Subtype Activation and Interaction of Protein Kinase C and Mitogen-Activated Protein Kinase Controlling Receptor Expression in Cerebral Arteries and Microvessels After Subarachnoid Hemorrhage

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Background and Purpose—The pathogenesis of cerebral ischemia associated with subarachnoid hemorrhage (SAH) still remains elusive. The aim of this study was to examine the involvement of mitogen-activated protein kinase (MAPK) and protein kinase C (PKC) subtypes in the pathophysiology of cerebral ischemia after SAH in cerebral arteries and microvessels and to examine temporal activation of the kinases. We hypothesize that treatment with a MAPK or PKC inhibitor will prevent the SAH-induced kinase activation in brain vessels.

Methods—SAH was induced by injecting 250 µL blood into the prechiasmatic cistern in the rat. The activation of different MAPK and PKC isotypes in large circle of Willis cerebral arteries and intracerebral microvessels was examined at 0, 1, 3, 6, 12, 24, and 48 hours after SAH and after intrathecal treatment with PKC or MAPK inhibitor by use of Western blot.

Results—Among the 8 investigated PKC isoforms, only PKCδ was activated at 1 hour and at 48 hours, whereas PKCo was activated at 48 hours after SAH. For the MAPKs, there was early phosphorylation at 1 hour of extracellular signal-regulated kinase 1/2, whereas c-jun N-terminal kinase and p38 showed enhanced phosphorylation only at 48 hours after SAH. The pattern was identical in large cerebral arteries and in intracerebral microvessels. Treatment with either the PKC (RO-31-7549) or the raf (SB386023-b) inhibitor prevented the kinase activation.

Conclusions—The results show that specific subtypes of the MAPK and PKC pathways are activated in cerebral arteries after SAH and the PKC and raf inhibitors are able to prevent this activation. (Stroke. 2008;39:185-190.)

Key Words: cerebral arteries ■ cerebral ischemia ■ mitogen-activated protein kinase (MAPK) ■ protein kinase C (PKC) ■ subarachnoid hemorrhage (SAH)

The pathogenesis of cerebral ischemia associated with subarachnoid hemorrhage (SAH) still remains elusive. The late phase of cerebral ischemia that occurs after SAH has resulted in numerous theories such as increased amounts of vasospastic substances such as endothelin-1, 5-hydroxytryptamine, and thromboxane A₂, enhanced levels of free radicals generated by the extravasation of blood, endothelial dysfunction, and a central nervous system dysfunction. In addition, there are data showing that SAH elicits a wide range of stress-associated responses, which result in activation of several intracellular pathways, for example, the protein kinase C (PKC) and mitogen-activated protein kinase (MAPK) pathways.

More recent studies have suggested that the upregulation of endothelin type B and 5-hydroxytryptamine type 1B receptors in large cerebral arteries after experimental SAH may partly be responsible for the late cerebral ischemia. The intracellular pathways responsible for this receptor upregulation remain unclear, but it has now been shown both in vivo and in vitro that PKC and MAPK are key elements in this upregulation. We suggest that the increase in cerebrovascular receptor expression that is seen after SAH in the vascular smooth muscle cells (endothelin type B and 5-hydroxytryptamine type 1B) occur through transcription/translation and results in increased vascular tone, which in turn ends up in late cerebral vasconstriction, reduced cerebral blood flow (CBF), and subsequent development of cerebral ischemia. We suggest that these cerebrovascular alterations are mediated through the intracellular signaling pathways that involve PKC and MAPK.

MAPK is a family of serine/threonine kinases that consists of 3 well-characterized MAPKs: the extracellular signal-regulated kinases (ERK)1/2, the p38, and the c-jun N-terminal kinase (JNK). JNK and p38 are cellular stress-activated protein kinases responding to different types of stress, whereas ERK1/2 is activated by, for example, growth factors and shear stress.
Protein kinase C is another family of serine/threonine kinases that comprises at least 12 different isoforms. PKCs are activated by different stimuli such as growth factors, hormones, and neurotransmitters and participate in cellular processes such as growth, differentiation, and apoptosis.

