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Impaired Feedback Regulation of the Receptor Activity and the Myofilament Ca^{2+} Sensitivity Contributes to Increased Vascular Reactiveness after Subarachnoid Hemorrhage

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Running headline; Mechanism of increased vascular response in SAH

RR1

Abstract

Cerebral vasospasm determines the prognosis of subarachnoid hemorrhage (SAH). The increased vascular reactiveness plays an important role in the development of cerebral vasospasm. This study investigated the roles of the receptor-mediated signaling and the myofilament Ca^{2+} sensitivity in the increased vascular reactiveness in SAH, using the basilar artery of a rabbit SAH model. Endothelin-1, thrombin and phenylephrine induced transient increases in $[Ca^{2+}]_i$, myosin light chain phosphorylation, and contraction in the control. All these responses were not only enhanced but also became sustained in SAH. In the sequential stimulation of thrombin receptor or α_1-adrenoceptor, the second response was substantially attenuated in the control, while it was maintained in SAH. The thrombin-induced contraction in SAH irreversibly persisted even after terminating the thrombin stimulation. This contraction was completely reversed by trypsin and a $G\alpha_q$ inhibitor YM254890, thus suggesting the sustained receptor activity during the sustained contraction. YM254890 also inhibited the endothelin-1- and phenylephrine-induced sustained contraction. Furthermore, the GTPγS-induced transient contraction in the control α-toxin-permeabilized strips was converted to a sustained contraction in SAH. The results provides the first evidence that the feedback inactivation of the receptor activity and the myofilament Ca^{2+} sensitivity was impaired in SAH, thus contributing to the increased vascular reactiveness.

Abbreviations

MLC, myosin light chain; PAR_1, proteinase-activated receptor 1; PAR_1-AP, PAR_1-activating peptide; PDBu, phorbol 12,13-dibutyrate; SAH, subarachnoid hemorrhage.

Key words

Subarachnoid hemorrhage, Cerebral vasospasm, Receptors, Ca^{2+} sensitivity, Smooth muscle

Introduction

Cerebral vasospasm after aneurysmal subarachnoid hemorrhage (SAH) is characterized by the delayed and prolonged contraction of cerebral arteries, which may cause cerebral ischemia, thereby leading to death or neurological deficit in SAH patients (Kassell et al., 1985). Therefore, the prevention and treatment of vasospasm play an important role for the management of SAH patients. The increased vascular reactiveness plays a key role in the pathogenesis of the delayed onset of cerebral vasospasm after SAH (Kai et al., 2008). The contractile reactiveness of the cerebral arteries to various putative spasmogens, including endothelin-1 (Ide et al., 1989; Vatter et al., 2007), thrombin (Kai et al., 2007; Maeda et al., 2007), platelet-derived growth factor (Maeda et al., 2009) and 5-HT (Hansen-Schwartz et al., 2003), have been shown to increase in SAH. In consistent with these increased contractile responses, the expression of ET_A receptor, proteinase-activated receptor 1 (PAR_1), and 5-HT_{1B} is up-regulated in SAH (Hansen-Schwartz et al., 2003; Ide et al., 1989; Kai et al., 2007; Maeda et al., 2007; Vatter et al., 2007). The receptor up-regulation therefore plays a very important role in the increased vascular reactiveness in SAH. However, this can explain the increased responses only to certain spasmogens that act on the up-regulated receptors. On the other hand, the changes in the expression of intracellular signaling proteins, such as RhoA and protein kinase C, have also been demonstrated in SAH, and this could explain the mechanism for the increased responses to various spasmogens (Laher and Zhang, 2001; Miyagi et al., 2000; Sato et al., 2000). However, the changes in the regulatory mechanisms of smooth muscle contraction remain controversial (Maeda et al., 2003; Miyagi et al., 2000; Sato et al., 2000). Therefore, the mechanism underlying the increased vascular reactiveness in SAH still needs to be elucidated.

In many cases, the contractile responses to agonists demonstrate diminished responsiveness during the persisted or repeated stimulations. These phenomena are referred to as desensitization or tachyphylaxis, respectively, which represent an important

physiological "feedback" mechanism that protects against both acute and chronic receptor overstimulation (Ferguson, 2001; Lurie et al., 1985; Miasiro and Paiva, 1990; Trejo, 2003). This feedback regulation could be attributable to not only the inactivation of receptors, but also down-regulation of signaling function at the post-receptor level (Ferguson, 2001; Gong et al., 1997). Importantly, the mechanism of receptor desensitization is impaired under various pathological conditions, such as hypoxia, cancer, and diabetes (Booden et al., 2004; Endo et al., 2005; Hinton et al., 2007). The impairment of feedback regulation could thus play an important role in cerebral vasospasm in SAH. White and Robertson proposed that the desensitization and tachyphylaxis of vascular reactiveness might help to prevent the development of cerebral vasospasm after SAH (White and Robertson, 1987). However, whether and how such feedback regulation of the vascular reactiveness is altered in SAH still remains to be investigated.

The present study used a rabbit SAH model to examine the changes in the contractile responses of the isolated basilar artery to endothelin-1, thrombin and phenylephrine, as representatives of putative spasmogens. The investigation was focused on the changes in the smooth muscle reactiveness; therefore, it was conducted in the absence of endothelium. This study evaluated not only the extent of the contractile responses, but also the temporal correlation among cytosolic Ca^{2+} concentrations ($[Ca^{2+}]_i$), myosin light chain (MLC) phosphorylation and tension development during the prolonged stimulation as well as the tachyphylaxis of contractile responses. The level of expression of receptors was also evaluated with an immunoblot analysis. Furthermore, any changes in the myofilament Ca^{2+} sensitivity were evaluated using α-toxin-permeabilized preparations. The result demonstrates, for the first time, the impairment of negative feedback regulation of receptor signaling and myofilament Ca^{2+} sensitivity to play an important role in the increased vascular reactiveness after SAH.

Materials and methods

Preparation of the rabbit subarachnoid hemorrhage model

This study protocol was approved by the Animal Care and Use Committee, Kyushu University. Adult male Japanese white rabbits (2.5 to 3.0 kg) were anesthetized with an intramuscular injection of ketamine (40 mg/kg weight) and an intravenous injection of sodium pentobarbital (20 mg/kg weight). On day 0, 0.5 mL of cerebrospinal fluid was aspirated percutaneously from the cisterna magna with use of a 23-gauge butterfly needle, and then 2.5 mL of autologous arterial blood obtained from the middle branch of the ear artery was injected. The animal was then kept in a prone position with the head tilting down at 30° for 30 min. On day 2, a second injection of autologous blood was similarly performed. The control animals received injections of the same volume of normal physiological salt solution (PSS) instead of the autologous blood.

Preparation of intact ring of basilar artery

On day 7, the rabbits were heparinized (1000 U) and then euthanized by intravenous injection of an overdose of sodium pentobarbital (120 mg/kg weight) and exsanguinated from the common carotid artery. Exposing the brain revealed that the clot formation was observed over the surface of the pons and the basilar artery in SAH. Immediately after the whole brain was excised *en bloc* and the clot was removed, the narrowing of the basilar artery in SAH was observed under a binocular microscope. The external diameter of the basilar artery was 0.58 ± 0.01 mm in the control (n=5) and 0.35 ± 0.03mm in SAH (n=5). The basilar artery of SAH was thus significantly ($P<0.001$) narrowed to 60.5 ± 5.1% of the control.

The basilar artery was then immediately excided and cut into ring preparations measuring 500 μm wide. To remove the endothelium, the internal surface of arteries was rubbed with a hair. The removal of endothelium was confirmed by the loss of the relaxant response to acetylcholine. The ring preparations were kept in normal PSS at room

temperature until use. These preparations were referred to as "intact" preparations, regardless of the presence or absence of endothelium, in contrast to the "permeabilized" preparations, which will be described below.

