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Simvastatin attenuates microglia, astrocyte activation and decreases IL-1 β Level following traumatic brain injury

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Abstract

Objective—Our previous studies demonstrated that simvastatin promotes neurological functional recovery after traumatic brain injury (TBI) in rat; however, the underlying mechanisms remain poorly understood. The purpose of this study was to investigate the anti-inflammatory effect of simvastatin by measuring the level of cytokines and activation of glial cells.

Methods—Controlled cortical impact injury was performed in adult male Wistar rats. The rats were randomly divided into three groups: sham, saline control group and simvastatin treatment group. Simvastatin was administered orally starting at day 1 after TBI until sacrifice. Animals were sacrificed at 1, 3, 7, 14, and 35 days after treatment. Functional outcome was measured using modified neurological severity scores (mNSS). ELISA and immunohistochemical staining were employed to measure the expression of IL-1 β , IL-6 and TNF- α , and to identify activated microglia and astrocytes.

Results—At days 1 and 3 after simvastatin or saline treatment, cytokine levels in the lesion boundary zone were significantly higher in the simvastatin-treated rats and saline-treated rats compared to the sham group, peaking at day 3. Simvastatin only reduced the level of IL-1 β but not IL-6 and TNF- α compared with the saline group. Also, simvastatin reduced significantly the number of activated microglia and astrocytes compared to the saline control animals. There was also a trend towards improvement of mNSS score, reaching statistical significance ($P=0.003$) towards the end of the trial.

Conclusion—Our data demonstrate that TBI causes inflammatory reaction, including increased levels of IL-1 β , IL-6 and TNF- α , as well as activated microglia. Simvastatin selectively reduces IL-1 β expression and inhibits the activation of microglia and astrocytes after TBI, which may be one of the mechanisms underlying the therapeutic benefits of simvastatin treatment of TBI.

Keywords

Astrocyte; Interleukin 1 beta; Microglia; Simvastatin; Traumatic brain injury

INTRODUCTION

Traumatic brain injury (TBI) remains a major public health problem globally. None of the current interventions including management of cerebral edema and intracranial hypertension has been demonstrated to significantly improve long-term functional outcome and there remains a compelling need for more effective therapeutic options in this patient population (4). Accumulating data suggest that brain trauma is associated with a neuroinflammatory response characterized by microglial and astrocytic activation, as well as the release of pro-inflammatory cytokines (22,23). This neuroinflammatory cascade is implicated in the development of cerebral edema, breakdown of the blood–brain barrier, and secondary neuronal injury. Cytokines are upregulated in the brain after a variety of insults including TBI, and are expressed not only in cells of the immune system, but also produced by resident brain cells, including glia and neurons (29). The most studied cytokines related to inflammation are interleukin-1 (IL-1), TNF- α , and interleukin-6 (IL-6). Among these cytokines, IL-1 and TNF- α exacerbate cerebral injury (14); however, the effect of IL-6 is controversial. Some studies indicate IL-6 may be neuroprotective (34). Using anti-inflammatory compounds for the treatment of TBI to attenuate the deleterious consequences of cerebral inflammation and reduce delayed cell death is a promising therapy (33). However, at present there are no clinically viable therapeutic strategies targeting this mechanism.

3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) have been developed as lipid-lowering drugs. Mevalonic acid, the product of HMG-CoA reductase, is the precursor for key cellular isoprenoid compounds in addition to cholesterol. By inhibiting the production of one or more of these compounds, statins have been demonstrated to exert anti-inflammatory effects, which may be palliative in the setting of acute brain injury (9). Recent studies show that statins can inhibit a number of inflammatory processes known to be important to brain damage and suppress the secretion of cytokines such as IL-1 and TNF- α in spinal cord injury and ischemic stroke (2,7). Statins also inhibit the activation of microglia and astrocytes after brain insults (19). These findings suggest that statins are promising candidates as anti-inflammatory compounds for TBI. Although there are some adverse effects with statin treatment such as myalgia, elevated liver enzymes and rare but severe rhabdomyolysis (17, 26), simvastatin is a medicine widely used in the clinic and at the same dose has been proved to be superior to atorvastatin in treating TBI (21), we used simvastatin as the intervention agent.

