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Evidence for the role of phosphatidylcholine-specific phospholipase in experimental subarachnoid hemorrhage in rats

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ABSTRACT

Neuron apoptosis and inflammatory responses contribute to subarachnoid hemorrhage (SAH)-induced early brain injury (EBI), which is the main aspect that affects patients' outcome. Previous research has demonstrated that phosphatidylcholine-specific phospholipase C (PC-PLC) plays critical roles in cell apoptosis and various inflammatory responses, and that tricyclodecan-9-yl-xanthogenate (D609), a well known PC-PLC inhibitor, is a powerful agent to protect brain from cerebral ischemic injury and SAH-induced cerebral vasospasm. However, the association between PC-PLC and SAH-induced EBI is undetermined. Therefore, we sought to investigate whether PC-PLC was implicated in SAH-induced EBI. Compared with sham group, an upregulation of PC-PLC activity was detected in the brain tissue and serum of SAH group. Pharmacological blockade of PC-PLC by D609 attenuated neurological behavior impairment, brain edema and blood–brain barrier (BBB) damage induced by SAH. In addition, D609 treatment significantly inhibited SAH-induced inflammatory response and neuron apoptosis. Furthermore, inhibition of PC-PLC in primary-cultured rat cortical neurons attenuated oxyhemoglobin (OxyHb)-induced apoptosis morphology and decrease in survival rate. In conclusion, our data suggest that PC-PLC participates in SAH-induced EBI.

Introduction

Currently, early brain injury (EBI) is considered as the primary cause of a poor prognosis for patients with aneurysmal subarachnoid hemorrhage (SAH) (Kikkawa et al., 2015). Inflammatory responses contribute to the pathophysiological processes of acute brain injuries such as stroke (An et al., 2014). As part of EBI associated with SAH is attributable to neuron apoptosis and inflammatory responses (Marbacher et al., 2014; Suzuki et al., 2010), pro-apoptosis and pro-inflammatory factors involved in the process may be potential therapeutic targets for SAH patients.

Phosphatidylcholine-specific phospholipase C (PC-PLC), an important member of phospholipase C family, has been implicated in several intracellular signal pathways of apoptosis and inflammatory responses, and is a promising therapy target for atherogenesis therapy (Li et al., 2010). As mammalian PC-PLC has not been cloned and its sequence and structural information are unavailable, PC-PLC activity assay and tricyclodecan-9-yl-xanthogenate (D609), a specific PC-PLC inhibitor, have been used as the tools for investigation of mammalian PC-PLC(Adibhatla et al., 2012; Zhang et al., 2010).

PC-PLC exists in rat cerebral cortex synaptosomes (Mateos et al., 2006). Pretreatment rat brain astrocytes with the specific PC-PLC inhibitor D609 could inhibit thrombin-induced matrix metalloproteinase-9 expression and astrocyte migration, which play a crucial role in pathological processes of brain inflammation and injury (Lin et al., 2013). PC-PLC mediated cigarette smoke extract-induced heme oxygenase expression in cultured cerebral endothelial cells, which has been recently demonstrated to exacerbate EBI produced by intracerebral hemorrhagic stroke (Shih et al., 2011). More interesting, as reported previously, D609 could reduce cerebral vasospasm after experimental SAH in rats (Yan et al., 2006), and that D609 could reduce the infarct volume and blood–brain barrier (BBB) extravasation, decrease the mortality and improve the neurological deficits in a middle cerebral artery occlusion-induced focal ischemia rat model (Chen et al., 2007), suggesting a potential brain-protective effect of D609. In conclusion, previous studies suggest that PC-PLC activation may contribute to brain injury and D609 may exert a protection effect on brain under several pathological conditions. However, none of the previous studies paid attention to the changes in PC-PLC activity during SAH or the role of PC-PLC in SAH-induced EBI.

Based on the evidence described above, in this study, we hypothesized and aimed to prove the hypothesis that PC-PLC activation may contribute to SAH-induced EBI, and that D609 would attenuate the development of EBI and promote neurobehavioral recovery after SAH.

Materials and methods

Animals and SAH model induction

As described in our previous study (Wang et al., 2014), a prechiasmatic cistern SAH model was induced in adult male Sprague–Dawley (SD, 250–300 g) rats in this study. All experiments were

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approved by the Ethics Committee of the First Affiliated Hospital of Soochow University and were performed in accordance with EU Directive 2010/63/EU for animal experiments. As shown in Fig. 1, different from sham group, inferior basal temporal lobe was always stained by blood in SAH group. Therefore, the brain tissue under and adjacent to the blood clots was taken for the analysis in our study. Schematic representation of the areas taken for assay was shown in Fig. 1.