Simultaneous measurement of changes in the cytosolic Ca^{2+} concentrations and developed tension in intact ring preparations of the basilar artery

The arterial rings were loaded with the Ca^{2+} indicator dye, fura-2, by incubation in Dulbecco's modified Eagle's medium containing 25 μmol/L fura-2 acetoxymethyl ester and 5v/v% fetal bovine serum for 90 min at 37ºC. The fura-2 loaded rings were washed with normal PSS to remove the dye in the extracellular space, and then were mounted horizontally between two tungsten wires in an organ bath containing 2 mL buffer. One of the wires was connected to the force transducer U gauge (Minebea, Nagano, Japan), while the other was fixed. The preparations were equilibrated in normal PSS at 37ºC for at least 60 min before starting the experimental protocol. During the 60-min equilibration period, the rings were stimulated with 118 mmol/L K^+ every 15 min, and the resting load was increased in a stepwise manner. The resting load was finally adjusted to 50 mg, which was the minimal load to give the maximal tension development in response to 118 mmol/L K^+. The measurement was then performed at 37ºC in PSS aerated with 95% O_2 and 5% CO_2. The changes in the fura-2 fluorescence intensities obtained with 340 nm (F340) and 380 nm (F380) excitation and their ratio (F340/F380) were monitored with a CAM-230 fluorometer (JASCO, Tokyo, Japan). The data were expressed as a percentage, assigning the values of fluorescence ratio and tension obtained in normal PSS and 118 mmol/L K^+ PSS to be 0% and 100%, respectively, unless otherwise specified. Loading of fura-2 was confirmed to have no effect on the contractility of ring preparations, since the degree of tension obtained with 118 mmol/L K^+ in the fura-2-loaded rings (333.7 ± 30.4 mg; n=6) did not differ significantly from that obtained without fura-2 loading (316.2 ± 55.9 mg; n=5). As specified in the figure

legends, tension development was therefore measured without fura-2 loading in some cases. The pH value of the tissues-bathing PSS, in both presence and absence of agonists, and the contractile response to high K^+-depolarization remained unchanged after a 2-hr observation period (data not shown), thus suggesting that there was no significant loss in the integrity of the arterial rings.

Tension measurement in the α -toxin-permeabilized preparations of basilar artery

The arterial rings were permeabilized with 5,000 units/mL staphylococcal α-toxin in cytosolic substitution solution (CSS) for 30 min at 25ºC, as previously described (Maeda et al., 2007). The rings were then treated with 10 μmol/L A23187, a Ca^{2+} ionophore, in Ca^{2+}-free CSS for 15 min to deplete the intracellular Ca^{2+} stores. These permeabilized preparations were then mounted between two tungsten wires, as described above, and then were stretched to 1.5-fold their resting length. After obtaining complete relaxation in Ca^{2+}-free CSS, the experimental protocols was started and the tension development was recorded at 25ºC.

Analysis of the MLC phosphorylation with Phos-tag SDS-PAGE

The phosphorylation of myosin light chain was analyzed using a new method based on a Phos-tag technology (Takeya et al., 2008). Phos-tag is a compound which specifically binds to a phosphate group. Therefore, SDS-PAGE containing polyacrylamide-bound Mn^{2+} Phos-tag (Phos-tag SDS-PAGE) causes a mobility shift of protein depending on the degree of phosphorylation (Takeya et al., 2008). The samples for analysis were obtained during the measurement of tension in the intact preparations. In brief, at the indicated time points, the bathing buffer was promptly changed to 90v/v% acetone, 10w/v% trichloroacetic acid and 10 mmol/L dithiothreitol (DTT) prechilled at –80ºC to stop the reaction. The specimens were then transferred to microcentrifuge tubes, and then were extensively washed and stored in

acetone containing 10 mmol/L DTT. After the specimens were air-dried to remove acetone, the cellular protein was extracted in the sample buffer (50 mmol/L Tris-hydroxymethyl aminomethane, 2w/v% SDS, 5v/v% glycerol, 0.01w/v% NaN_3, 0.01w/v% bromophenol blue, 5v/v% β-mercaptoethanol, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μmol/ml 4-aminidophenylmethane sulphonyl fluoride, and 5 μmol/L microcystin). The supernatant was heated to 100ºC for 5 min prior to electrophoresis. Electrophoresis was performed in 0.1w/v% SDS, 25 mmol/L Tris-hydroxymethyl aminomethane, and 192 mmol/L glycine at 12 mA constant current/8 cm x 5 cm x 0.75 mm gel for 100 min. After electrophoresis, the gel was soaked in transfer buffer (25 mmol/L Tris, 192 mmol/L glycine, 10v/v% methanol) containing 2 mmol/L EDTA to remove Mn^{2+} for 30 min, and then in transfer buffer without EDTA for 15 min. Proteins were then transferred to polyvinylidene difluoride membrane (0.2 μm pore size; Bio-Rad, Hercules, CA, U.S.A) in transfer buffer for 2 hr at room temperature. The membranes were then washed in phosphate buffered saline (PBS; 136.9 mmol/L NaCl, 2.7 mmol/L KCl, 8.1 mmol/L Na_2HPO_4, 1.47 mmol/L KH_2PO_4) for 5 min, and treated with 0.5w/v% formaldehyde in PBS for 45 min (Takeya et al., 2008). After a brief wash in PBS containing 0.1% Tween 20 (T-PBS), the membrane was blocked with 5w/v% skimmed milk in T-PBS overnight at 4ºC. In the immunoblot detection, all forms of 20 kDa MLC were detected using a rabbit polyclonal anti-MLC antibody (sc-15370; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) and horseradish peroxidase conjugated goat anti-rabbit IgG antibody (Sigma, St. Louis, MO, U.S.A.). Primary and secondary antibodies were diluted at 500- and 1000-folds, respectively, in immunoreaction enhancer solution (Can Get Signal; Toyobo, Osaka, Japan). The immune complex was detected using enhanced chemiluminescence technique (ECL plus kit; Amersham, Buckinghamshire, U.K.). The light emission was detected and analyzed with ChemiDoc XRS-J and the computer program Quantity One (BioRad). The percentage of the phosphorylated forms in total MLC (sum of unphosphorylated and phosphorylated forms) was calculated to indicate the extent of MLC

phosphorylation. The extract from one ring preparation was sufficient to permit the quantification of MLC phosphorylation.

Immunoblot analysis of the expression of ET_A receptor, PAR_1 and α_1-adrenoceptor

The isolated basilar arteries were homogenized in 50 mmol/L HEPES, pH 7.4, 150 mmol/L NaCl, 0.5v/v% Nonidet P-40, 1 mmol/L EDTA, 1 mmol/L DTT, 0.5 mmol/L Na_3VO_4, 10 μg/mL leupeptin, 10 μg/mL aprotinin and 10 μmol/L 4-aminidophenylmethane sulphonyl fluoride. Five μg of total protein were separated by SDS-PAGE and then were transferred to the polyvinylidene difluoride membrane (BioRad). The membranes were blocked with 5w/v% skimmed milk in T-PBS overnight at 4ºC. Next, the membrane was incubated for 1 hr at room temperature with anti-ET_A receptor antibody (sc-33535; Santa Cruz Biotechnology), anti-PAR_1 antibody (sc-5605; Santa Cruz Biotechnology) or anti-α_1-adrenoceptor antibody (sc-28982; Santa Cruz Biotechnology) diluted 200-fold in Can Get Signal (Toyobo), followed by the 1-hr incubation with the secondary antibody conjugated with horseradish peroxidase (1000-fold dilution). The immune complex was detected using an ECL plus kit. The detection and analysis of the chemiluminescence signals were as noted above. After chemiluminescence detection, the membranes were stained with naphthol blue black to visualize the band corresponding to actin. The optical density of the each receptor band was normalized to that of actin.

Drugs and solution

The composition of normal PSS was 123 mmol/L NaCl, 4.7 mmol/L KCl, 1.25 mmol/L $CaCl_2$, 1.2 mmol/L $MgCl_2$, 1.2 mmol/L KH_2PO_4, 15.5 mmol/L $NaHCO_3$ and 11.5 mmol/L D-glucose. PSS was aerated with a mixture of 95% O_2 and 5% CO_2, with the resulting pH being 7.4. High K^+ PSS was prepared by replacing NaCl with equimolar KCl. The composition of Ca^{2+}-free CSS was 100 mmol/L potassium methanesulphonate, 2.2 mmol/L

Na_2ATP, 3.38 mmol/L $MgCl_2$, 10 mmol/L EGTA, 10 mmol/L creatine phosphate, and 20 mmol/L Tris-malate (pH 6.8). The CSS containing the indicated concentration of free Ca^{2+} was prepared by adding an appropriate amount of $CaCl_2$, while assuming the Ca^{2+}-EGTA binding constant to be 10^6 (L/mol) (Maeda et al., 2007). Thrombin (bovine plasma; 1178 NIH units/mg protein), *Staphylococcus aureus* α-toxin, GTPγS, phorbol 12,13-dibutyrate (PDBu), phenylephrine and 4-amidinophenylmethanesulfonyl fluoride were purchased from Sigma (St. Louis, MO, U.S.A.). Endothelin-1 was obtained from Peptide Institute Inc. (Osaka, Japan). TFLLR-NH_2 (PAR_1 activating peptide) was obtained from Bachem (Budendorf, Switzerland). Trypsin was purchased from Difco Laboratories (Detroit, MI, U.S.A). Fura-2 acetoxymethyl ester was purchased from Dojindo Laboratories (Kumamoto, Japan). Rabbit polyclonal anti-phospho-MLC (Ser19; #3671) and phospho-MLC (Thr18/Ser19; #3674) antibodies were purchased from Cell Signaling Technology Inc. (Beverly, MA, U.S.A). YM254890 was a kind gift from Astellas Pharma Inc. (Tokyo, Japan).

