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

Saema Ansar, DMSci; Lars Edvinsson, MD, PhD

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 PKCε 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)
Protein kinase C is another family of serine/threonine kinases that comprises at least 12 different isoforms. Depending on their activity and structure, they are divided into 3 groups: the novel, the conventional, and the atypical PKCs. 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 activate MAPK or PKC 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 using 5% halothane (Halocarbon Laboratories, River Edge, NJ) or vehicle in conjunction with the operation and after the induced SAH. Fifty microliters 10⁻³ M of the inhibitor or vehicle was injected intracisternally 30 minutes before the induced SAH and after the SAH, 20 μL 10⁻³ 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 

**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 720 g 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 1300 g for 30 minutes at 4°C. The resulted supernatant was discarded, and the pellets containing cerebral blood vessels were collected and washed with ice-cold phosphate-buffered saline over a nylon mesh (50 μm).

**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 4500 g 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 1 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).

The membrane was blocked with 6.5% nonfat milk in Tween-TBS overnight 4°C. Membranes were incubated with the primary antibody of interest: pPKCα, pPKCβ, pPKCε, pPKCγ, pPKCβII, pPKCβI, or pPKCa (1:1000 dilution; Biosource, Camarillo, Calif); pPKCa, p-p38, or pJNK (1:1000 dilution; Cell Signaling Technology, Beverly, Mass); 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...
Table. Activation of Protein Levels in Cerebral Microvessels and Circle of Willis After SAH

<table>
<thead>
<tr>
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<th>Microvessel</th>
<th>Circle of Willis</th>
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<tbody>
<tr>
<td>pPKC6 1 hour after SAH</td>
<td>132±17</td>
<td>187±53</td>
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<tr>
<td>pPKCα 48 hours after SAH</td>
<td>154±21</td>
<td>130±17</td>
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<tr>
<td>pERK1/2 48 hours after SAH</td>
<td>170±7</td>
<td>187±4</td>
</tr>
<tr>
<td>pJNK 48 hours after SAH</td>
<td>175±36</td>
<td>268±96</td>
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<tr>
<td>p-p38 48 hours after SAH</td>
<td>173±30</td>
<td>141±24</td>
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</table>

Activation of the different protein levels measured with Western blot in cerebral microvessels and circle of Willis after SAH. Values are expressed as percentage of control and given as mean±SEM, n=3 to 4, *P<0.05. There was no significant difference between the microvessel and circle of Willis arteries; pPKC6 (P=0.4), pPKCα (P=0.4), pERK1/2 (P=0.1), pJNK (P=0.4), and p-p38 (P=0.5).

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.

Figure 1. Activation of pPKC6 (A) and pPKCα (B) protein levels in cerebral arteries after 0, 1, 3, 6, 12, 24, and 48 hours of SAH together with β-actin as a loading control are shown. Data are presented as the pPKC/β-actin mean optical density ratio relative to control. Data are expressed as mean±SEM. *P<0.05.

Supersignal Dura kit (Pierce) and visualized using a Fujifilm LAS-1000 Luminiscent Image Analyzer (Stamford, Conn).

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 had a score of 0. In all operated rats, mean arterial blood pressure (98±4 mm Hg), partial pCO2 (39±1 mm Hg), and partial pO2 (106±3 mm Hg) values were within acceptable limits during the operation. As a result of injecting the blood, the cortical CBF dropped over both hemispheres to 16±4% of resting flow and the intracranial pressure increased from 8±1 to 122±9 mm Hg. The laser Doppler blood flow and the intracranial pressure returned to the basal value within 1 hour of the insult.

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 were increased only at 48 hours after SAH (Figure 2A–C).

PKC isotypes during the time course 0 to 48 hours after SAH. The study revealed that there was only enhanced phosphorylation of PKC6 (Figure 1A) and PKCα (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 pPKCδ (52±3%) and pPKCα.
(59±5%) protein levels in the cerebral arteries as compared with the SAH (152±13%) and (156±13%) for pPKCβ and α, respectively. The raf inhibitor also prevented the activation of pPKCβ (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 PKCδ that was activated at 1 hour and at 48 hours, whereas PKCα 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 activated PKC has been observed after SAH.7,29 In a previous study using the same SAH model as the present one, angiographic examinations of the cerebral arteries revealed a biphasic vasospasm with a maximal acute vasoconstriction at 10 minutes and a late spasm (vasoconstriction) that was maximal at 2 days after SAH.37 Our study suggests that PKCδ plays a role in the early initiation of the cerebrovascular event, whereas PKCα seems to be involved in the late phase of cerebral vasospasm. The PKC subtype may have different roles in the pathophysiology—an early transient vasoconstriction phase and a late phase involving several processes, eg, late spasm, inflammation, and increased amounts of vasospastic substances. Our findings are in agreement with another study on dogs in which PKCδ was demonstrated to play a role in the initiation of cerebral vasospasm and PKCα in its maintenance.7 Recent evidence supports a role for brain PKCδ in reperfusion injury, and its inhibition was found to reduce histopathological damage and improve behavioral outcome after stroke.30,31 PKCδ has in particular been implicated in mediating oxidative stress, apoptosis, and inflammation, which all are hallmarks of cerebral ischemia,32-34 but it 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.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 death51; 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 reduction13,22 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-31–754922 or SB386023-b13 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 activity of PKCδ and PKCα at the 48-hour time point after SAH. However, treatment with a PKC inhibitor prevents the increased activity of PKCδ and PKCα 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 PKCδ 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 PKCδ 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 PKCδ 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 PKCα, JNK, and p38 were increased only at 48 hours after SAH. These findings suggest that ERK1/2 and PKCδ are key pathways for the initiation of cerebral ischemia after SAH. PKCα, 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.

Sources of Funding
This work was supported by the Swedish Research Council, the Heart and Lung Foundation (Sweden), the Royal Physiographic
Disclosures

References