Published in final edited form as:

J Neurosurg. 2010 September ; 113(3): 591–597. doi:10.3171/2009.9.JNS09859.

Simvastatin Attenuates Astrogliosis after Traumatic Brain Injury through the Modulation of EGFR in Lipid Rafts

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Abstract

Objective—Our previous studies demonstrated that simvastatin treatment promotes neuronal survival and reduces inflammatory cytokine release from astrocytes after traumatic brain injury (TBI) in rats. Since reactive astrocytes produce inflammation mediators, in the current study we investigated the effect of simvastatin on astrocyte activation after TBI and its underlying signaling mechanisms.

Methods—Saline or simvastatin (1 mg/kg) was orally administered to rats starting at Day 1 after TBI and then daily for 14 days. Rats were sacrificed at 1, 3, 7, 14 days after treatment. Brain sections and tissues were prepared for immunohistochemical staining and Western blot analysis, respectively. Cultured astrocytes were subjected to oxygen-glucose deprivation (OGD) and followed by immunocytochemical staining with GFAP/caveolin-1 and Western blot analysis. Lipid rafts were isolated from the cell lysate and Western blot was carried out to detect the changes in epidermal growth factor receptor (EGFR) expression and phosphorylation in the lipid rafts.

Results—Simvastatin significantly promoted neuronal survival after TBI and attenuated activation of astrocytes. Simvastatin modified the caveolin-1 expression in lipid rafts in astrocyte cell membrane, suppressed the phosphorylation of EGFR in lipid rafts of astrocytes after OGD, and inhibited the OGD-induced interleukin-1 (IL-1) production.

Conclusions—These data suggest that simvastatin reduces reactive astrogliosis and rescues neuronal cells after TBI. These beneficial effects of simvastatin may be mediated by inhibiting astrocyte activation after TBI through modifying the caveolin-1 expression in lipid rafts and the subsequent modulation of EGFR phosphorylation in lipid rafts.

Keywords

simvastatin; EGFR; lipid rafts; astrocyte; traumatic brain injury

Traumatic brain injury (TBI) is a leading cause of serious, long-term disability worldwide. Widely distributed neuronal damage has been well documented in the cerebral cortex,

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Disclaimer The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

hippocampus, and dentate gyrus of the brain during the first few days after experimental TBI in rats.¹³ TBI results in a rapid response from resident astrocytes, a process often referred to as reactive astrogliosis which plays a key role in the process of neuro-inflammation.⁸ Although its role after neuronal injury is still controversial, reactive astrogliosis is believed to be detrimental to the injured neurons in the early phase after TBI.¹⁸ Our previous studies have demonstrated the beneficial effects of statins on functional recovery and neuronal survival after TBI in rats.²⁸ Although the mechanism of the neuroprotective effects of statins is still not clear, previous studies suggest that simvastatin has anti-inflammatory effects after TBI, including reducing the secretion of inflammatory mediators IL-1 β , interleukin-6 (IL-6) and tumor necrosis factor- α (TNF- α).^{2,9} Since the cytokines are mostly secreted by reactive astrocytes, inhibiting reactive astrocytes has become an appealing approach to attenuate neuro-inflammation and thereby protect neurons after TBI. Targeting the astrogliosis pathways hours or even days after the initial injury may prevent the progression of neuronal damage and be a promising therapeutic strategy.¹⁶

The epidermal growth factor receptor (EGFR) pathway is a key signaling pathway in the activation of astrocytes after brain injury.¹¹ Activation of EGFR triggers astrocytes into a visibly active phenotype with significantly increased cell motility, which is one of the characteristics of reactive astrocytes. Blocking EGFR activation in adult astrocytes promotes the survival of neurons after glaucomatous optic neuronal injury.¹⁰ In all cell types examined so far, EGFR selectively partitions into the cholesterol-rich lipid rafts. The presence within lipid rafts of a variety of membrane proteins involved in cell signaling, including EGFR, suggests that these lipid domains play important roles in the process of signal transduction.¹⁹ Lipid rafts may function as platforms that allow local accumulation of raft-associated proteins, which promote the interaction of protein complexes, and modulate neurotransmitter signaling.¹⁴ Simvastatin, a clinically widely used cholesterol synthesis inhibitor, lowers raft cholesterol content in various types of cells, reduces association of N-methyl-D-aspartate (NMDA) receptors to lipid rafts²² and affects raft-dependent signaling such as the Akt-mediated signaling pathway.²⁹ Therefore simvastatin may affect the functionality of proteins associated with lipid rafts (e.g., EGFR).