Data analysis

The data are expressed as the mean ± SEM of the indicated experimental number. One basilar arterial preparation obtained from one animal was used for each experiment, and therefore the number of experiments (n value) indicates the number of animals. Unpaired Student's *t*-test was used to determine statistical differences between the two groups. A value of $P<0.05$ was considered to be statistically significant.

Results

Enhanced contractile response of the rabbit basilar artery to endothelin-1, thrombin and phenylephrine after SAH

Depolarization with 118 mmol/L K^+ induced a sustained increase in tension in the rabbit basilar artery both in the control and SAH (**Figure 1a**). The absolute values of the tension induced by 118 mmol/L K^+ in SAH did not significantly differ from that seen in the control (**Figure 1b**). Therefore, the contractile response to 118 mmol/L K^+ was recorded as a reference response at the beginning of each measurement and thus assigned to be 100%. The level of tension obtained with different concentrations of K^+ did not significantly differ between the control and SAH at any concentrations (**Figure 1c**).

The evaluation of the concentration-dependent responses to endothelin-1 revealed significant enhancement of contractile responses at 3 nmol/L, 10 nmol/L and 30 nmol/L in SAH compared to those seen in the control, while the contraction obtained with 100 nmol/L endothelin-1 in SAH was similar to that seen in the control (**Figure 1d**). The contractile response to thrombin and phenylephrine was significantly enhanced after SAH at concentrations higher than 0.01 unit/mL and 3 μmol/L, respectively (**Figure 1d**). An immunoblot analysis of the expression of ET_A receptor, PAR_1 and α_1-adrenoceptor showed the level of the expression of all the receptors examined to significantly increase in SAH (**Figure 1e**).

In the control, 100 nmol/L endothelin-1 induced a contraction, which reached the peak (143.6 ± 13.8% of the 118 mmol/L K^+-induced contraction, n=5) at 12 min, and thereafter gradually and significantly declined, reaching the level of 85.0 ± 16.3% (n=5) at 45 min (**Figure 2a and 2b**). In SAH, endothelin-1 induced a contraction, which reached a similar peak level with a similar time course as that seen in the control (**Figure 2a and 2b**). However, there was no significant decline in the level of tension even at 45 min (**Figure 2a and 2b**). Thrombin (1 unit/mL) induced a significant, but small, contraction, which reached a

peak at 15 min and thereafter declined to the significantly lower level at 45 min in the control (**Figures 2a and 2b**). In SAH, thrombin induced not only a greater tension development at 15 min than that seen in the control, but also a sustained contraction without a significant decline at 45 min (**Figures 2a and 2b**). Phenylephrine (10 μmol/L) induced a transient contraction with a peak at 6 min in the control, while it induced not only an enhanced, but also a sustained contraction in SAH (**Figures 2a and 2b**). The time course of the changes in $[Ca^{2+}]_i$ correlated with that seen with tension for all agonists (**Figures 2a and 2b**). Endothelin-1 increased $[Ca^{2+}]_i$ to a similar peak level in both control and SAH. $[Ca^{2+}]_i$ thereafter slightly declined in the control, while it remained elevated in SAH. Both thrombin and phenylephrine induced a small transient elevation of $[Ca^{2+}]_i$ in the control, while they induced an enhanced and sustained elevation in SAH.

The phosphorylation of MLC during the contraction induced by endothelin-1, thrombin and phenylephrine in the rabbit basilar artery

Phos-tag SDS-PAGE followed by immunoblot detection with anti-MLC antibody yielded three bands with an apparent molecular size of 20, 23 and 29 kDa in the extract of the basilar arteries obtained before and after the stimulation with endothelin-1 (**Figure 3a**). The upper and middle bands were also detected by anti-phospho-MLC (Thr18/Ser19) and phospho-MLC (Ser19) antibodies, respectively (**Figure 3a**). On the other hand, the conventional SDS-PAGE without Phos-tag yielded only a single immunoreactive band with anti-MLC antibody (data not shown). These observations thus indicated the upper, middle and lower bands to represent di-, mono-, and non-phosphorylated forms of MLC, respectively (**Figure 3a**).

The control basilar arteries contained 39.5 ± 1.0% and 17.6 ± 1.5% of mono- and di-phosphorylated forms of MLC before the contractile stimulation (n=15), while those of SAH contained 44.7 ± 2.5% and 15.1 ± 1.6% (n=15), respectively. There was no significant

difference in the resting level of MLC phosphorylation (the sum of di- and mono-phosphorylation) between the control and SAH. Endothelin-1 (100 nmol/L) significantly increased the MLC phosphorylation to a similar level in the control and SAH, at the peak of the tension development (12 min; **Figure 3b**). The level of MLC phosphorylation thereafter declined in the control, while it remained sustained in SAH (**Figure 3b**). Thrombin (1 unit/mL) and phenylephrine (10 μmol/L) induced no significant increase in the MLC phosphorylation at any time in the control, while they induced a significant and sustained increase in the MLC phosphorylation in SAH (**Figure 3b**). As a result, the changes in the agonists-induced MLC phosphorylation correlated to the changes in $[Ca^{2+}]_i$ and tension both in the control and SAH.

As a result, the conversion of a transient response to a sustained response in SAH was common to all agonists examined. Notably, this conversion was observed with all parameters: $[Ca^{2+}]_i$, MLC phosphorylation and tension.

Changes in the intracellular mechanisms of smooth muscle contraction in the rabbit basilar artery after SAH

To elucidate the mechanism for enhanced and sustained contractile responses in SAH, any changes in the intracellular mechanisms involved in smooth muscle contraction were evaluated. For this purpose, the contractile response to the direct activation of protein kinase C by phorbol 12,13-dibutyrate (PDBu) was first examined in intact preparations. In both control and SAH, 300 nmol/L PDBu induced a precipitous initial contraction, followed by a steady sustained contraction (**Figure 4a**). There was no significant difference in the level of tension development between the control and SAH at the initial peak and 30 min after the initiation of the contraction (**Figure 4b**). The concentration-dependent effects of PDBu also did not significantly differ between the control and SAH (**Figure 4c**).

Next, any alteration of the Ca^{2+}-dependent contractile mechanism or the

myofilament Ca^{2+} sensitivity after SAH was investigated by using α-toxin-permeabilized preparations. In this experiment, GTPγS, a nonhydrolyzable GTP analog, which is known to directly activate G proteins by skipping the receptor-mediated activation (Cockcroft and Gomperts, 1985; Gilman, 1984), was used to increase the Ca^{2+} sensitivity. A stepwise increase in Ca^{2+} concentrations induced a similar stepwise development of tension in the control and SAH (**Figure 4d**). In the presence of 10 μmol/L GTPγS, the pCa^{2+}-tension curves shifted to the left to a similar extent in both the control and SAH (**Figure 4d**). During the 180 nmol/L Ca^{2+}-induced sustained contraction, 10 μmol/L GTPγS induced a further development of tension in both the control and SAH (**Figure 4e**). The level of peak contraction seen in SAH was similar to that seen in the control (**Figure 4e**). In the control, the tension thereafter gradually decreased and the level seen at 25 min was significantly lower than that seen at the peak (**Figure 4e**). In contrast, there was no significant decline in the level of tension in SAH (**Figure 4e**). As a result, the transient contractile response to GTPγS in the control was converted to the sustained response in SAH.

Impairment of the tachyphylaxis of agonist-induced contractile responses in the rabbit basilar artery after SAH

To further elucidate the mechanism of the enhanced and sustained contraction after SAH, the tachyphylaxis of the agonist-induced contractile responses was investigated by examining the reduction of the second response during the consecutive stimulation of the arteries with the same agonist. For this purpose, PAR_1-AP and phenylephrine was used at 100 μmol/L and 50 μmol/L, respectively, to induce a significant contraction in the control. PAR_1-AP induced little contraction at 10 μmol/L, while it induced an enhanced contraction in SAH (data not shown). However, the contraction induced by 100 μmol/L PAR_1-AP in the control (331 ± 25 mg, n=5) was similar to that seen in SAH (304 ± 37 mg, n=5; **Figure 5a**). On the other hand, the contraction induced by 50 μmol/L phenylephrine in the control (108.8 ± 11.5 mg, n=5)

was significantly enhanced in SAH (183.4 ± 21.8 mg, n=5; **Figure 5b**). After recording the first response to 100 μmol/L PAR_1-AP or 50 μmol/L phenylephrine for 5 min, the rings were equilibrated in PSS without stimulation for 10 min, and then they were challenged to the second stimulation with same agonists (**Figure 5**). In the control, the second response to PAR_1-AP and phenylephrine was significantly reduced to 29.9 ± 5.1% (n=5) and 51.7 ± 7.2% (n=5) of the first response, respectively (**Figure 5**). This reduction of the second response was significantly and substantially attenuated in SAH (**Figure 5**).

