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Research Report

# Simvastatin attenuates axonal injury after experimental traumatic brain injury and promotes neurite outgrowth of primary cortical neurons

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ABSTRACT

The beneficial effects of simvastatin on experimental traumatic brain injury (TBI) have been demonstrated in previous studies. In this study, we investigated the effects of simvastatin on axonal injury and neurite outgrowth after experimental TBI and explored the underlying mechanisms. Wistar rats were subjected to controlled cortical impact or sham surgery. Saline or simvastatin was administered for 14 days. A modified neurological severity score (mNSS) test was performed to evaluate functional recovery. Immunohistochemistry studies using synaptophysin, neurofilament H (NF-H) and amyloid-β precursor protein (APP) were performed to examine synaptogenesis and axonal injury. Primary cortical neurons (PCNs) were subjected to oxygen glucose deprivation (OGD) followed by various treatments. Western blot analysis was utilized to assess the activation of phosphatidylinositol-3 kinase (PI-3 K)/Akt/mammalian target of rapamycin (mTOR) and glycogen synthase kinase 3β (GSK-3β)/adenomatous polyposis coli (APC) pathways. Simvastatin decreased the density of APP-positive profiles and increased the density of NF-H -positive profiles. Simvastatin reduced mNSS, which was correlated with the increase of axonal density. Simvastatin treatment stimulated the neurite outgrowth of PCNs after OGD, which was attenuated by LY294002 and enhanced by lithium chloride (LiCl). Simvastatin activated Akt and mTOR, inactivated GSK-3β and dephosphorylated APC in the injured PCNs. Our data suggest that simvastatin reduces axonal injury, enhances neurite outgrowth and promotes neurological functional recovery after experimental TBI. The beneficial effects of simvastatin on neurite outgrowth may be mediated through manipulation of the PI-3 K/Akt/mTOR and PI-3 K/GSK-3β/APC pathways.



## 1. Introduction

Diffuse axonal injury is well documented after head injury and is a major cause of long-term severe disability after traumatic brain injury (TBI) (Adams et al., 1989; Graham et al., 2000). Secondary injury, defined as a cascade of downstream events following initial mechanical injury, is a leading cause of axonal degeneration after TBI (Fitzpatrick et al., 1998).

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Axons undergo a sequence of changes following a primary insult that culminate in a secondary axotomy over a period of time (Maxwell et al., 1997). Theoretically, this process could be arrested or slowed, making therapies directed against axonal injury applicable during the time course of injury. Amyloid-$\beta$ precursor protein (APP) is a membrane spanning glycoprotein of nerve cells which is transported by fast axoplasmic flow and accumulates after axonal damage (Koo et al., 1990). APP immunohistochemistry has been shown to be an effective marker for axonal damage (Blumbergs et al., 1995; Bramlett et al., 1997).

Axons in the central nervous system (CNS) of mature animals undergo little spontaneous regeneration (Di Giovanni, 2009). The failure of severed adult CNS axons to regenerate could be attributed to both a reduced intrinsic capacity to grow and a heightened susceptibility to inhibitory factors of the CNS extracellular environment (Teng and Tang, 2006). Recent data support the hypothesis that diminished intrinsic regenerative ability of mature neurons is a major contributor to regeneration failure (Sun and He, 2010). As axonal injury and regeneration play a major role in functional recovery after TBI, several therapeutic strategies to date have been advanced targeting this important pathological process (Buki et al., 1999; Marion and White, 1996; Mills et al., 2011; Singleton et al., 2001).

Given the heterogeneous nature of TBI involving complex primary and secondary events, an ideal TBI therapy should target multiple injury factors. Our previous studies, and those of other researchers, have shown that simvastatin, independent of its cholesterol-lowering activity, mediates pleiotropic effects including modulation of neuroinflammation (Wu et al., 2010) and increase of cerebral blood flow (Cucchiara and Kasner, 2001) in the acute phase, suppression of apoptosis (Wu et al., 2008b) and reduction of excitotoxic death (Zacco et al., 2003) in the subacute phase, and induction of neurogenesis (Wu et al., 2008a) and angiogenesis (Wu et al., 2011) in the chronic phase after TBI. Recent studies also indicate that combination therapies of simvastatin with other agents attenuate axonal damage after CNS injury (Chauhan and Gatto, 2010; Shehadah et al., 2010). In light of these previous studies, simvastatin may be a promising candidate for TBI therapy.