The present study was designed to understand the signal transduction responsible for receptor upregulation and the subsequent reduction in CBF after SAH. Therefore, we asked the following questions: which of the different PKC isoforms and MAPK pathways are activated in cerebral ischemia after SAH and what is their temporal relation? In addition, we suggested that MAPK or PKC inhibitor will prevent the activated intracellular pathways after SAH and that both large cerebral arteries and intracerebral microvessels will show similar specific protein kinase activation.

Materials and Methods

All animal procedures were carried out strictly within national laws and guidelines and approved by the University Animal Experimentation Inspectorate.

Rat Subarachnoid Hemorrhage Model

Subarachnoid hemorrhage was induced by a model carefully described by Prunell et al. Male Sprague-Dawley rats were anesthetized using 5% halothane (Halocarbon Laboratories, River Edge, NJ) in N2O/O2 (30:70). The rat was intubated and artificially ventilated with inhalation of 0.5% to 1.5% halothane in N2O/O2 (70:30) during the surgical procedure. An arterial catheter to measure blood pressure was placed in the tail artery and a catheter to monitor intracranial pressure was placed in the subarachnoid space under the suboccipital membrane. At either side of the skull, holes were drilled through the skull bone down to dura mater allowing the placement of 2 laser Doppler flow probes to measure cortical CBF. Finally, a 27-G blunt canula with a side hole was introduced stereotactically 6.5 mm anterior to bregma in the midline at an angle of 30° to the vertical placing the tip of the needle just anterior to the chiasma. After 30 minutes of equilibration, 250 μL blood was withdrawn from the tail catheter and injected intracisternally 30 minutes before the induced SAH and after SAH. Fifty microliters 10-6 M of the inhibitor or vehicle was injected intracranially 30 minutes before the induced SAH and after the SAH, 20 μL 10-5 M of the inhibitor was given repeatedly after 3, 6, 24, and 32 hours from the first injection. This dose was chosen on the basis of previous detailed work on isolated cerebral arteries: the dose was chosen at near maximum inhibition and calculation of cerebrospinal fluid volume/turnover. After 48 hours, the rats were anesthetized with CO2 and decapitated.

Harvest of Cerebral Arteries

The rats from the various groups were anesthetized with CO2 and decapitated. The brains were quickly removed and chilled in ice-cold bicarbonate buffer solution. The circle of Willis arteries were dissected free from the brain. Circle of Willis arteries and the brain were immediately snap-frozen at −80°C and used for subsequent Western blot.

Cerebral Microvessel Isolation

Isolated brains from the different groups were gently Dounce homogenized in ice-cold phosphate-buffered saline and centrifuged at 720g for 10 minutes at 4°C. The supernatant was discarded, and pellets were resuspended in ice-cold phosphate-buffered saline. The resuspended pellet was layered over a 15% dextran solution (35 to 40 kDa) and centrifuged in a swinging bucket rotor at 1300g for 30 minutes×2 at 4°C. The aqueous supernatant was discarded and the pellet containing cerebral blood vessels was collected and washed with ice-cold phosphate-buffered saline over a nylon mesh (50 μm). The isolated vessel preparation consists of a mixture of arteries, arterioles, veins, venules, capillaries, and perivascular elements as confirmed before.

Tissue Lysis and Protein Content Determination

After microvessel isolation or dissection of the circle of Willis arteries, the vessels were collected and placed on ice and homogenized in lysis buffer with protease and phosphatase inhibitors. After 20 minutes incubation in lysis buffer on ice, homogenates were centrifuged at 4500g for 10 minutes at 4°C and supernatant collected. Total protein concentration was determined using a BioRad DC kit (Hercules, Calif) and measuring absorbance at 750 nm on a Genesys 10 spectrophotometer (Thermo, Waltham, Mass). Lysates were used immediately or stored at −80°C.