The effect of trypsin on the thrombin-induced sustained contraction in the rabbit basilar artery after SAH

Thrombin activates its receptor by proteolytically cleaving the extracellular domain of PAR_1 at the residues 41-42, thereby unveiling a new N-terminus which then acts as a tethered ligand to activate the receptor. Therefore, the thrombin activation of PAR_1 is an irreversible process (Macfarlane et al., 2001). The mechanism of feedback regulation helps to terminate its signaling activity (Hollenberg and Compton, 2002; Macfarlane et al., 2001). Otherwise, PAR_1 signaling would persist. In SAH, the thrombin-induced contraction persisted even after removing thrombin and adding 10 μmol/L 4-amidinophenylmethanesulfonyl fluoride, a protease inhibitor (**Figure 6a**). The contribution of the persisted signaling activity of PAR_1 to this irreversible contraction was first investigated by examining the effect of trypsin on the sustained phase of the contraction. Trypsin has been shown to cleave the extracellular domain of PAR_1 at the residues 71-72 and/or 82-83 (in human), thereby removing the tethered ligand region (Nakayama et al., 2003). The addition of 10 μmol/L trypsin during the thrombin-induced sustained contraction completely inhibited the contraction to the resting level (**Figures 6b**). However, trypsin had no effect on the sustained contraction induced by 118 mmol/L K^+ or endothelin-1 (**Figure 6c and 6d**).

The effect of YM254890 on the agonist-induced contraction in the rabbit basilar artery after SAH

Next, the persistent receptor activity during the sustained contraction was then investigated by examining the effect of YM254890, a selective $G\alpha_q$ inhibitor, on the sustained phase of the contraction. YM254890 inhibited the thrombin-induced sustained contraction, with a complete inhibition at 100 nmol/L (**Figures 7a and 7b**). YM254890 also completely inhibited the contraction induced by 100 nmol/L endothelin-1 and 30 μmol/L phenylephrine at 100 nmol/L (**Figures 7a and 7b**). Furthermore, the $[Ca^{2+}]_i$ elevations induced by these agonists were completely inhibited by 100 nmol/L YM254890 (data not shown). The IC_{50} values of YM254890 for the inhibition of the contraction induced by thrombin, endothelin-1 and phenylephrine were 69.8 nmol/L, 69.7 nmol/L and 84.9 nmol/L, respectively. On the other hand, YM254890, even at 1 μmol/L, had no significant effect on the level of $[Ca^{2+}]_i$ (data not shown) and tension (**Figure 7a**) during the contraction induced by 300 nmol/L PDBu or 118 mmol/L K^+.

Discussion

The most noticeable observation of the present study is that the contractile response of the isolated basilar artery to three G protein-coupled receptor agonists was not only enhanced but also sustained after SAH. In the control, the contractions induced by endothelin-1, thrombin and phenylephrine declined more or less after reaching the peak of contraction despite the continuous presence of the receptor stimulation. In SAH, those contractions sustained with no significant decline. In addition, the experiments of the consecutive stimulations demonstrated that the responsiveness to the second stimulation was substantially attenuated in the control artery, while it was maintained in SAH. These observations therefore suggest that some negative feedback regulation of the contractile response was impaired in SAH, thus converting the transient response to the sustained response and also maintaining the responsiveness to the second stimulation. In this respect, it is noteworthy that the conversion of the transient response to the sustained response was observed with not only tension but also $[Ca^{2+}]_i$ and MLC phosphorylation. This observation therefore suggests the mechanism that was impaired in SAH to reside at the upstream of the Ca^{2+} signal, and presumably at a receptor level. In addition, the GTPγS-induced contraction seen at the fixed Ca^{2+} concentration in the permeabilized rings also declined in the control artery, while it remained sustained in SAH. This observation thus suggest another feedback regulation to reside at a level of regulation of the myofilament Ca^{2+} sensitivity, which was also impaired in SAH. As a result, the present study suggests the impaired feedback regulation of the receptor activity and the myofilament Ca^{2+} sensitivity, to play a critical role in the increased vascular reactiveness in SAH.

The mechanism for cerebral vasospasm can be attributable to either increased production of spasmogens or increased vascular reactiveness (Kai et al., 2008). The increase in the vascular reactiveness may result from either endothelial dysfunction (Sasaki et al., 1985; Sasaki et al., 1986) or an increase in the smooth muscle contractility (Kassell et al., 1985). The present study proposes the impairment of the feedback regulation of the

contractile response as a novel mechanism contributing to the increased vascular reactiveness in SAH. The receptor up-regulation contributes to the increased contractile responses in SAH (Hansen-Schwartz et al., 2003; Itoh et al., 1994; Kai et al., 2007; Maeda et al., 2007; Vatter et al., 2007). However, this could explain the increased responses to certain spasmogens. In contrast, the impaired feedback regulation of the receptor activity seems to affect a broad range of G protein-coupled receptors, thus contributing to the reported increase in the contractile response to various spasmogens (Hansen-Schwartz et al., 2003; Itoh et al., 1994; Kai et al., 2007; Kai et al., 2008; Maeda et al., 2009; Maeda et al., 2007; Vatter et al., 2007).

The impairment of the feedback regulation of the receptor activity in SAH is convincingly suggested for PAR_1. The thrombin activation of PAR_1 is an irreversible process (Macfarlane et al., 2001). The subsequent receptor phosphorylation, β-arrestin binding, endocytotic internalization and lysosomal degradation thus help to terminate the signaling activity of PAR_1 (Hollenberg and Compton, 2002; Trejo, 2003). Otherwise, PAR_1 signaling would persist even after the termination of thrombin stimulation, as previously reported in Sf9 cells or metastatic breast cancer cells (Booden et al., 2004; Chen et al., 1996; Trejo, 2003). The observation that the thrombin-induced contraction irreversibly persisted after terminating the thrombin stimulation therefore suggest the impairment of the receptor inactivation. Furthermore, the observations with trypsin and YM254890 support the persistent signaling activity of PAR_1 during the thrombin-induced contraction. The removal of the tethered ligand region of PAR_1 by proteinases converts PAR_1 to the inactive conformation, thereby terminating the irreversibly persisted signals (Holinstat et al., 2009). Trypsin is one such proteinase and it removes the tethered ligand region of PAR_1 (Nakayama et al., 2004; Nakayama et al., 2003). The inhibition of the thrombin-induced sustained contraction by trypsin thus suggests the requirement of the active conformation of PAR_1 for the thrombin-induced sustained contraction. Since the contractile responses to endothelin-1 and phenylephrine were also converted from the transient response in the control to the

sustained response in SAH, and YM254890 also inhibited the sustained phase of those contractions, the feedback regulation of ET_A receptor and α_1-adrenoceptor is also suggested to be impaired after SAH.

The general impairment of the receptor inactivation may be consistent with previous reports showing the enhanced contractile response to various receptor ligands (Hansen-Schwartz et al., 2003; Itoh et al., 1994; Kai et al., 2007; Kai et al., 2008; Maeda et al., 2009; Maeda et al., 2007; Vatter et al., 2007). However, the impaired receptor inactivation exerts a significant effect especially on the activity of ET_A receptor and PAR_1. The affinity of ET_A receptor for endothelin-1 is extremely high, and therefore their binding is practically irreversible under physiological conditions (Endo et al., 2005; Freedman et al., 1997). On the other hand, PAR_1 activation is a irreversible process. The impaired receptor inactivation therefore causes a greater impact to the signaling activity of PAR_1 than other receptors. The massive hemorrhage around cerebral arteries is the fundamental pathology of SAH, which causes the extensive production of thrombin. The increased reactiveness of the basilar artery to thrombin is characteristic of SAH. Thrombin and its receptor PAR_1 are therefore suggested to play an important role in the development of cerebral vasospasm.

The mechanism for the impairment of the receptor inactivation in SAH still remains to be elucidated, with regard to how SAH causes such impairment and which step of the feedback regulation is impaired. The phosphorylation of receptor by G protein-coupled receptor kinases and the binding of β-arrestin to receptor contribute to the rapid termination of receptor signaling, while the endocytotic internalization and the lysosomal degradation also contribute to the subsequent termination (Drake et al., 2006; Moore et al., 2007). The relative contribution of each step and precise molecular mechanism varies depending on the type of receptor (Abe et al., 2000; Chalothorn et al., 2002; Freedman et al., 1997). Therefore, the step common to ET_A receptor, PAR_1 and α_1-adrenoceptor is considered to be impaired in SAH, and the molecule(s) positively or negatively involved in such a step may be up- or

down-regulated in the cerebral artery after SAH. Those molecules still remain to be identified.