In axon regeneration some distinct intracellular signaling pathways are recruited including the PI-3K/Akt pathway (Goold and Gordon-Weeks, 2004; Read and Gorman, 2009). In this pathway, GSK-3$\beta$ is one of the key downstream regulators of axon growth. By formation of GSK-3$\beta$/APC complex, GSK-3$\beta$ controls the microtubule-associated protein APC and regulates the microtubule dynamics during axon growth (Votin et al., 2005). As another important downstream target of PI-3K/Akt pathway, mTOR also plays a vital role in the intrinsic axonal regrowth of adult neurons. Activating mTOR induces extensive axon regeneration by promoting protein synthesis in the injured neurons (Park et al., 2008).

In this study we investigate the effect of simvastatin on axonal injury and neurite outgrowth after experimental TBI. To examine possible mechanisms underlying the beneficial effects of simvastatin, we focus on the PI-3K/Akt/mTOR and PI-3K/GSK-3$\beta$/APC pathways, which have been implicated as key intrinsic signaling pathways for axon regeneration (Barth et al., 2008; Park et al., 2008).

## 2. Results

### 2.1. Neurological outcomes after experimental TBI

Neurological functional deficits caused by injury in the left hemispheric cortex of rats were measured by mNSS (Fig. 1). Injury induced an mNSS score of approximately 12 at 1 day post TBI. Recovery began on day 3 after TBI and persisted for up to 14 days. Simvastatin treatment significantly reduced the mNSS compared to the saline treatment group at day 7 (ANOVA $P=0.001$; Sim vs Saline: $6.1\pm0.6$vs $8.4\pm0.7$, $P=0.028$) and day 14 (ANOVA $P<0.001$; Sim vs Saline: $5.2\pm0.4$vs $7.3\pm0.5$, $P=0.012$) post TBI (Fig. 1).

### 2.2. Change of axonal damage and synaptic density after simvastatin treatment

APP immunohistochemical staining was performed to measure the traumatic axonal injury (Culmsee et al., 2003) in the injured brain. TBI induced a marked appearance of APP immunoreactivity in cortical perikarya and axons. Simvastatin significantly decreased the density of APP positive axons in the ipsilateral hemisphere compared with saline-treated group (Fig. 2I–L, ANOVA $P<0.001$, simvastatin vs saline, $P=0.021$). In addition, axons were identified by fluorescent immunostaining of a pan-axonal NF-H marker, SMI-312, and by Bielschowsky silver staining. Simvastatin treatment significantly increased the density of NF-H-positive axons in the lesion boundary zone (Fig. 2E–H, ANOVA $P<0.001$, simvastatin vs saline, $P=0.007$) and density of Bielschowsky silver-positive axons in the striatal bundles (Fig. 2M–P, ANOVA $P<0.001$, simvastatin vs saline, $P=0.015$). Synaptophysin was employed to measure the level of synapses after TBI. TBI led to loss of synapses in the lesion boundary zone, which was partially reversed by treatment of simvastatin. Quantitative analysis revealed that simvastatin increased the density of synaptophysin in the ipsilateral hemisphere compared to the saline-treated

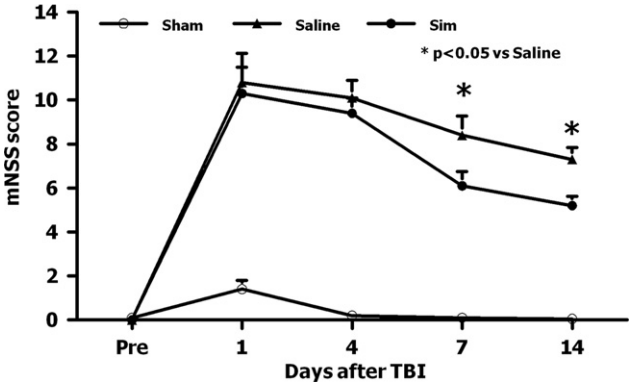


**Fig. 1 – Graph showing the effect of simvastatin on functional recovery after TBI. TBI significantly impairs sensory motor function at days 1–14 compared with sham controls. Treatment with simvastatin improves sensorimotor function measured by mNSS at days 7–14 compared with the saline group. Pre=pre-injury level. Data represent the mean±SD. $n$=8/group.**