The purpose of this study is to determine whether simvastatin protects neurons by attenuating reactive astrogliosis, thereby exerting beneficial effects after TBI. We hypothesize that simvastatin promotes neuronal survival by inhibiting the activation of astrocytes after brain injury through the modification of lipid rafts, and regulation of EGFR phosphorylation in the astrocyte membrane.

Methods

Experimental Groups

Adult male Wistar rats were randomly divided into three groups. Rats in the first group were subjected to TBI and given saline orally 1 day later and consecutively for 14 days. Rats in the second group were subjected to TBI and simvastatin was administered orally at a dose of 1 mg/kg/day 1 day later and then for 14 consecutive days. Rats in the third group were subjected to sham surgery. Rats in the first and second groups were sacrificed at 3 and 14 days after saline or simvastatin administration, and rats in the sham group were sacrificed at 1 day after sham surgery. Sixteen animals were sacrificed at each time point; eight rats were used for immunohistochemistry and the other eight rats for Western blot analysis.

Surgery and TBI

We used a modified controlled cortical injury (CCI) model of TBI in this study.¹⁵ Rats were anesthetized intraperitoneally with 350 mg/kg body weight of chloral hydrate. A CCI device

was used to induce injury. Rats were placed in a stereotactic frame. Two 10-mm diameter craniotomies were performed adjacent to the central suture, midway between the lambda and the bregma. The second craniotomy allowed for lateral movement of cortical tissue. The dura mater was kept intact over the cortex. Injury was induced by striking the left cortex (ipsilateral cortex) with a pneumatic piston at a rate of 4m/second and 2.5 mm of compression. Sham-injured animals were similarly anesthetized and subjected to craniectomies without injury.

Immunohistochemistry

For immunostaining, rats were anesthetized with chloral hydrate at 350 mg/kg body weight and perfused transcardially with saline followed by 4% paraformaldehyde. Brains were isolated, post-fixed in 4% paraformaldehyde for 2 days at room temperature, and then processed for paraffin sectioning. A series of 6- μ m-thick sections were cut with a microtome through each of seven standard sections. After rehydration, tissue sections were boiled in 1% citric acid buffer (pH 6) in a microwave oven for 10 minutes, cooled to room temperature, and incubated in 1% saponin for 1 hour. The sections were then incubated in 1% BSA to block the nonspecific signals. Using the same buffer solution, the sections were incubated overnight at 4°C with primary antibody (i.e., monoclonal anti-NeuN, glial fibrillary acidic protein (GFAP) or MAP-2 dilution 1:200; Millipore, Temecula, CA), and then for 2 hours at room temperature with corresponding fluorochrome-conjugated secondary antibody (fluorescein isothiocyanate; FITC, Jackson ImmunoResearch, West Grove, PA). Each step was followed by three 5-minute rinses in phosphate-buffered saline (PBS). Tissue sections were mounted on slides with Vectashield mounting medium (Vector laboratories, Burlingame, CA). Sections were observed with a fluorescent microscope.

Isolation of Lipid Rafts

All procedures were carried out on ice. After cells were collected, 1 ml of lysis buffer containing 1% Triton X-100 was added and incubated on ice for 30 minutes. The density gradient consisted of 5 layers of OptiPrep at the following concentrations: 35%, 30%, 25%, 20%, and 0%. The lower layer (35% OptiPrep) contained the cell lysates. The density gradient was centrifuged at $200,000 \times g$ (70.1 Ti rotor, Beckman Coulter, Fullerton, CA) for 4 hours at 4°C. One-millimeter fractions of the lysate were collected from top to bottom of the ultracentrifuge tube and transferred to a microcentrifuge tube. The protein concentration of each fraction was determined by a BCA (bicinchoninic acid) protein assay kit (Pierce, Rockford, IL). The total cholesterol of each fraction was determined using a Wako CII Total Cholesterol assay kit (Wako Chemicals, Inc., Richmond, VA). Lipid raft-containing fractions were tracked by the enrichment of the cholesterol-binding protein caveolin-1.