Our hypothesis is that oral administration of simvastatin after TBI in rats attenuates activation of microglia and astrocytes, suppresses the secretion of cytokines including IL-1, IL-6 and TNF α , and thereby inhibits post-injury inflammation at the lesion boundary zone and reduces tissue damage, which leads to recovery of neurological function.

MATERIALS AND METHODS

All procedures have been approved by the Henry Ford Hospital Animal Care Committee and Institutional Review Board.

Animal models

A controlled cortical impact model in rat was used (20). Male Wistar rats were anesthetized with chloral hydrate 350 mg/kg body weight, intraperitoneally. Rectal temperature was controlled at $37^{\circ} \pm 0.5^{\circ}\text{C}$ with a feedback-regulated water-heating pad. A controlled cortical impact device was used to induce the injury. Rats were placed in a stereotactic frame. Two 10-mm diameter craniotomies were performed adjacent to the central suture, midway between lambda and bregma. The contralateral craniotomy allowed lateral movement of cortical tissue. The dura was kept intact over the cortex. Injury was induced by impacting the left cortex (ipsilateral cortex) with a pneumatic piston containing a 6-mm diameter tip at the rate of 4 m/

s and 2.5 mm of compression. Velocity was measured with a linear velocity displacement transducer.

Experimental Groups

A total of 88 Male Wistar rats were randomly divided into three groups. Rats in the first group (n = 40) were exposed to TBI and given saline orally 1 day later and consecutively for 14 days. Rats in the second group (n = 40) were subjected to TBI, and 1 day later, simvastatin was administered orally at a dose of 1 mg/kg/day for 14 consecutive days. This dose was selected based on our previous study (6). Rats in the third group (n = 8) were subjected to sham surgery. Rats in the first and second groups were sacrificed at 1, 3, 7, 14 and 35 days after simvastatin or saline administration, and rats in sham group were sacrificed at 1 day after TBI. At each time point, eight animals in each group were sacrificed and their brains were harvested. Four out of eight rat brains were used for immunohistochemistry. The other four rat brains were homogenized for enzyme-linked immunosorbent assay (ELISA).

Brain sample preparation

Half of the brain tissue of the rats from each group was processed for preparation of paraffin-embedded sections, which were used for immunostaining analysis. Rat brains were removed after transcardiac perfusion with PBS and 4% paraformaldehyde. The brains were stored in 4% paraformaldehyde for 48–72 hours. Standard 2 mm-thick blocks of rat brain were cut on a rodent brain matrix (a total of 7 blocks from A to G) and embedded with paraffin. A series of adjacent 6 μ m-thick sections were cut for immunohistochemistry.

Immunohistochemical Staining

The brain sections, after being deparaffinized, were incubated in 2% bovine serum albumin (BSA)-phosphate buffered saline (PBS) at room temperature for 30 minutes, and subsequently incubated with mouse anti-CD68, anti-gial fibrillary acidic protein (GFAP) antibody (Dako, Carpinteria, CA) at 1:200 dilutions in PBS at 4°C overnight. Following sequential incubation with biotin-conjugated anti-mouse IgG (1:100 dilution, Dakopatts, Carpinteria, CA), the sections were incubated with an avidin-biotin-peroxidase system (ABC kit, Vector Laboratories, Inc., Burlingame, CA). DAB was then used as a sensitive chromogen for light microscopy.

Enzyme-Linked ImmunoSorbent Assay (ELISA)

Using ELISA kits (R&D Systems, Minneapolis, MN), IL-1- β , TNF- α and IL-6 expression were measured using equal amounts of lysate from tissue samples of the lesion boundary zone at different time points.