The present study also suggests the impairment of the feedback regulation at the step regulating the myofilament Ca^{2+} sensitivity in SAH. However, the importance of this impairment may vary with the contractile stimulation, depending on the degree of contribution of the myofilament Ca^{2+} sensitization to the contractile response. The evaluation of the $[Ca^{2+}]_i$-tension relationship based on the observations in **Figure 2** suggests the Ca^{2+} sensitization to make a relatively greater contribution to the endothelin-1-induced contractions than other contractions. Rho kinase and protein kinase C are two major signaling molecules known to contribute to the myofilament Ca^{2+} sensitization (Hirano, 2007). The activation of protein kinase C by PDBu induced a sustained contraction in cerebral artery. However, this contractile response remained unchanged after SAH, thus ruling out the major contribution of protein kinase C to the feedback regulation of the myofilament Ca^{2+} sensitivity. On the other hand, the activity of Rho kinase is up-regulated in SAH (Miyagi et al., 2000; Sato et al., 2000). The increased Rho kinase activity may therefore contribute to the persistent increase in the myofilament Ca^{2+} sensitivity in SAH, especially in the endothelin-induced contraction.

In SAH, the maximal contractile responses to thrombin and phenylephrine were greatly enhanced, while the concentration-response curve of endothlin-1 was shifted to the left. The present study demonstrated the up-regulation of receptors for all these agonists in SAH. The up-regulation of ET_A receptor and PAR_1 is consistent with the observations of the previous reports (Ide et al., 1989; Itoh et al., 1994; Maeda et al., 2007; Vatter et al., 2007), while no study has previously reported the up-regulation of α_1-adrenoceptor in SAH. The up-regulation of PAR_1 and α_1-adrenoceptor may be consistent with the enhancement of the maximal response. However, the up-regulation of ET_A receptor was not associated with the enhancement of the maximal response to endothelin-1. The reason for this, however, remains

unknown. It may be related to large population of the spare receptor for ET_A receptor or a relatively lower degree of the up-regulation in comparison to that seen with PAR_1 and α_1-adrenoceptor (**Figure 1e**).

In conclusion, the present study provides the first evidence that the feedback regulation of the smooth muscle contraction is impaired in SAH. The impairment of this feedback regulation is suggested to contribute to the persistent receptor signaling and the sustained increase in the myofilament Ca^{2+} sensitivity. The impaired feedback regulation is thus suggested to contribute to the increased vascular reactiveness, and therefore the pathogenesis of cerebral vasospasm.

Disclosure/Conflict of Interest

none

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the genesis of vasospasm. *Neurosurgery* 21:523-531.

Titles and Legends to Figures

Figure 1. Contractile responses of the rabbit basilar artery to high K^+ depolarization, endothelin-1, thrombin and phenylephrine and the expression of ET_A receptor, PAR_1 and α_1-adrenoceptor in the control and SAH

(a) Representative recordings showing the 118 mmol/L K^+-induced contractions in the fura-2-unloaded basilar artery of control and SAH. (b) The level of tension induced by 118 mmol/L K^+ depolarization in the control and SAH, as expressed in absolute value (n=5). (c) Concentration-dependent effect of high K^+ depolarization on the tension development in the control and SAH (n=5). (d) The concentration-response curves of the contraction induced by endotheliln-1, thrombin and phenylephrine in the basilar artery of the control and SAH (n=5~7). (e) Immunoblot analysis of the expression of ET_A receptor, PAR_1 and α_1-adrenoceptor in the basilar artery of the control and SAH (n=4~5). The level of tension obtained in normal PSS and 118 mmol/L K^+ PSS were assigned values of 0% and 100%, respectively. The level of expression seen in the control was assigned to be 100%. The data represent the mean ± SEM. n.s., not significantly different; *, $P<0.05$ vs. control.

Figure 2. The time course of the change in $[Ca^{2+}]_i$ and tension induced by endothelin-1, thrombin and phenylephrine in the rabbit basilar artery of the control and SAH.

Representative recordings (a) and summaries of the time course (b) of $[Ca^{2+}]_i$ and tension induced by 100 nmol/L endothelin-1, 1 unit/mL thrombin and 10 μmol/L phenylephrine in the fura-2-loaded basilar artery of the control and SAH. In panel (b), the time course of $[Ca^{2+}]_i$ was evaluated at three time points; just before the stimulation (0 min), at the peak of the tension development seen with each agonist (12, 15 or 6 min) and 45 min after the stimulation. The data represent the mean ± SEM (n=5~7). The levels of $[Ca^{2+}]_i$ and tension obtained at the rest and 5 min after initiating a contraction by 118 mmol/L K^+-PSS were assigned values of 0 and 100%, respectively. *, $P<0.05$ vs. resting level (0 min); #, $P<0.05$;

n.s., not significantly different.

Figure 3. Change in MLC phosphorylation induced by endothelin-1, thrombin and phenylephrine in the rabbit basilar artery of the control and SAH.

(a) Representative immunoblots obtained with anti-MLC, anti-phospho-MLC (Ser19) and anti-phospho-MLC (Thr18/Ser19) antibodies. The samples were obtained before and 12 min after the stimulation with 100 nmol/L endothelin-1. The upper, middle and lower bands detected with anti-MLC antibody represent di-, mono-, and non-phosphorylated forms of MLC. (b) Summary of the MLC phosphorylation (% of total MLC) induced by 100 nmol/L endothelin-1, 1 unit/mL thrombin and 10 μmol/L phenylephrine, as evaluated at three time points; just before the stimulation (0 min), at the peak of contraction (12, 15 or 6 min) and 45 min after the stimulation. The data represent the mean ± SEM (n=5). *, $P<0.05$ vs. 0 min.

Figure 4. Contractile responses of the intact artery to phorbol 12,13-dibutyrate (PDBu) and the α-toxin-permeabilized artery to GTPγS in the control and SAH.

(a-c) The contractile responses to PDBu in the intact basilar artery of the control and SAH; (a) Representative recordings showing the tension development induced by 300 nmol/L PDBu in the fura-2-unloaded intact basilar artery of the control and SAH, (b) The levels of tension obtained at the peak and 30 min after the stimulation, and (c) The concentration-response curves for the PDBu-induced tension development in the control and SAH. The levels of tension obtained at the rest and 5 min after initiating a contraction by 118 mmol/L K^+-PSS were assigned values of 0 and 100%, respectively, in the intact artery. (d, e) The contractile responses to GTPγS in the α-toxin-permeabilized artery of the control and SAH; (d) The pCa^{2+}-tension curves of the contraction induced by stepwise increment of Ca^{2+} concentrations in the absence and presence of 10 μmol/L GTPγS, and in the control and SAH, and (e) Representative recordings and summary of the contractions induced by 10 μmol/L

GTPγS during the 180 nmol/L Ca^{2+}-induced contractions in the control and SAH. The level of tension was evaluated at the peak, 15 min and 25 min after initiating the contraction by GTPγS, as indicated by arrowheads in the traces. The response to 10 μmol/L Ca^{2+} was recorded as a reference response at the end of each experiment, and this level of tension was assigned value of 100%, while that obtained in Ca^{2+}-free CSS was assigned values of 0% in the permeabilized artery. The data represent the mean ± SEM (n=5). n.s., not significantly different; *, P<0.05; #, P<0.05 vs. control.

Figure 5. Contractile response of basilar artery to the consecutive stimulation with PAR_1-AP and phenylephrine in the control and SAH.

(a, b) Representative recordings and summary showing the contractile responses to the consecutive stimulation with 100 μmol/L PAR_1-AP (a) and 50 μmol/L phenylephrine (b) in the fura-2-unloaded ring preparations. The rings were consecutively stimulated with PAR_1-AP or phenylephrine for 5 min with a 10-min interval of incubation without contractile stimulation. The response to the second stimulation was evaluated by assigning the response to the first stimulation to be 100 %. The data represent the mean ± SEM (n=5). n.s., not significantly different; *, P<0.05.