Animal treatment

Firstly, 72 rats were randomly divided into four groups: sham group (n = 18), SAH + vehicle group (n = 18), D609-L (low-dosage, 5 mg/kg) group (n = 18) and SAH + D609-H (high-dosage, 10 mg/kg) group (n = 18). As described in previous study (Zhang et al., 2010), D609 was purchased from Sigma (M-5250) and dissolved in normal saline as the vehicle. D609 or vehicle at equal volume was injected intraperitoneally at 0.5 h after SAH. In all of the groups describe above, the blood and brain tissue samples were obtained separately from rats at 12 h, 24 h and 48 h after SAH (n = 6 for each time point) for the time course study of PC-PLC activity and the effect of D609 on PC-PLC activity. Secondly, 96 rats were randomly divided into four groups: sham group, SAH + vehicle group, D609-L group and SAH + D609-H group (n = 24 per group). Based on the results of the time course study, the blood and brain tissue samples were obtained from rats at 48 h after SAH. Among the 24 rats per group, 12 rats per group were separately used for blood–brain barrier (BBB) integrity assay (n = 6) and brain edema evaluation (n = 6), while brain tissues of six rats per group were embedded in paraffin and cut into paraffin sections at 4 μm thickness for terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining and immunofluorescence analysis, and brain tissues of another six rats per group were homogenized and lysed in ice-cold RIPA lysis buffer (Beyotime, P0013) for Western blot analysis. The blood of rats was collected by retro-orbital puncture using glass Pasteur pipettes, and subsequently, the blood serum was obtained and stored at −80 °C until used for PC-PLC activity and inflammatory cytokines analysis. Then, all the rats were deeply anesthetized by chloral hydrate (36 mg/100 g body weight), and the brain tissues, except that for brain edema evaluation, were collected after transcardially perfusion with ice-cold PBS. Flow chart and the experimental design were shown in Fig. 2.

Cell culture and treatment

Primary rat cortical neurons were obtained from 17-day-old Sprague–Dawley rat embryos as described previously (Pacifici and Peruzzi, 2012). To mimic SAH and evaluate the effect of D609 in vitro, cells were exposed to OxyHb (Ruibio, O7109) at concentration of

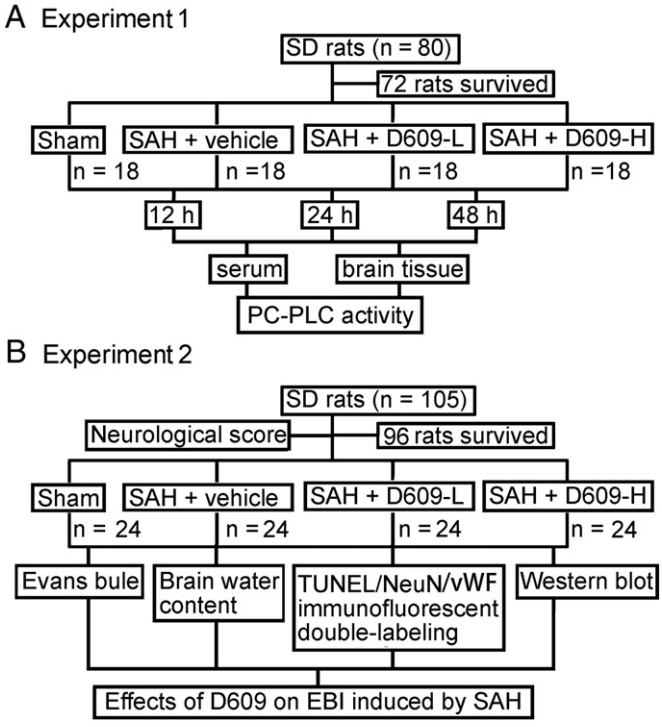

Fig. 2. Experimental design.

10 μM in the presence or absence of 5 μM (low dose) or 10 μM (high dose) D609 for 24 h before doing the following assays (Meguro et al., 2001). After these treatments, cellular morphology was observed by inverted phase contrast microscope, and the total protein of the cells was collected and stored at −80 °C until tested.

PC-PLC activity assay

PC-PLC activity in the serum, brain tissue homogenate and cultured neurons was measured by the Amplex Red PC-PLC-specific assay kit (Molecular Probes, A12218) according to the manufacturer's protocol as described previously (Wang et al., 2013; Zhang et al., 2010).

Neurological impairment

After treatment, all the rats were examined for behavioral impairment using a previously published scoring system and monitored for appetite, activity, and neurological defects (Table 1) (Wang et al., 2014).

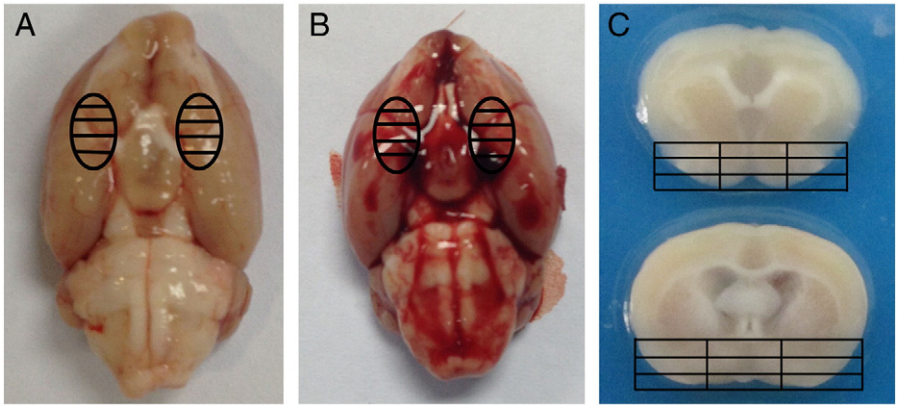

Fig. 1. The representative areas taken for assay. (A) Sham group. (B) Subarachnoid hemorrhage (SAH) group. (C) Schematic representation of the regions taken for Western blot and immunofluorescence assay.