Statistical Analysis

All data are presented as means $\pm$ standard deviation. Data were analyzed by a one-way analysis of variance (ANOVA). Differences were determined to be significant with $P < 0.05$. All measurements were performed by observers blinded to individual treatments

RESULTS

1. Sensory-motor function after treatment of TBI with simvastatin

A modified Neurological Severity Score (mNSS) was used to assess posttraumatic neurological impairment, as previously described (5). The score consists of several clinical parameters, including tasks on sensory function, motor function, beam balance and reflex, whereby a point is given for failure of the task and no point for succeeding. A high score of mNSS indicates severe neurological dysfunction. In the present study, the mNSS was assessed at days 1, 3, 7,

14, and 35 after treatment with saline or simvastatin. Evaluation of task performance was performed by an investigator blinded to the treatment groups. TBI rats presented with high scores on day 1 after treatment. On day 3, recovery began, which persisted at all subsequent evaluation time points in both saline-treated and simvastatin-treated groups. The mNSS scores for the simvastatin-treated group were significantly decreased at day 35 after TBI when compared with the saline-treated groups (1.86 ± 1.08 versus 0.33 ± 0.18 , $P = 0.003$) (Fig. 1). These data suggest a trend towards functional improvement reaching statistical significance towards the end of the trial.

2. TBI Induces the Expression of IL-1 β , IL-6 and TNF- α Early in the Injured Cortex

To examine the temporal profile of cytokine expression in the injured cortex, lysates from the ipsilateral cortex of the saline-treated rats and sham surgery rats were collected. Levels of IL-1 β , IL-6 and TNF- α were tested by ELISA at different time points. The levels of IL-1 β , IL-6 and TNF- α were significantly higher compared to sham control animals at days 1 and 3 after saline treatment, (IL-1 β $P=0.002$, IL-6 $P=0.001$ and TNF- α $P=0.0004$ versus sham), with a peak at day 3. Seven days after saline treatment, the level of cytokines declined to the sham control levels (Fig. 2).

3. Simvastatin selectively downregulates the expression of IL-1 β without altering expression of IL-6 and TNF- α in the injured cortex

As the expression of cytokines peaked at day 3 after treatment, this time point was chosen to analyze the effect of simvastatin. Simvastatin significantly suppressed the level of IL-1 β from the injured cortex when compared to the saline group at day 3 after treatment ($P=0.040$) (Fig. 3A). There were no significant differences in the levels of IL-6 ($P=0.868$) and TNF- α ($P=0.538$) between the simvastatin- and saline-treated groups at day 3 (Fig. 3B, C). These data demonstrate that simvastatin selectively downregulates the production of IL-1 β but not IL-6 and TNF- α after TBI.

4. Simvastatin inhibits the activation of microglia after TBI

Since pro-inflammatory IL-1 β is predominantly expressed by activated microglia, and partially by astrocytes in the injured brain, we wanted to investigate the effect of simvastatin on the activation of microglia and astrocytes. CD68 is a specific marker for activated microglia. Immunostaining of CD68 shows that simvastatin significantly reduced the number of CD68-positive cells at the lesion boundary zone. There was a significant decrease in microglia activation in the simvastatin-treated group compared to the saline-treated group (956 ± 347 cells/mm² in the simvastatin-treated group versus 1754 ± 112 cells/mm² in the saline treated-group, $n = 4$, $P = 0.001$) (Fig. 4). These data suggest that simvastatin suppresses the activation of microglia.

5. GFAP-positive astrocytes were reduced after treatment with simvastatin

Aside from traditional inflammatory cells, astrocytes are known to express different kinds of inflammatory mediators and play an important role in secondary injury post TBI. Reactive astrocytes are identified by increased immunoreactivity of GFAP, which is a distinct marker for astrocytes (12). In normal brain tissue (sham group), few astrocytes expressed GFAP (Fig. 5A, D). However, a robust increase in GFAP immunoreactivity and hypertrophic morphology of astrocytes were observed at the lesion boundary zone following TBI (Fig. 5B, E). The increased GFAP immunoreactivity was markedly attenuated in the simvastatin-treated rats compared to saline-treated rats (796 ± 147 cells/mm² in the simvastatin-treated group versus 1327 ± 156 cells/mm² in the saline treated-group, $n = 4$, $P = 0.001$) (Fig. 5C, F). These data show that treatment with simvastatin suppresses the proliferation and hypertrophy of reactive astrocytes at the lesion boundary zone after TBI.