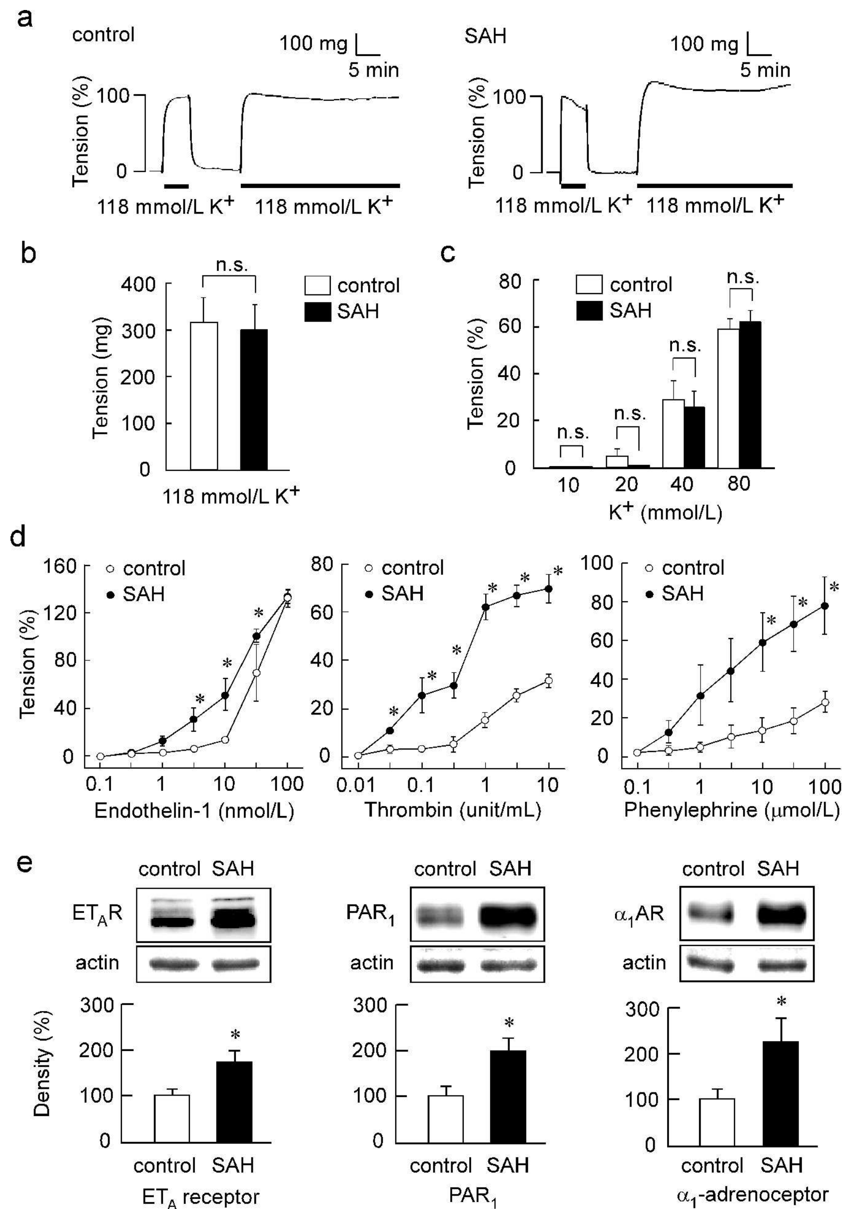
Figure 6. The effects of trypsin on the sustained contractions induced by thrombin in the rabbit basilar artery with SAH.

Representative recordings of at least three independent experiment, showing the effect of the removal of thrombin on the subsequent time course of the thrombin-induced contraction (a) and the effect of 10 μmol/L trypsin on the contraction induced by 1 unit/mL thrombin (b), 100 nmol/L endothelin-1 (c) and 118 mmol/L K^+ (d) in the fura-2-unloaded ring preparations of SAH. In panel (a), when thrombin was washed out, 10 μmol/L 4-amidinophenylmethanesulfonyl fluoride was added to the bathing buffer to ensure the

complete inhibition of any residual thrombin activity.

Figure 7. The effects of YM254890 on the contractions induced by thrombin, endothelin-1, phenylephrine, PDBu and 118 mmol/L K^+ depolarization in the rabbit basilar artery with SAH.

(a) Representative recordings showing the effect of YM254890 on the contraction induced by 100 nmol/L endothelin-1, 1 unit/mL thrombin, 30 μmol/L phenylephrine, 300 nmol/L PDBu and 118 mmol/L K^+ in the fura-2-unloaded ring preparations of SAH. (b) Concentration-dependent effects of YM254890 on the contraction induced by 1 unit/mL thrombin, 100 nmol/L endothelin-1 and 30 μmol/L phenylephrine in SAH. The relaxant effect of YM254890 was evaluated by assigning the level of tension obtained just prior to the application of YM254890 and that obtained in the normal PSS before initiating the pre-contraction to be 100% and 0%, respectively. The data represent the mean ± SEM (n=5).



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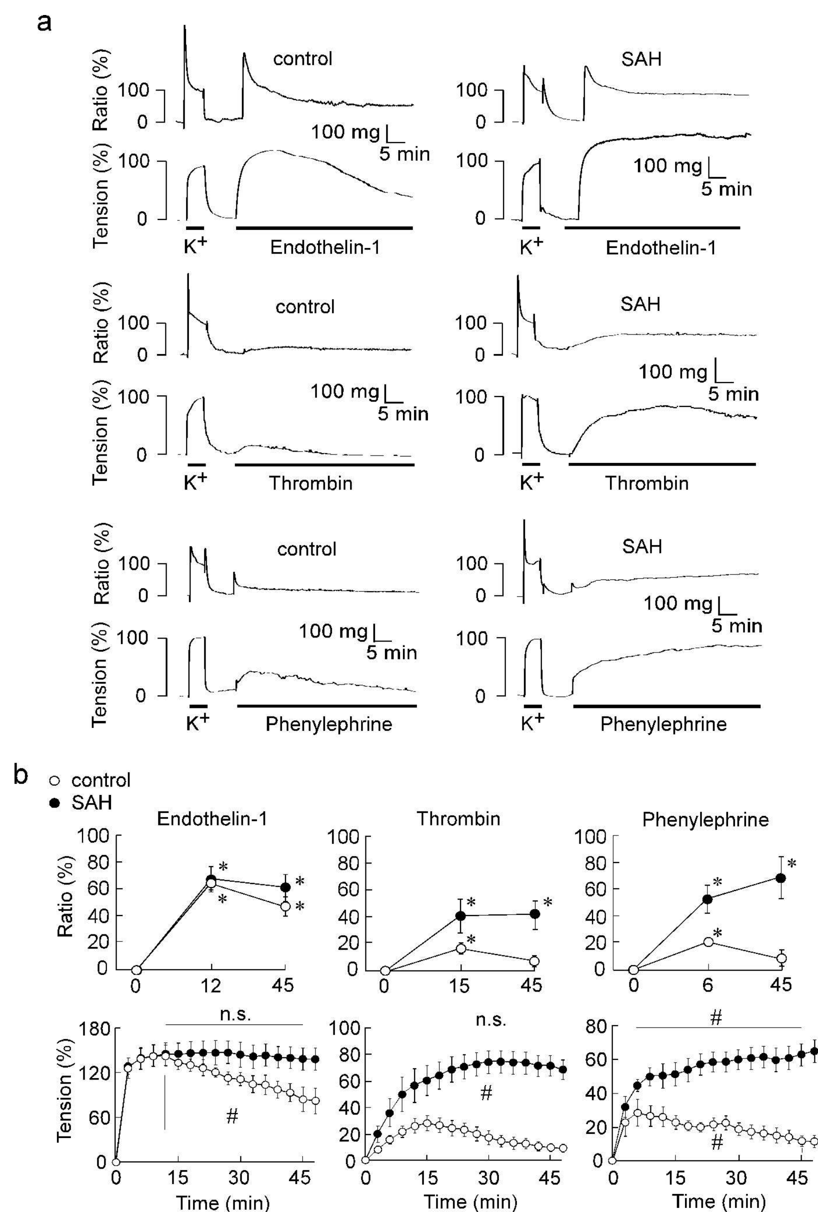

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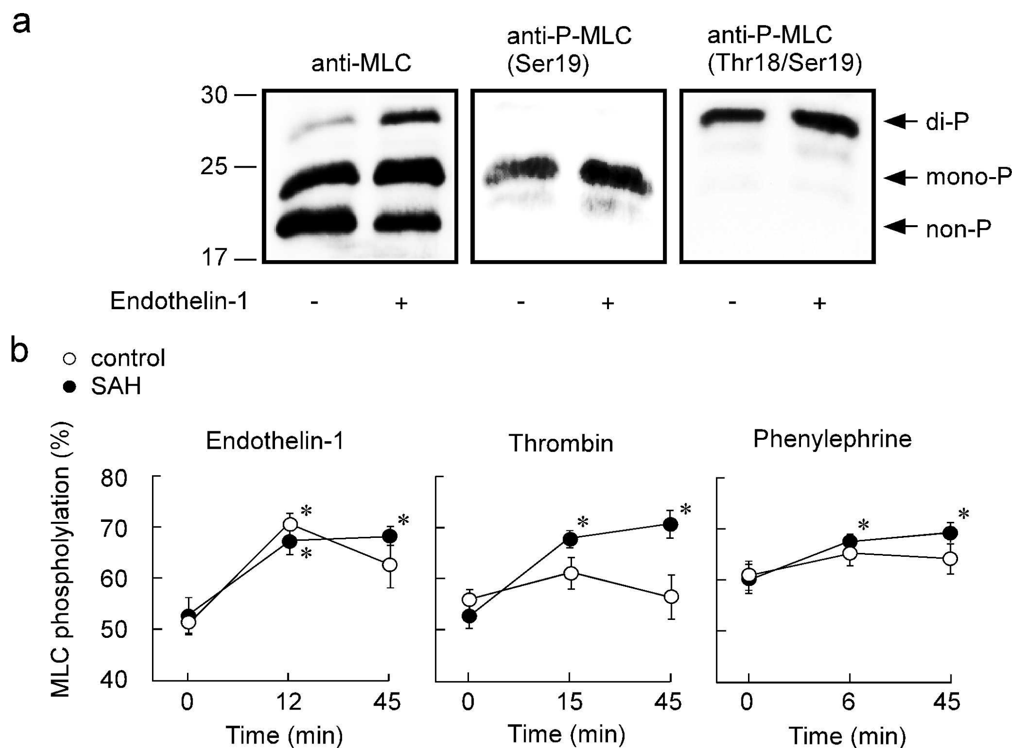

