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Simvastatin activates Akt/glycogen synthase kinase-3β signal and inhibits caspase-3 activation after experimental subarachnoid hemorrhage

Gao Cheng *, Wang Chunlei [1], Wu Pei [2], Liu Zhen [3], Liu Xiangzhen [4]

Department of Neurosurgery, The First affiliated hospital of Harbin Medical University, No.23, Youzheng Street, Nangang District, Harbin, China

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ABSTRACT

This study was designed to explore the role of simvastatin and its effects on the Akt/GSK3β survival signal and apoptosis pathway after experimental subarachnoid hemorrhage (SAH). SAH was induced by blood injection into the cisterna magna in New Zealand white rabbits. Increased expression of phospho-Akt and phospho-GSK3β was observed in brain tissue after SAH. Apoptosis and related proteins, including P53, apoptosis-inducing factor (AIF), cytochrome *C*, and cleaved caspase-3, were also activated. Simvastatin, at both low dose (10 mg/kg) and high dose (40 mg/kg), further increased expression of phospho-Akt and phospho-GSK3β, decreased activation of caspase-3, and inhibited apoptosis. Preserved blood-brain barrier and attenuated brain edema were observed following simvastatin treatment. In addition, the neuroprotective effects of simvastatin were blocked by wortmannin (2.5 μg/kg/min), an irreversible PIK3 inhibitor. P53, AIF, and cytochrome *C* were not affected by simvastatin treatment. Findings from the present study suggest that simvastatin ameliorates acute brain injury after SAH. The potential mechanisms of action include activation of the Akt/GSK3β survival signal and inhibition of caspase-dependent apoptosis pathway.

1. Introduction

Aneurismal subarachnoid hemorrhage (SAH) is associated with high mortality and morbidity despite sophisticated medical management and neurosurgical techniques. Twelve percent of patients with SAH die before reaching the hospital, and 40% die within one month of admission (Schievink, 1997). Acute brain injury (ABI) after SAH caused by aneurismal rupture is the most important factor in determining the prognosis of patients suffering from SAH (Ogungbo et al., 2001; Cahill et al., 2006), and severity of ABI is a key factor contributing to overall mortality related to SAH (Ostrowski et al., 2005). ABI has also been thought to play an important role in persistent cognitive deficits, psychosocial impairment, and decreased quality of life for patients who survive the initial hemorrhage and overcome vasospasms (Hütter et al., 2001). Many factors, such as acute cerebral ischemia (Park et al., 2004), subarachnoid blood toxicity (Matz et al., 2000a), and acute vasospasm (Bederson et al, 1998), are involved in the complicated mechanism of ABI. These factors lead to apoptotic cell death after SAH (Matz et al., 2000b; Park et al., 2004; Prunell et al., 2005). Accumulating evidence indicates that apoptosis plays an important role in ABI after SAH in both experimental and clinical settings (Matz et al., 2001; Nau et al., 2002; Park et al., 2004; Prunell et al., 2005). Apoptosis is at least – if not more – important than vasospasm as a major cause of mortality and morbidity in SAH patients (Cahill et al., 2007). Therefore, apoptotic cell death may be a potential therapeutic target for ABI after SAH.

Akt, also known as proteinase B, is a member of the serine/threonine kinase family and plays a crucial role in the cell death/survival pathway (Datta et al., 1997). It is activated by phosphorylation at the Ser473 residue and acts downstream of the phosphoinositide 3-kinase (PI-3K) pathway (Alessi et al., 1996). Activated Akt phosphorylates and inhibits downstream substrates, including glycogen synthase kinase-3β (GSK3β) (Cross et al., 1995), which makes neurons resistant to apoptotic stimuli by promoting cell survival and suppressing apoptosis (Hetman et al., 2000). In cerebral ischemia, traumatic brain injury, spinal cord injury, and SAH (Endo et al., 2006a, b; Noshita et al., 2001, 2002; Yu et al., 2005), the Akt survival pathway has been established to be involved in the mechanisms of apoptotic neuronal death.

HMG-CoA reductase inhibitors, also known as statins, were initially developed as cholesterol-lowering agents but have additional effects independent of their cholesterol-lowering mechanisms, which are known as pleiotropic effects (Miida et al., 2007; Sillberg et al., 2008). Statins reduce vasospasm and improve cerebral autoregulation, decreasing vasospasm-related delayed ischemic deficits after SAH (Tseng et al., 2005; 2006; McGirt et al., 2009). In our previous study, neuroprotective and anti-apoptotic effects of atorvastatin were

* Corresponding author. Tel.: +86 13604888921.
E-mail addresses: doctorgaocheng@yahoo.com.cn (G. Cheng), wangchunlei@163.com (W. Chunlei), wupei001@163.com (W. Pei), liuzhen8028@163.com (L. Zhen), liuxzhen001@yahoo.com.cn (L. Xiangzhen).
[1] Tel.: +86 15945695475.
[2] Tel.: +86 451 85555098.
[3] Tel.: +86 451 85555090.
[4] Tel.: +86 13904606614.

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observed in experimental SAH in rats; however, the mechanisms underlying atorvastatin-dependent inhibition of the apoptotic pathway were not elucidated (Cheng et al., 2009). The present study was designed to explore the role of simvastatin in SAH and the effects of simvastatin on the Akt/GSK3β survival signal and apoptosis pathway. Several apoptosis-related proteins were examined, including cytochrome *C* (representing the mitochondrial pathway), apoptosis-inducing factor (AIF, representing the caspase-independent pathway), and caspases 3 (representing the caspase-dependent pathway) (Cahill et al., 2007). P53 levels were also measured because P53 has been found to play an orchestrating role in apoptosis after SAH (Cahill et al., 2007). By examining these proteins, we hoped to provide an overview of the role of simvastatin in regulating apoptotic pathways after SAH.