DISCUSSION

The primary findings in the present study are: 1) Simvastatin selectively reduces secretion of IL-1 β but not IL-6 and TNF- α in the lesion boundary zone of cortex after TBI, and 2) Simvastatin suppresses the activation of microglia and astrocytes at day 3 after treatment in the injured cortex of rats after TBI.

The mNSS data shows statistically significant improvement only at day 35 after TBI. However, a clear trend towards improvement was seen as early as day 1 after treatment and this persisted till the end of the trial. Additional long-term functional studies after simvastatin treatment of TBI are needed to confirm these beneficial effects.

Cytokines are upregulated in the brain after a variety of insults including TBI. The most studied cytokines related to inflammation after TBI are IL-1 β , TNF- α , and IL-6 (18,22). Among those cytokines, IL-1 β and TNF- α appear to exacerbate cerebral injury. However, there is controversy about the effects of IL-6 with some studies reporting that IL-6 may be neuroprotective (8). As previous studies have demonstrated, simvastatin can reduce the inflammation response in damaged tissue post spinal cord injury and in ischemic stroke via the suppression of TNF- α , iNOS, and IL-1 β (28). We thereby investigated the levels of these cytokines in the lesion boundary zone after TBI. Measuring temporal changes of these cytokines after TBI, we found that the levels of IL-1 β , IL-6 and TNF- α were significantly elevated compared to sham animals at days 1 and 3 after saline treatment, with a peak at day 3. Seven days after TBI, the expression declined to the sham control levels. Since the levels of all cytokines peak at day 3, this time point was selected for further study. Our data demonstrate that treatment with simvastatin significantly reduces the level of IL-1 β in the injured cortex compared to the saline group at day 3 after treatment. There were no significant differences in the levels of IL-6 and TNF- α between simvastatin- and saline-treated groups at this time point. These data demonstrate that simvastatin selectively downregulates the production of IL-1 β instead of IL-6 and TNF- α after TBI.

Previous studies have demonstrated that elevated levels of inflammatory cytokines such as IL-1 β and TNF- α predict secondary brain injury and poor long-term outcome after brain injury (11,15), and pharmacological strategies aimed at reducing the activity of these pro-inflammatory cytokines attenuate tissue damage (35). In the current study, we found robust upregulation of IL-1 β , TNF- α and IL-6 in traumatized brain. Simvastatin selectively attenuated the upregulation of IL-1 β at day 3 after treatment. These data are similar to an earlier report that statins inhibit induction of pro-inflammation cytokines such as TNF- α and IL-1 β in primary glial cell culture (27). IL-1 β is the key mediator of the acute inflammatory host response. It has multiple pro-inflammatory and cell growth modulatory actions (3). Single bolus injection or parenchymal expression of IL-1 β in rodents increases expression of pro-inflammatory cytokines, chemokines and cell surface adhesion molecules within the brain parenchyma (31). While elevated expression of IL-1 β leads to a poor functional outcome post neuronal injury (1), blockage of IL-1 β signaling by central overexpression of IL-1 receptor antagonist results in delayed proinflammatory cytokine induction and improved neurological recovery after TBI, indicating a central role of IL-1 β in neuroinflammation (35).

IL-1R1 knockout mice lack IL-1 β signaling in the setting of penetrating brain injury which causes dramatic attenuation in microglial and astrocytic activation (30), suggesting that there is a close interrelationship between IL-1 β and microglia and astrocytes. IL-1 β can induce activation of astrocytes and microglia, and IL-1 β , TNF- α and IL-6 are mainly produced by activated microglia and astrocytes after brain insult (13). We therefore further investigated the change of microglia and astrocytes after treatment with simvastatin. Microglia plays a critical role as resident immuno-competent and phagocytic cells in the CNS. Once activated, microglia

can undergo morphologic transformation into phagocytes and serve as scavenger cells. Microglial activation may be induced by brain trauma, causing a release of a variety of proinflammatory substances including cytokines, chemokines, and trophic factors (16). These substances in turn may exacerbate the secondary injury in the lesion boundary zone after TBI. Our data demonstrate that simvastatin significantly attenuates the activation of microglia after TBI compared to the saline control group ($P = 0.001$) in the lesion boundary zone. Astrocytes can respond to activated microglia by receiving their paracrine signals. Astrocytes which contribute to the blood brain barrier may weaken tight junctions (36), resulting in the extravasation of inflammatory signaling molecules into the CNS capillary bed as well as the migration of other leukocytes into the site of microglia activity. In the current study, we find that activation of astrocytes marked by GFAP was significantly suppressed by simvastatin compared to saline control ($P = 0.001$). These data indicate that treatment with simvastatin significantly reduces the number of activated microglia and astrocytes within the lesion boundary zone at the early phase after TBI.