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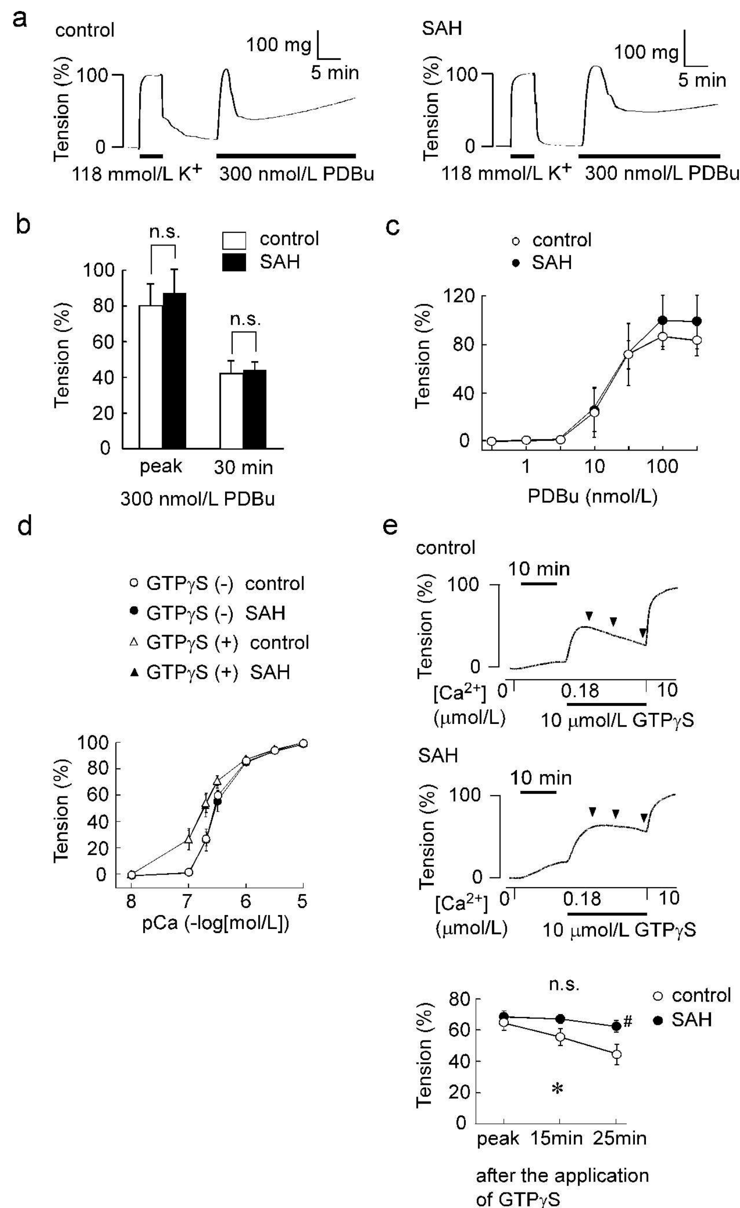

Figure 4. Contractile responses of the intact artery to phorbol 12,13-dibutyrate (PDBu) and the α-toxin-permeabilized artery to GTPγS in the control and SAH.
(a-c) The contractile responses to PDBu in the intact basilar artery of the control and SAH; (a) Representative recordings showing the tension development induced by 300 nmol/L PDBu in the fura-2-unloaded intact basilar artery of the control and SAH, (b) The levels of tension obtained at the peak and 30 min after the stimulation, and (c) The concentration-response curves for the PDBu-induced tension development in the control and SAH. The levels of tension obtained at the rest and 5 min after initiating a contraction by 118 mmol/L K^+-PSS were assigned values of 0 and 100%, respectively, in the intact artery. (d, e) The contractile responses to GTPγS in the α-toxin-permeabilized artery of the control and SAH; (d) The pCa^{2+}-tension curves of the contraction induced by stepwise increment of Ca^{2+} concentrations in the absence and presence of 10 μmol/L GTPγS, and in the control and SAH, and (e) Representative recordings and summary of the

contractions induced by 10 μmol/L GTPγS during the 180 nmol/L Ca^{2+}-induced contractions in the control and SAH. The level of tension was evaluated at the peak, 15 min and 25 min after initiating the contraction by GTPγS, as indicated by arrowheads in the traces. The response to 10 μmol/L Ca^{2+} was recorded as a reference response at the end of each experiment, and this level of tension was assigned value of 100%, while that obtained in Ca^{2+}-free CSS was assigned values of 0% in the permeabilized artery. The data represent the mean ± SEM (n=5). n.s., not significantly different; *, $P<0.05$; #, $P<0.05$ vs. control.
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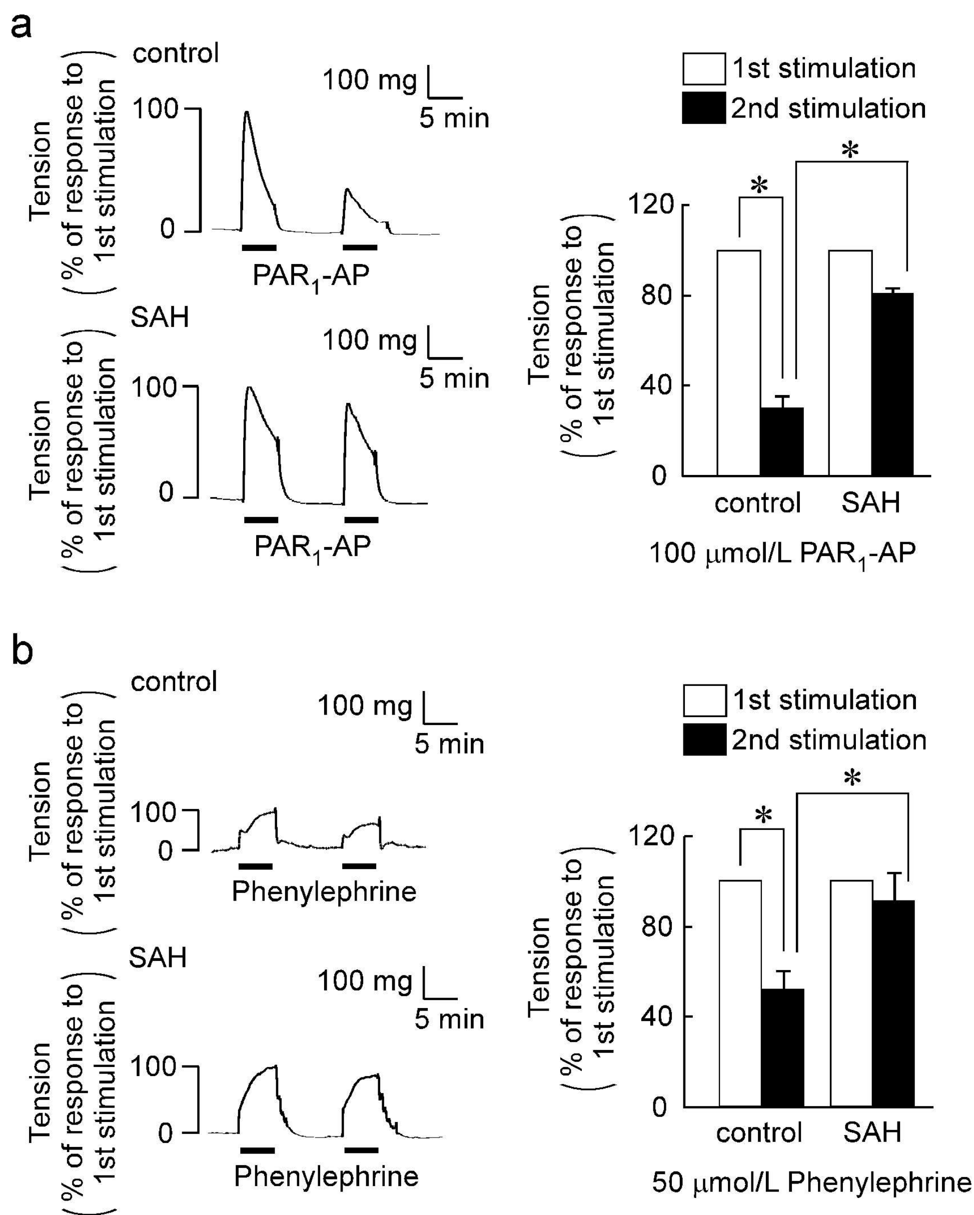

Figure 5. Contractile response of basilar artery to the consecutive stimulation with PAR_1-AP and phenylephrine in the control and SAH.
(a, b) Representative recordings and summary showing the contractile responses to the consecutive stimulation with 100 μmol/L PAR_1-AP (a) and 50 μmol/L phenylephrine (b) in the fura-2-unloaded ring preparations. The rings were consecutively stimulated with PAR_1-AP or phenylephrine for 5 min with a 10-min interval of incubation without contractile stimulation. The response to the second stimulation was evaluated by assigning the response to the first stimulation to be 100 %. The data represent the mean ± SEM (n=5). n.s., not significantly different; *, P<0.05.