Cell Culture

Rat astrocytes were obtained from American Type Culture Collection (ATCC) and cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 100 units/ml of penicillin and 100 μ g/ml of streptomycin (Invitrogen) at 37°C in a humidified incubator containing 5% CO₂.

In Vitro Oxygen Glucose Deprivation/reoxygenation model (OGD)

Astrocytes were seeded in each well of a 6-well plate containing normal medium until they attached to the plate. Normal growth medium was then replaced with DMEM without glucose and the cells were incubated in an anaerobic chamber (model 1025; Forma Scientific) for 3 hours. The oxygen level within the anaerobic chamber was routinely measured with a BD Disposable Anaerobic Indicator (Becton, Dickinson and Company, Sparks, MD), which confirmed that the oxygen level remained below 0.2%. After OGD,

glucose-free DMEM was replaced with normal growth medium and the cells were incubated under normal culture conditions. Simvastatin was added at the start of OGD and maintained during the reoxygenation process. After 24 hours, cells were detached and collected for further study.

Immunocytochemistry

Cells were fixed with a fixing solution (i.e., 4% paraformaldehyde, 4% sucrose, 0.01 M PBS, and 50 mM HEPES, pH 7.5) at 25°C for 15 minutes. Cells were then washed three times for 5 minutes with PBS and permeabilized with a solution containing 1% bovine serum albumin (BSA) and 0.3% Triton X-100 in PBS for 30 minutes. Cells were incubated with a rabbit polyclonal GFAP antibody (diluted 1:200; Millipore) and a monoclonal anti-caveolin-1 antibody (diluted 1:100; Sigma) in 1% BSA overnight at 4°C. Cells were then washed three times for 5 minutes with PBS. GFAP immunoreactivity was visualized with a goat anti-rabbit secondary antibody conjugated to FITC and the caveolin-1 antibody was visualized with a goat anti-mouse secondary antibody conjugated to CY-3 (Jackson ImmunoResearch). Slides were mounted with Fluoromount-G® mounting media on glass and analyzed under a fluorescence microscope (Nikon, Tokyo, Japan).

Western Blot Analysis

After various treatments, cells were collected and washed once with 1X PBS, lysed in lysis buffer on ice for 30 minutes, and briefly sonicated. Protein concentrations were determined. Equal amounts of cell lysate were subjected to SDS-polyacrylamide electrophoresis on Novex tris-glycine gels (Invitrogen) and separated proteins were then electrotransferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA). After exposure to various antibodies, specific proteins were detected using the SuperSignal West Pico chemiluminescence substrate system (Pierce, Rockford, IL). The band intensity was analyzed using Scion image software (Scion, Frederick, MD). Antibodies used for Western blot included anti-caveolin-1 antibody (Sigma), actin (Santa Cruz Biotechnology, Santa Cruz, CA), phospho-EGFR (Ser1046/1047) (Cell Signaling), EGFR (C74B9) (Cell Signaling) and Phospho-I κ B α (Ser32) (Cell Signaling).

IL-1 β ELISA

IL-1 β expression was measured using equal amounts of lysate from each sample and an ELISA kit (R&D System, Minneapolis, MN).

Statistical Analysis

All data are presented as means $\pm$ standard deviation (SD). Data are analyzed by a one-way analysis of variance (ANOVA) followed by post hoc Student–Newman–Keuls (SNK) tests. Differences are determined to be significant with $p < 0.05$.

Results

Simvastatin Promotes Neuronal Survival in the Hippocampal CA3 region and Dentate Gyrus after TBI