Western Blot Analysis

Proteins of interest were evaluated in microvessels and circle of Willis arteries from the various groups. Lysates were dissolved in Tris-glycine sodium dodecyl sulfate sample buffer and boiled for 5 minutes. Equal amounts of protein (50 μg/lane) were loaded on a 8% Tris-glycine gel (Invitrogen A/S, Taastrup, Denmark) and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Molecular weight markers (New England BioLabs, Ipswich, Mass) were loaded on each gel for protein band identification. After separation, proteins were transferred to a nitrocellulose membrane (BioRad). Subsequently, the membrane was blocked with 6.5% nonfat milk in Tween-TBS overnight 4°C. Membranes were then incubated with the primary antibody of interest: pPKCα, pPKCβ, pPKCδ, pPKCB, pPKCD, pPKCγ, or pPKCη (1:1000 dilution; Biosource, Camarillo, Calif); pPKCb, p-p38, or pJNK (1:1000 dilution; Cell Signaling Technology, Beverly, Mass); pERK1/2 (1:5000; Promega, Madison, Wis); or β-actin (1:1000 dilution; Sigma, St Louis, Mo) for 1 hour at 37°C followed by 3×5 minutes wash with Tween-TBS. Subsequently, the membranes were incubated with the appropriate secondary antibody: goat anti-rabbit IgG–horseradish peroxidase or goat anti-mouse IgG–horseradish peroxidase (1:5000; Pierce, Rockford, Ill) for 1 hour at room temperature followed by 5×5 minutes wash with Tween-TBS. Levels of β-actin were used to confirm equal loading of the lanes. The membranes were developed using the...
Supersignal Dura kit (Pierce) and visualized using a Fujifilm LAS-1000 Luminiscent Image Analyzer (Stamford, Conn).

Calculations and Statistics
Data are expressed as mean ± SEM. Statistical analyses were performed with Kruskal-Wallis nonparametric test with Dunn’s post hoc test in which P < 0.05 was considered significant.

Cerebrovascular protein lysates from the different groups were compared. Cerebral arteries from 2 animals were pooled for each group of experiment and each experiment was repeated 3 times for both microvessels and circle of Willis arteries. Quantitation of band density was performed with the electrophoresis computer analysis program Fujifilm Science Laboratory Image Gauge 4.0. The immunoblot optical density values were determined with repeated measurement and presented as percentage activity in the treated groups compared with the sham or 0-hour groups, in which the 0-hour or sham group was set to 100%.

Results

Subarachnoid Hemorrhage Model
The mortality rate of the animal model of SAH was 5% and there was no difference in the mortality rate between the groups. On the neurological examination, all SAH animals received a score of 1 and the sham and PKC- or raf-treated groups. The phosphorylated ERK1/2 levels were activated at 1 hour (162 ± 31%) and at 48 hours (122 ± 18%) after SAH and the PKCα (123 ± 18%) was increased only at 48 hours after SAH compared with the control. The activation of PKCβ at time point 48 hours was not significant (P = 0.15). There was no activation in PKCe, PKCβI, PKCβII, PKCγ, PKCη, and PKCθ protein expression in cerebral blood vessels, neither in large cerebral arteries nor in brain microvessels after SAH as compared with control vessels (data not shown). The same pattern of activation was seen in circle of Willis arteries and in cerebral microvessels (Table).

Western Blot
Because the results from the 2 types of cerebral vessels were identical (Table), the results from the separate Western blots of circle of Willis arteries and cerebral microvessels (n = 3 to 4 in each group) were pooled together for the further statistical analysis. The sham-operated and 0-hour rats showed similar results and were used as controls.