The mechanisms by which simvastatin suppresses induction of IL-1 β and activates microglia and astrocytes remain elusive. Some studies propose that the suppression may be mediated by interference with nuclear factor- κ B (NF- κ B) transcription pathway (37) or reduction of isoprenylation of protein involved in cellular signaling and inflammation (10). The transcription factor NF- κ B is a crucial mediator in the IL-1 signaling pathway and acts as a major driving force behind the induction of adhesion molecules and chemokines (24). It has recently been shown that simvastatin treatment abolishes the activation of NF- κ B observed in vehicle control animals and then inhibits the IL-1 β expression (32).

The reduction in the early expression of IL-1 β , attenuation of microglia and astrocytes activation, and improved neurological function observed in this study support the hypothesis that early suppression of the inflammatory cascade by simvastatin may result in a marked reduction in secondary neuronal damage after TBI.

In conclusion, our data demonstrate that TBI causes significant inflammatory reaction, including increased levels of IL-1 β , IL-6 and TNF- α as well as activated microglia and astrocytes. Simvastatin selectively reduces IL-1 β expression and inhibits activation of microglia and astrocytes after TBI, which may be one of the mechanisms underlying the therapeutic benefit of treating TBI with simvastatin. Although both animal model experiments and some phase-2 clinical trials (25) have suggested the beneficial effects of statins, large scale, randomized, double-blind clinical trials are needed to validate the beneficial effects of simvastatin after TBI.

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Figure 1.

Plot shows the temporal profile of the modified neurological severity score in sham, saline- or simvastatin-treated rats post TBI. The score was significantly lower in the simvastatin-treated group compared with the saline-treated group at 35 days after treatment. Data are represented as mean $\pm$ SD. * $P=0.0028$ versus the saline-treated group, $n=8$.

Figure 2.

Plots show the temporal profiles of ELISA of IL-1 β (a), TNF (b), and IL-6(c) in the lesion boundary zone of rats treated with saline after TBI. These cytokines increase on day 1 and peak on day 3 after treatment. Data are represented as mean $\pm$ SD. * $P < 0.05$ versus sham group.

Figure 3.

Bar graphs show the level of IL-1 β (a), TNF (b), and IL-6(c) in the lesion boundary zone of rats at day 3 after treatment with simvastatin or saline. Data are represented as mean $\pm$ SD. *P < 0.05 versus sham group, # P = 0.040 versus the saline group. IL-1 β expression was decreased at day 3 after simvastatin treatment compared to the saline-treated group. However, simvastatin did not suppress the expression of IL-6 and TNF compared to the saline-treated group.

Figure 4.

Immunohistochemical staining for CD68 at day 3 in the lesion boundary zone of the sham group (a, d), saline-treated group (b, e) and simvastatin-treated group (c, f). CD68 positive cells are stained brown. * $P < 0.05$ versus sham group, # $P = 0.001$ versus saline-treated group. Scale bar = 100 μm (a, b, c), 50 μm (d, e, f). Simvastatin significantly reduces the number of CD68-positive cells after TBI.

Figure 5.

Immunohistochemical staining of GFAP for the detection of reactive astrocytes on day 3 at the lesion boundary zone. This image shows a representative section of the sham group (a, d), saline-treated group (b, e) and simvastatin-treated group (c, f). The bar graph shows the statistic analysis of the number of GFAP positive cells between the saline control group and the simvastatin-treated group (g). *P < 0.05 versus sham group, # P = 0.001 versus saline group. Scale bar = 100µm (a, b, c), 50 µm (d, e, f). The GFAP positive cells (brown) were significantly decreased after treatment with simvastatin post TBI.