In the CA3 region of hippocampus of the sham group, the neurons in the pyramidal cell layer had large cell bodies with long processes (Fig. 1A). Fourteen days after TBI, the number of MAP-2-positive cells declined significantly in the CA3 region of the saline-treated group compared with the sham group (491 ± 58 neurons/mm²) (Fig. 1B), with surviving neurons showing a loss of processes (Fig. 1B). After treatment with simvastatin, the number of surviving neurons in the CA3 region (214 ± 22 neurons/mm²) was significantly increased with augmentation of processes of the neuronal cells (Fig. 1C)

compared to the saline-treated group (368 ± 32 neurons/mm², $p = 0.012$) (Fig. 1G). After TBI, the density of NeuN-positive cells (neurons) in the dentate gyrus was significantly higher in the simvastatin-treated group (541 ± 32 neurons/mm²) than in the saline-treated group (342 ± 29 neurons/mm², $p = 0.037$) (Fig. 1G), but both are still significantly lower than sham group (687 ± 51 neurons/mm²). These data demonstrate that treatment with simvastatin after TBI promotes neuronal survival in both the CA3 region and dentate gyrus, possibly related to the rescue of damaged pyramidal cells.

Simvastatin Attenuates Astrogliosis in the Lesion Boundary Zone, Hippocampus and Dentate Gyrus after TBI

Since neuro-inflammation is an important part of the pathophysiology of TBI and it is deleterious to neuronal survival in the early phase, we investigated the effects of simvastatin on astrogliosis after TBI. Immunostaining showed that scattered GFAP-positive astrocytes in the cortex, hippocampus and dentate gyrus were present in the brain tissue of the sham control group. These astrocytes have small bodies and dendrites, and the expression of GFAP was relatively low (Fig. 2A, D and G). Four days after TBI, the astrocytes were hypertrophic and GFAP expression was upregulated in the lesion boundary zone, ipsilateral hippocampus and dentate gyrus (Fig. 2B, E and H). Those astrocytes with enlarged cell bodies and highlighted cell processes are deemed as reactive astrocytes. These activated astrocytes produce numerous cytokines which can induce neuronal injury in the early phase after TBI.¹⁸ Treatment with simvastatin not only decreased the hypertrophy, but also downregulated GFAP expression as compared to the saline control group. Statistical analysis confirmed significant differences in the number of reactive astrocytes between the saline-treated and simvastatin-treated groups in the ipsilateral cortex ($p = 0.003$), hippocampus ($p = 0.013$) and dentate gyrus ($p = 0.034$). These data suggest that simvastatin suppresses the activation of astrocytes at four days after TBI.

Simvastatin suppresses activation of astrocytes after OGD and modifies the lipid rafts

In light of the important role of ischemia and hypoxia in secondary injury after TBI, OGD was used to activate astrocytes and the effects of simvastatin on lipid rafts in astrocytes were evaluated. Astrocytes were double stained by immunohistochemistry with anti-GFAP and anti-caveolin-1 (red) antibodies.⁵ Arrows show the signal of caveolin-1 - the specific marker of lipid rafts - in cells under normal conditions (Fig. 3A), cells under OGD conditions (Fig. 3B), cells treated with simvastatin (Fig. 3C) and cells treated with simvastatin and cholesterol (Fig. 3D). OGD upregulated caveolin-1 (marker of lipid raft) expression in the reactive astrocytes and simvastatin inhibited such an increase. Addition of cholesterol partially reversed the effect of simvastatin in the lipid rafts. Western blot analysis further confirmed the immunohistochemistry results with a significant caveolin-1 band density change between OGD and control ($p = 0.002$) and between simvastatin and OGD ($p = 0.021$) (Fig. 3 E).

Simvastatin reduces the IL-1 β production by suppressing the expression and phosphorylation of EGFR in lipid rafts in reactive astrocytes

Lipid rafts were identified by their enrichment of cholesterol and the presence of the raft marker caveolin (Fig. 4A). Cholesterol and caveolin were concentrated between fractions 2 and 4, and peaked in fraction 2 and 3. Thus fractions 2-4 were deemed "lipid raft fractions" and used in subsequent studies. In contrast, protein and the non-lipid raft protein actin were concentrated in fractions 8 and 9 (Fig. 4A). To measure the association of EGFR with lipid rafts after OGD, lipid raft fractions (fraction 2-4) were collected and pooled. Western blot analysis showed that OGD induced a dramatic increase of EGFR expression and phosphorylation in the lipid raft fractions (Fig. 4B). Treatment with simvastatin downregulated the phosphorylation of EGFR in lipid raft fractions after OGD (Fig. 4B).