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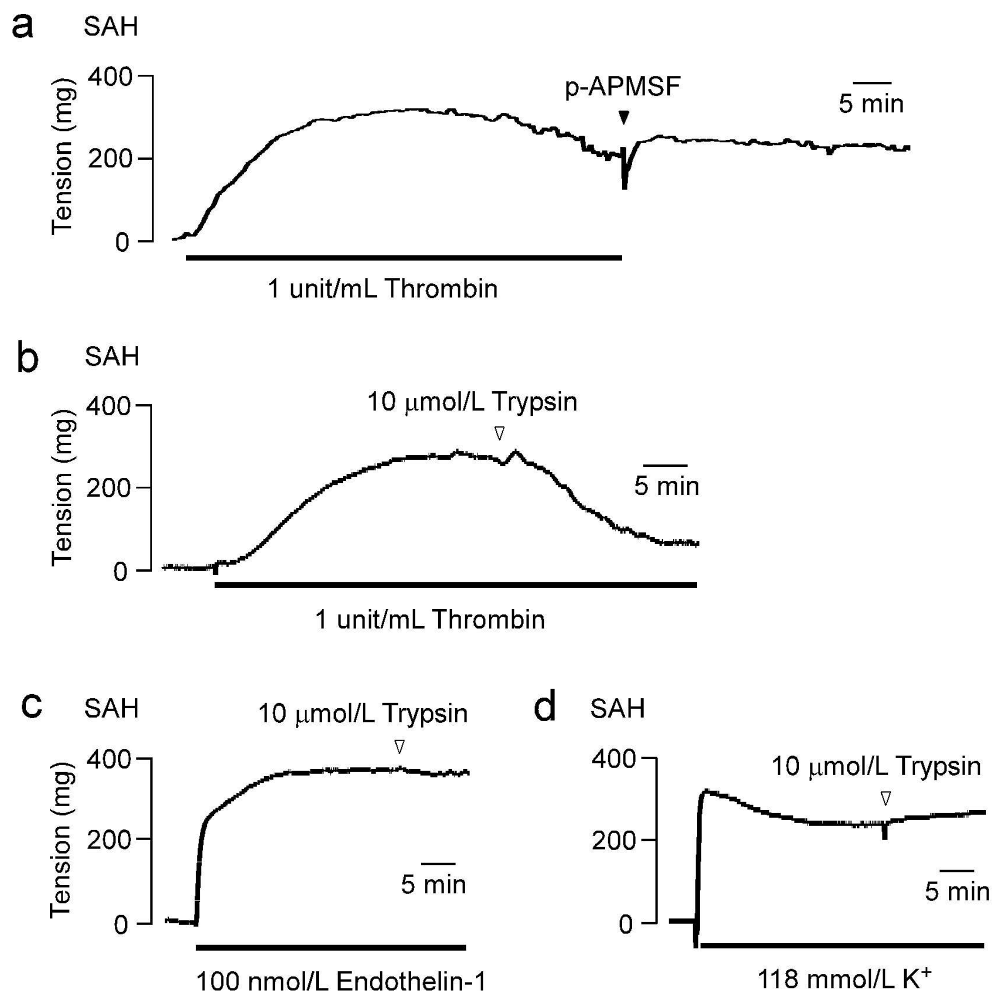

Figure 6. The effects of trypsin on the sustained contractions induced by thrombin in the rabbit basilar artery with SAH.
Representative recordings of at least three independent experiment, showing the effect of the removal of thrombin on the subsequent time course of the thrombin-induced contraction (a) and the effect of 10 μmol/L trypsin on the contraction induced by 1 unit/mL thrombin (b), 100 nmol/L endothelin-1 (c) and 118 mmol/L K^+ (d) in the fura-2-unloaded ring preparations of SAH. In panel (a), when thrombin was washed out, 10 μmol/L 4-amidinophenylmethanesulfonyl fluoride was added to the bathing buffer to ensure the complete inhibition of any residual thrombin activity.
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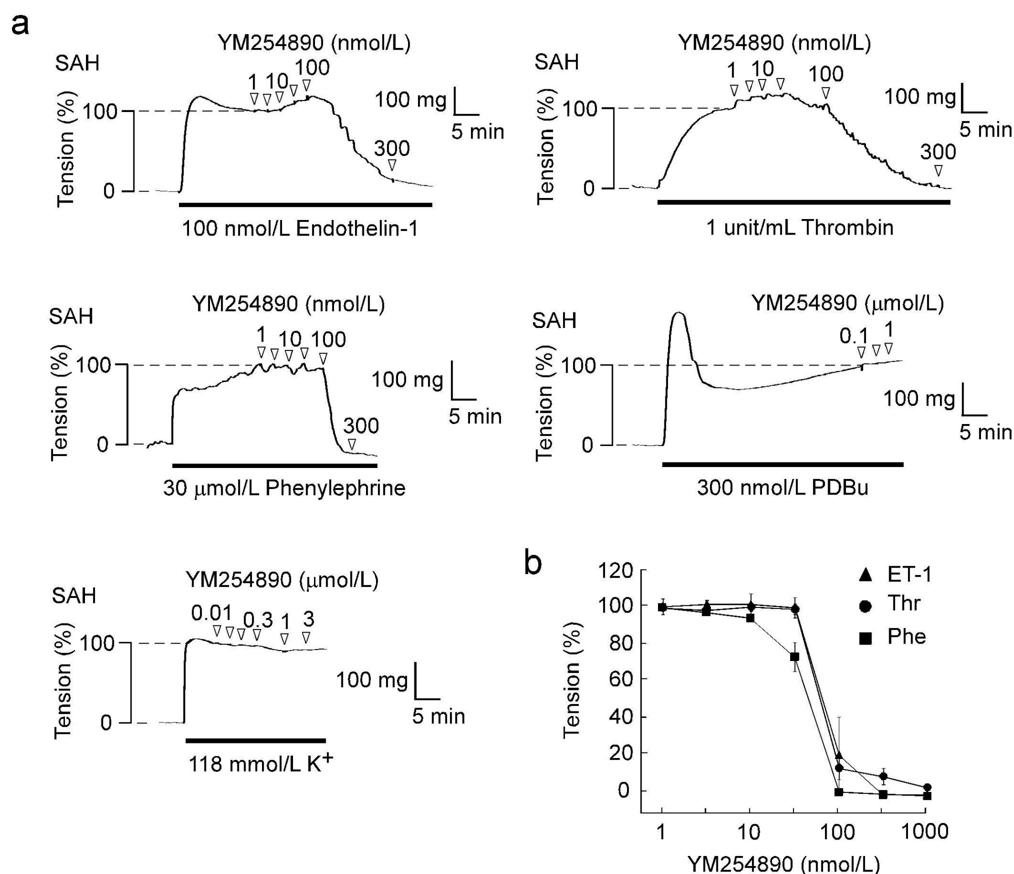

Figure 7. The effects of YM254890 on the contractions induced by thrombin, endothelin-1, phenylephrine, PDBu and 118 mmol/L K^+ depolarization in the rabbit basilar artery with SAH. (a) Representative recordings showing the effect of YM254890 on the contraction induced by 100 nmol/L endothelin-1, 1 unit/mL thrombin, 30 μmol/L phenylephrine, 300 nmol/L PDBu and 118 mmol/L K^+ in the fura-2-unloaded ring preparations of SAH. (b) Concentration-dependent effects of YM254890 on the contraction induced by 1 unit/mL thrombin, 100 nmol/L endothelin-1 and 30 μmol/L phenylephrine in SAH. The relaxant effect of YM254890 was evaluated by assigning the level of tension obtained just prior to the application of YM254890 and that obtained in the normal PSS before initiating the pre-contraction to be 100% and 0%, respectively. The data represent the mean ± SEM (n=5).

177x152mm (600 x 600 DPI)