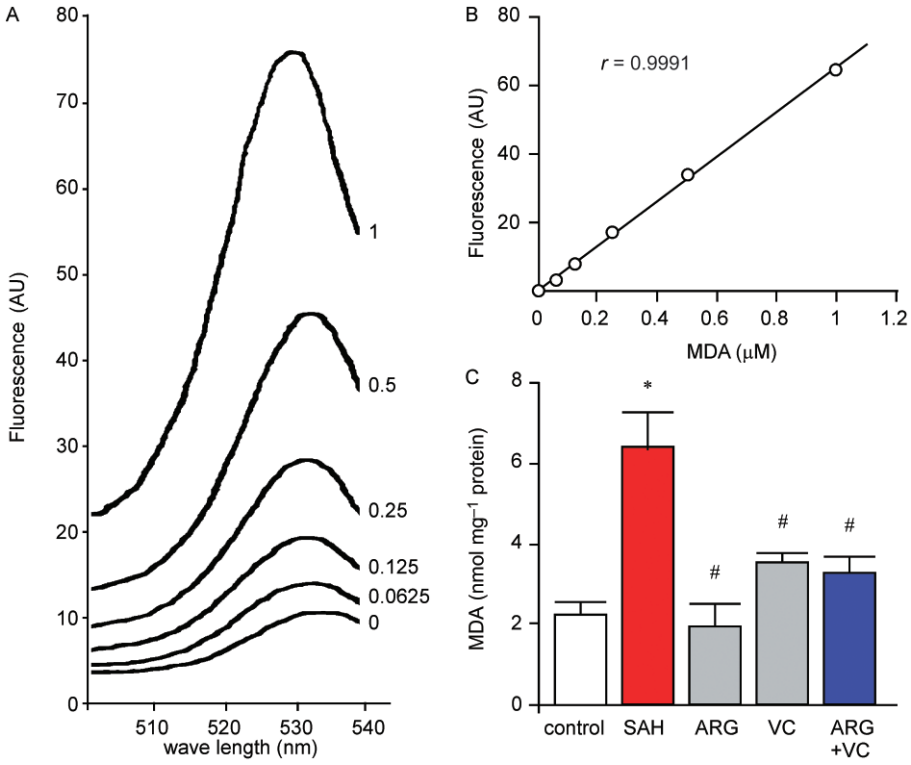

Figure 8

Effect of intrathecal treatment with argatroban and vitamin C on the levels of oxidative stress in brain tissues. (A) Excitation spectra of fluorescence obtained with the indicated concentrations (in μM) of the standard substance malondialdehyde (MDA). (B) A standard relationship between the concentrations of MDA and the fluorescence intensity of its reaction product with thiobarbituric acid. (C) The level of oxidative stress, as expressed by the amount of MDA, in the brain tissues obtained from the control and SAH animals without (SAH) and with treatment with 1 μg argatroban·kg^{-1} per injection (ARG), 0.6 mg vitamin C·kg^{-1} per injection (VC) or their combination (ARG + VC). The fluorescence intensity was converted to the amount of MDA according to the standard relationship shown in (B). The data are expressed as the mean ± SEM (n = 4–6). *P < 0.05 versus control; #P < 0.05 versus SAH.

contribute to the complete restoration of the feedback regulation of the contractile response. Furthermore, although the TBARS analysis of brain tissues provided evidence for the presence of oxidative stress during SAH, the degree of oxidative stress in the vascular tissues, especially in smooth muscle cells, still remains to be determined. Precisely how oxidative stress contributes to the impairment of the feedback regulation therefore remains to be elucidated.

A previous report demonstrated that ROS down-regulated the expression of G protein-coupled receptor kinase (GRK) 2 in glial cells (Cobelens *et al.*, 2007). GRK plays a key role especially in the early phase of the desensitization of the G protein-coupled receptor. GRK might therefore be one of the key targets involved in the impairment of receptor desensitization during SAH. However, PAR1 was shown to be phosphorylated by GRK3 and GRK5, but not GRK2 (Soh *et al.*, 2010), and there have so far been no reports showing the inactivation of GRK3 and GRK5 by ROS. The target of ROS, which is involved in the impairment of receptor desensitization during SAH, still remains unclear.

The observations with the three additional agonists demonstrated that the effects of pathological conditions and the treatments on the receptor activity differed depending on the type of receptor being targeted. Enhancement and prolongation of the contractile responses during SAH were commonly observed, but with some quantitative differences. The effects of treatment with argatroban and vitamin C were the most apparent with the vasopressin receptor. The angiotensin II receptor, vasopressin receptor and $PGF_{2\alpha}$ receptor exhibited three different patterns with regard to the alteration of contractile effects during SAH and the response to treatment. The angiotensin II receptor was the most strongly influenced by the desensitization mechanism, while the $PGF_{2\alpha}$ receptor was the least affected. The vasopressin receptor exhibited a response intermediate between the two, and exhibited a phenomenon similar to that seen with PAR1. Hence, the phenomenon observed with PAR1 is not unique to PAR1 but appears to be applicable to a subset of receptors, including the vasopressin receptor, while it cannot be generalized to a broad range of G protein-coupled receptors.

Thrombin is undetectable in cerebrospinal fluid under physiological conditions, while prothrombin, a thrombin precursor, exists at 10 nM, which is capable of generating a sufficient amount of thrombin to induce a contractile effect (Smirnova *et al.*, 1997). During SAH, prothrombin derived from blood also contributes to the generation of thrombin, thereby resulting in a massive generation of thrombin in the subarachnoid space. The incidence and severity of cerebral

vasospasm have been shown to correlate with thrombin activity in the cerebrospinal fluid or the amount of clot, thus suggesting the critical role of thrombin in cerebral vasospasm (Fisher *et al.*, 1980; Inagawa *et al.*, 1990; Kasuya *et al.*, 1998; Tsurutani *et al.*, 2003). In addition, the enhancement and prolongation of the contractile response to thrombin, which was revealed by the present study and the previous reports (Kai *et al.*, 2007; Maeda *et al.*, 2007; Kikkawa *et al.*, 2010), suggest a special importance of thrombin as a spasmogen. The irreversible persistent contraction induced by thrombin appeared to mimic the persistent narrowing of the cerebral artery seen after SAH.

In conclusion, the present study demonstrated that the contractile response of basilar artery to thrombin is not only enhanced but also prolonged after SAH. The impaired receptor desensitization is suggested to contribute to the irreversible contraction. Thrombin inhibition by argatroban prevented the enhancement of the contractile response, without an apparent effect on the coagulation system. However, the combination of thrombin inhibition and administration of an anti-oxidative agent was required to prevent both the enhancement and prolongation of the contractile response, thereby restoring the normal vascular reactivity. The present study therefore suggests the potential effectiveness of a combination of thrombin inhibition and anti-oxidative treatment as a new therapeutic strategy for the prevention and treatment of cerebral vasospasm. The preventive effect of the delayed treatment suggests that the proposed treatment may have therapeutic potential for clinical situations, where patients may receive medical care later, after the incidence of SAH.

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Conflict of interest

None.

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