AG1478, a selective inhibitor of p-EGFR, served as a positive control. AG1478 almost completely inhibited EGFR phosphorylation in the lipid raft fractions. Since NF- κ B is one of the main downstream targets in the EGFR signaling pathway, its activity was examined indirectly by the phosphorylation of I κ B, an inhibitory protein of NF- κ B. Phosphorylation of I κ B leads to the activation of NF- κ B. Simvastatin inhibited the phosphorylation of I κ B in reactive astrocytes after OGD (Fig. 4B). Blocking of EGFR by AG1478 led to a greater inhibition of phosphorylation of I κ B in astrocytes after OGD. To explore the effect of simvastatin on the production of cytokines in reactive astrocytes, ELISA of interleukin 1 (IL-1 β) was performed. OGD induced the production of IL-1 β in astrocytes ($p = 0.001$ vs. control), and simvastatin significantly suppressed this induction ($p = 0.035$ vs OGD) (Fig. 4C). AG1478, a potent EGFR inhibitor, also drastically decreased the concentration of IL-1 β in astrocytes after OGD ($p = 0.022$ vs. OGD), which indicated that EGFR played a pivotal role in regulating production of IL-1 β in reactive astrocytes.

Discussion

The primary findings in the present study are: 1) Treatment of TBI with simvastatin promotes neuronal survival and attenuates reactive astrogliosis in the early phase after TBI; 2) Simvastatin modulates lipid rafts in astrocytes after OGD; and 3) Simvastatin downregulates EGFR and reduces phosphorylation of EGFR in lipid rafts, leading to the suppression of astrocyte activation and IL-1 β production.

Recently, there has been much interest in statins because they show neuroprotective effects and promote CNS neurite outgrowth independent of their effect on serum cholesterol.^{3,21} It has been suggested that statins produce anti-inflammatory effects, including attenuation of TBI-induced inflammatory mediators TNF α and IL-1 β .^{20,26} Our data show that simvastatin promotes neuronal survival in the hippocampus CA3 area and the dentate gyrus 14 days after TBI (Fig. 1). These data are consistent with the previous findings using atorvastatin.¹³

TBI leads to secondary tissue damage including the blood brain barrier breakdown that activates astrocytes and microglia.¹ This cascade results in up-regulation of inflammatory cytokine production by activated astrocytes.²³ Elevated levels of inflammatory cytokines such as IL-1 and IL-6 promote secondary brain injury and poor long-term outcome after TBI.^{17,27} Inflammation exacerbates neuronal injury, inhibits axonal growth and reduces recovery, therefore it is reasonable to investigate the etiology of astrogliosis and the mechanisms by which it can be reduced.

In light of the potential benefits of simvastatin on TBI, the effects of simvastatin on astrocyte activation were investigated *in vivo* as well as *in vitro*. Immunohistochemical staining with GFAP shows that simvastatin attenuates the TBI-induced reactive astrogliosis in the lesion boundary zone, ipsilateral hippocampus and dentate gyrus at 4 days after TBI (Fig. 2). Immunocytochemical staining with GFAP/caveolin-1 demonstrates that simvastatin reduces the expression of caveolin-1, the specific marker of lipid rafts, in reactive astrocytes after OGD (Fig. 3). Western blot analysis of caveolin-1 expression in astrocytes after OGD confirms the simvastatin modulation of lipid rafts in reactive astrocytes (Fig. 3).

Lipid rafts are plasma membrane microdomains rich in cholesterol and sphingolipids, as well as proteins involved in signal transduction.¹⁴ In neuronal cells, lipid rafts act as platforms for the signal transduction and regulate phosphorylation cascades originating from membrane-bound proteins.²⁴ Thus, lipid rafts are structurally unique components of plasma membranes, crucial for neural function.⁷

To evaluate the putative role of simvastatin in modulating lipid rafts in astrocytes, we next isolated the lipid rafts from cell membrane and investigated the mechanism underlying this

modulation (Fig. 4). EGFR pathway is important in controlling the phenotypic characteristics of adult astrocytes.¹¹ Recent reports show that EGFR activation is a master signal transduction pathway of the astrocyte activation process in response to different neural injuries such as ischemia⁵ or electrolytic lesion.⁶ The EGFR pathway regulates a remarkable number of genes related to reactive astrocytes. Some of these genes are cytokines/cytokine receptors, suggesting that EGFR pathway regulates production of inflammatory cytokines in reactive astrocytes following neural injury. Inhibiting EGFR activation may be a way of blocking the activities of reactive astrocytes.¹² EGFR is closely associated with lipid rafts. This localization of the receptor appears to affect its signaling capacity.⁴ When lipid rafts are altered by cholesterol depletion, both the binding and tyrosine kinase activity of EGFR is affected. In addition, the EGFR pathway is inhibited by raft disruption,²⁵ which indicates that modifying lipid rafts may affect functioning of its downstream signaling pathway.