2. Materials and Methods

These experiments conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85/23, revised 1996).

2.1. SAH Model and Grouping

The SAH model was induced as described previously (McGirt et al., 2006). New Zealand white rabbits, weighing 2.0-.5 kg, were anesthetized by intramuscular injection of a mixture of ketamine (50 mg/kg) and xylazine (10 mg/kg). After induction of anesthesia, ceftriaxone (20 mg/kg) was administered intramuscularly, and the atlanto-occipital membrane was exposed through a midline incision. After 1 ml of cerebrospinal fluid was aspirated by cisternal puncture, 2.5 ml of non-heparinized autoblood from the central ear artery was injected into the cisterna magna over 1 min. The animals were kept in the head-down position for 30 min and were then allowed to recovery. In the sham group, animals were irrigated with saline solution, and the incision was closed. During the operation, the animal's body temperature was maintained at 37 ± 0.5 °C with a heating blanket, and a 4F catheter was inserted into the left femoral artery to continuously monitor the mean arterial blood pressure (MABP) and blood gases.

The rabbits were randomly allocated into one of the following groups as described: Group A, the sham-operated control (Sham) group; Group B, the low-dose simvastatin treatment group (referred to as the L + T group), in which 10 mg/kg of simvastatin was injected subcutaneously 30 min, 12 hs, and 24 hs after SAH; Group C, the high-dose simvastatin treatment group (referred to as the H + T group), in which 40 mg/kg of simvastatin was administered in the same manner as in group B; Group D, the vehicle treatment group (referred to as the DMSO group), in which the same volume of DMSO was administered; Group E, wortmannin treatment plus high-dose simvastatin group (referred to as the W + S group), in which wortmannin (2.5 μg/kg/min) was injected intravenously 30 min before blood injection (Sanada et al., 2004) and the high-dose simvastatin was injected as described above.

Simvastatin (Zocor; Merck and Co., Inc.) was chemically activated by alkaline hydrolysis before use, as described previously (Madonna et al., 2005). A lower dose than is used in humans was selected. The high dose was chosen as the dose of simvastatin that reportedly attenuates cerebral vasospasm (McGirt et al., 2006). The dose of wortmannin was chosen based on a previous report showing abolishment of PI3-K activity with this dose (Sanada et al., 2004).

For analysis of brain edema and blood–brain barrier (BBB) permeability, four rabbits were used from each group. Animals underwent neurological scoring before being sacrificed. For Western blot, cell death assay, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL), and immunohistochemistry analyses, five animals were used from each group at each time point.

2.2. Intracranial Pressure Monitoring

A 2-mm skull hole was drilled in the right parietal bone and a microsensor ICP transducer (Codman, Cordis Corp.) was inserted into the brain. The transducer was connected with the intracranial pressure monitor (Codman ICP Express™, Professional, INC. PAYNHAM MA, USA), and continuous ICP monitoring was performed during the experimental procedure.

2.3. Neurological Scoring

Animals in each group were recorded for neurological scores, as described previously with slight modifications, by two independent researchers blinded to the groups (Endo et al., 1988; Zhou et al., 2007). Grading of neurologic deficit was as follows: 1, no neurologic deficit (scores = 0); 2, minimum or suspicious neurologic deficit (scores=1); 3, mild neurologic deficit (scores=2–3); 4, severe neurologic deficit (scores = 4–6). The scores of animals were recorded by using the modified scoring table (Table 1) as described previously (Kusaka et al., 2003; Zhou et al., 2007).

2.4. BBB Permeability

BBB permeability was evaluated as described previously (Cheng et al., 2009). Briefly, 2 mg/kg of Evan's blue was injected via the ear vein and circulated for 60 min. The leakage of Evan's blue was calculated as described previously (Gao et al., 2008).

2.5. Brain Water Content

The animals were sacrificed with overdose anesthesia and the brain was removed with the arachnoid layer intact. The wet weight of the brain was calculated, then the brain was dried in a 104°C oven for 24 h, and the dry weight was calculated. The water in the brain was calculated as [(wet weight dry weight)/ (wet weight)×100%].

2.6. Western Blotting Analysis

Fresh brain tissue was carefully removed from the basal cortex, brain stem, and hippocampus. The brain tissues were immediately frozen in liquid nitrogen and stored at 80 °C until used. The frozen tissues were homogenized for 20 min at 4 °C with an ultrasonic wave (10 seconds, 3 times) in 100 mL of an extraction buffer containing 50 mmol/L Tris-HCl (pH 7.5), 1% nonylphenol ethoxylate (Igepal), 0.25% sodium deoxycholate, 150 mmol/L NaCl, 1 mmol/L EGTA, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mg/mL aprotinin, plus leupeptin and pepstatin, 1 mmol/L Na_3VO_4, and 1 mmol/L NaF. The insoluble material was removed by centrifugation at 16 000 *g* at 4 °C for 15 min. The samples (20 to 30 μg protein) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis with a 7.5% polyacrylamide gel. After electrophoretic transfer of the separated polypeptides to nitrocellulose membranes, the membranes were blocked with 3% nonfat milk. The membranes were then washed and incubated with primary antibodies at 4 °C.

Table 1
Behavior scores.

Category	Behavior	Score
Appetite	Finished meal	0
	Left meal unfinished	1
	Scarcely ate	2
Activity	Active or standing	0
	Lying down,will stand and walk some stimulation	1
	Almost always lying down	2
Deficits	No deficits	0
	Unable walk due to ataxia or paresis	1
	Impossible to walk and stand due to ataxia and paresis	2