Activation of Protein Kinase C Pathway
An initial study (data not shown) revealed that a positive PKC subtype signal was only recorded of the 8 studied PKC subtypes. Therefore, we studied henceforth activation of 8 PKC isotypes during the time course 0 to 48 hours after SAH. The study revealed that there was only enhanced phosphorylation of PKCδ (Figure 1A) and PKCa (Figure 1B). The protein levels of PKCδ were activated at 1 hour (162 ± 31%) and at 48 hours (122 ± 18%) after SAH and the PKCα (123 ± 18%) was increased only at 48 hours after SAH compared with the control. The activation of PKCδ at time point 48 hours was not significant (P = 0.15). There was no activation in PKCe, PKCβI, PKCβII, PKCγ, PKCη, and PKCθ protein expression in cerebral blood vessels, neither in large cerebral arteries nor in brain microvessels after SAH as compared with control vessels (data not shown). The same pattern of activation was seen in circle of Willis arteries and in cerebral microvessels (Table).

Activation of Mitogen-Activated Protein Kinase Pathway
The ERK1/2 protein levels were activated at 1 hour after SAH (178 ± 5%), whereas JNK (215 ± 62%) and p38 (194 ± 27%) were increased only at 48 hours after SAH (Figure 2A–C). The phosphorylated ERK1/2 levels were activated at the time points 1, 6, and 48 hours after SAH. The same pattern of activation was seen in circle of Willis arteries and in cerebral microvessels (Table).

Treatment With Protein Kinase C or raf Inhibitor
Intrathecal administration of the PKC inhibitor RO-31-7549 prevented the activation of PKCδ (52 ± 3%) and PKCa...
(59±5%) protein levels in the cerebral arteries as compared with the SAH (152±13%) and (156±13%) for pPKC8 and α, respectively. The raf inhibitor also prevented the activation of pPKC8 (89±14%) and pPKCα (73±12%) protein levels as compared with the SAH (Figure 3A–B). The PKC inhibitor prevented the activity of pERK1/2 (96±13%), whereas the raf inhibitor SB386023-b prevented the activity of pERK1/2 (78±12%) to a higher degree as compared with SAH (146±7%; Figure 3C). The PKC inhibitor RO-31–7549 was not able to inhibit the activation of p-p38 (189±26%) or pJNK (156±13%) as compared with the SAH (178±23%) and (184±48%) for p-p38 and pJNK, respectively, at the time point 48 hours. The raf inhibitor was not able to inhibit the activation of p-p38 (169±25%) and pJNK (157±29%) as compared with SAH at the time point 48 hours.

**Discussion**

We have demonstrated that SAH results in a general reduction of regional CBF and this is associated with enhanced expression of endothelin and 5-hydroxytryptamine receptors in the smooth muscle cells of cerebral arteries.13,22 After the brief initial rise in intracranial pressure and drop in CBF, which normalized within 1 hour, there is a subsequent reduction in CBF and cerebral metabolism26 that occurs in parallel with angiographic vasoconstriction.27 This latter phase is more rapid in rodents than in people and is associated with the clinical deterioration. Our hypothesis suggests that
the late cerebral ischemia is due in part to vascular receptor upregulation. In the present study, we hypothesize that subtypes of PKC and MAPK are initiators and relate to changes in CBF. The results are the first to reveal the time course for activation of PKC isoforms and MAPK pathways in cerebral arteries after experimental SAH. It is widely accepted that the degree of MAPK phosphorylation directly correlates with MAPK activity. However, this may not necessarily be the case with PKC phosphorylation; however, the general rule is that there is a correlation between activity and phosphorylation. \(^{28}\) We found that 8 PKC isoforms are expressed in cerebral arteries. Notably, it was only PKC\(\delta\) that was activated at 1 hour and at 48 hours, whereas PKCo was increased only at 48 hours after SAH. The other PKC isoforms showed no activation. Thus, it may be concluded that the novel and classical PKCs are the primary PKCs involved in the pathogenesis of cerebral ischemia after SAH but at different time points, putatively having different roles in cerebral vessels during cerebral ischemia. This agrees well with other studies on cerebral ischemia; an increased level of PKC activation is so far unreported for cerebral arteries.