Our data show that treatment with simvastatin reduces the phosphorylation of EGFR in lipid raft fractions (Fig. 4B). Furthermore, simvastatin reduced the phosphorylation of I κ B in reactive astrocytes, indicating that the activation of NF- κ B had been suppressed. NF- κ B pathway is the main signaling pathway responsible for the production of proinflammatory cytokines from astrocytes after neuronal injury.⁸ Our data also show that simvastatin reduces the production of cytokine IL-1 in astrocytes after OGD. Blocking of EGFR with AG1478 confirms the central role of the EGFR pathway in the activation of astrocytes. Therefore, the neuroprotective effect of simvastatin treatment after TBI may be associated with modification of lipid rafts, inhibition of EGFR phosphorylation, attenuation of reactive astrocytes and the consequent reduction of inflammatory cytokines.⁹

Conclusions

Our data demonstrate that simvastatin inhibits reactive astrogliosis and promotes neuronal survival. Modulation of lipid rafts and co-localized EGFR may be one of the mechanisms underlying the neuroprotective effects of simvastatin in TBI.

Acknowledgments

Financial Support: This work was supported by National Institutes of Health (NIH) grants R01NS052280-01A1 (Asim Mahmood, PI) and PO1NS23345 (Michael Chopp, PI).

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Fig. 1. Simvastatin promotes neuron survival in hippocampus CA3 and Dentate Gyrus fourteen days after TBI. Immunostaining with MAP2 antibody (A~C) or NeuN antibody (D-F) shows the survival of neurons. (G) The number of neurons was counted and averages were calculated. Data are the mean $\pm$ SD. * $p < 0.05$ vs. sham. # $p < 0.05$ vs. saline. Scale bar = 100 μ m (A~C), 50 μ m (D-F).

Fig. 2.

Effects of simvastatin on the activation of astrocytes after TBI. Immunofluorescent staining with GFAP antibody shows simvastatin suppresses the injury induced activation of astrocytes in the lesion boundary zone, ipsilateral hippocampus (D-F) and dentate gyrus (G-I) in four days after TBI. The number of reactive astrocytes was counted and calculated as cells/mm² (J). Data are presented as mean $\pm$ SD. N = 4. *p < 0.05 vs. sham. #p < 0.05 vs. saline. Scale bar = 50 μ m.

Fig. 3. Effects of simvastatin on lipid rafts in normal and reactive astrocytes after OGD. (A-D) Immunofluorescent double staining with GFAP⁵/caveolin-1 (red) shows that astrocytes were activated after OGD and simvastatin modified the lipid rafts expression on cell membrane. (E) Representative Western blot shows the levels of caveolin-1, the lipid rafts marker, change in OGD and after simvastatin treatment. Data are presented as mean $\pm$ SD. N = 4. *p < 0.05 vs. control. #p < 0.05 vs. OGD. Scale bar = 50 μ m.

Fig. 4.

Simvastatin downregulates phosphorylation of EGFR in lipid rafts fractions after OGD and lowers the IL-1 β production in astrocytes. (A). Identifying lipid rafts fractions in cultured astrocytes. Representative Western blot shows fractions 1 to 9 obtained from cultured astrocytes after OGD. Caveolin-1 antibody was used to detect lipid rafts in astrocytes. Total cholesterol and protein content were determined in fractions 1 to 9 (B). Western blot analysis for p-EGFR, EGFR, caveolin-1 and p-I κ B in collections of lipid raft fractions or cell lysate. (C). ELISA for concentration of IL-1 β in astrocytes under various treatments. Data are presented as mean $\pm$ SD. N = 4. *p < 0.05 vs. control. #p < 0.05 vs. OGD.