In this study, we observed that ERK1/2, JNK, and p38 were activated after SAH, however, at different time points. The phosphorylated ERK1/2 level was activated at the time points 1, 6, and 48 hours after SAH. We hypothesize that ERK1/2 activation is essential to trigger the subsequent cerebrovascular effects of SAH—the late cerebral ischemia with flow reduction and endothelin type B and 5-hydroxytryptamine type 1B receptor upregulation. In fact, treatment with a raf inhibitor abolished both these responses as well as normalizing the neurological outcome and CBF. \(^{11,13}\) The other 2 MAPKs, JNK and p38, were first activated 48 hours after SAH. These 2 MAPKs are associated with stress, apoptosis, and cell death; thus, such mechanisms may have increasing importance in the late part of ischemia after SAH. Several other studies have reported increased activity of all 3 groups of MAPK in cerebral arteries after SAH, but none has examined the time course. \(^{36}\) It is therefore important to unravel the temporal changes and sequential activation of MAPK. In addition, the event is associated not only with the basal circle of Willis activation (with the blood deposition), but also with the intracerebral microvessels, which may shed more light on the reasons behind the general regional CBF reduction \(^{13,22}\) and the ischemia which, in people, sometimes is observed despite no angiographic vasospasm. \(^{37}\) We have observed that both the larger cerebral arteries and the cerebral microvessels are involved in cerebral ischemia after SAH. One possibility may be that the microvessels are involved in the ischemia that occurs without angiographic vasospasm and the larger arteries might be involved in the ischemia where vasospasm takes place.

Recent in vivo studies revealed that RO-31–7549\(^{22}\) or SB386023-b\(^{13}\) treatment at the start of the SAH abolished the vascular receptor changes, the regional CBF reduction, and neurological deterioration. Therefore, we hypothesize that the PKC and MAPK signaling pathways may interact in the cerebral blood vessels. Our study revealed that treatment with a raf inhibitor in part prevents the increased activation of PKC\(\delta\) and PKCo at the 48-hour time point after SAH. However, treatment with a PKC inhibitor prevents the increased activity of PKC\(\delta\) and PKCo to a much higher degree compared with a raf inhibitor. At the same time, a PKC inhibitor prevents the increased activity of ERK1/2, whereas a specific raf inhibitor prevents the activity to a much higher degree. Neither the PKC inhibitor RO-31–7549 nor the raf inhibitor SB386023-b was able to inhibit the activation of p38 and JNK at the time point of 48 hours. This implicates that the inhibitor is selective for the PKC and ERK1/2 pathway. This agrees with the growing evidence of molecular crosstalk between the PKC and MAPK pathways. However, this is not surprising considering that cerebral ischemia after SAH is a multifactorial process that involves different pathological changes at different periods. \(^{38}\)

The ERK1/2 and PKC\(\delta\) are activated at 1 hour after SAH and we have demonstrated that the CBF is unchanged at this time point because CBF is returning to baseline within 1 hour. So there is no link in the change in ERK1/2 and PKC\(\delta\) relative to change in CBF at early time points after SAH. However, there is a link at the late time point 48 hours in which we have shown a decrease in CBF. \(^{11,13}\)

In conclusion, we have shown that PKC\(\delta\) is activated at 1 hour and at 48 hours after SAH. The ERK1/2 was similarly activated early and remained elevated at the time points 6 and 48 hours, whereas PKCo, JNK, and p38 were increased only at 48 hours after SAH. These findings suggest that ERK1/2 and PKC\(\delta\) are key pathways for the initiation of cerebral ischemia after SAH. PKCo, JNK, and p38 may have a role in the late phase of events in cerebral arteries after SAH. In addition, the study revealed that treatment with a PKC or raf inhibitor prevents the activation of the kinase that occurs after SAH. These findings suggest that the PKC and MAPK ERK1/2 pathway may be a novel therapeutic target in treatment of cerebral vasospasm and ischemia after SAH.

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Disclosures

None.

References

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