Experimental Study

Neural stem cells neuroprotection by simvastatin via autophagy induction and apoptosis inhibition

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ABSTRACT
OBJECTIVE: This study was conducted to investigate the effects of Simvastatin (SIM), a member of statin family, on the cellular antioxidant system, autophagy and apoptosis in NSCs exposed to hydrogen peroxide.

BACKGROUND: Reduction in cellular oxidative stress increases the survival of neural stem cells (NSCs) after transplantation into the damaged area of the affected central nervous system.

MATERIAL AND METHODS: NSCs derived from bone marrow stromal cells (BMSCs) were exposed to H2O2 (100 μM) for 48 hours after pretreatment with SIM (2 μM). Next, the expressions of the master antioxidant transcription factor, Nrf2/nuclear factor erythroid 2 (NFE2)-related factor 2, autophagy-related proteins (microtubule-associated proteins 1A/1B light chain 3B known as LC3I and LC3II and also p62/Sequestosome), and apoptosis (Bcl-2/ B-cell lymphoma 2 and Bax/BCL2 associated X protein) were analyzed.

RESULTS: SIM caused Nrf2 over-activation (more localizations in the cellular nucleus), reduction in reactive oxygen species (ROS), induction of autophagy (decrease in p62 expression and increase in LC3II/LC3I ratio) and inhibition of apoptosis (decrease in Bax protein and increase in Bcl-2) in NSCs exposed to H2O2-induced oxidative stress, thereby prolonging the cell viability within 48 hours at low concentration (2 μM).

CONCLUSION: SIM protects NSCs against H2O2-induced apoptosis in a pleiotropic signaling manner (Fig. 7, Ref. 35).

KEY WORDS: simvastatin, neural stem cells, autophagy, apoptosis.

Introduction

Due to the low capacity of NSCs residing in the subventricular zone and dentate gyrus of the brain to replenish damaged cells, other alternative methods have been considered (1, 2). One of the approaches is cell therapy using other available sources of stem cells such as BMSCs, and then their differentiation into NSCs (1, 3–5). However, such cells disappear after being transplanted into the affected area due to oxidative stress, hypoxia and serum deficiency (6). Therefore, it is important to pass the way to augment cell survival after transplantation (2, 7). Beside their lowering effects on cholesterol, statins have yet other advantageous effects associated namely with their anti-inflammatory, anti-apoptotic and anti-oxidative properties (8, 9). On the other hand, it is well accepted that autophagy deficiency plays a pivotal role in neurodegenerative diseases (10). Recent study reported that SIM was effective in the treatment of multiple sclerosis, Parkinson’s disease, Alzheimer’s disease, ischemic stroke and rheumatoid arthritis (11). Clinical trials have proven that these beneficial effects are not related to the lipid-lowering nature of SIM (12). In this regard, the pleiotropic effects of SIM to promote NSCs’ survival have been investigated in the current study.

Materials and methods

BMSCs isolation and culture

This experimental research was carried out on 5 female adult Wistar rats, aged 6–8 weeks, from the Pasteur Institute of Iran. The animals were kept in a 12/12 hours of light/dark cycle and under standard conditions of the animal house of Qazvin University of Medical Sciences in compliance with ethical consi-
The BMSCs were isolated from the lower limb long bones and washed with saline buffer phosphate until the third passage in DMEM/F12 medium (Dulbecco modified Eagle’s medium) containing 10% FBS (fetal bovine serum). 100 μg ml–1 of penicillin and 100 U ml–1 of streptomycin. The medium was placed in the incubator with 5% CO₂ at 95% humidity and 37°C.

Differentiation of BMSCs into NSCs

After trypsinization of BMSCs at the third passage, 10,000 cells were seeded equally into each well of 12-well plates (with one coverslip in each well). At this stage, the BMSCs were cultured in DMEM/F12 medium (GIBCO-BRL, Germany) containing 2% B27 (Invitrogen, Scotland) and 20 ng ml–1 of basic fibroblast growth factor (bFGF, Chemicon, Germany) and 20 ng ml–1 of epidermal growth factor (EGF, Sigma, Steinheim). After a week, neurospheres with spherical structure were collected by centrifugation. After removing the culture medium, by trypsinization and mechanical digestion, the neurosphere cells were singled out and re-seeded to form neuroepithelial-like cells, which were then incubated to induce BMSCs-derived-NSCs. Immunocytochemistry technique was used to evaluate the expression of the nestin protein (marker of NSCs).

Immunocytochemistry technique

To confirm the mesenchymal origin of BMSCs in the third passage and their differentiation into NSCs, the cells were assessed in two stages, before and after differentiation into the NSCs, for expressing the immunocytochemical markers of CD31 (endothelial stem cells’ marker), CD34 (hematopoietic stem cells’ marker), CD106 (BMSCs’ marker), and nestin (a NSCs’ marker). After trypsinization, 5,000 cells were seeded equally into each well of 6-well plates (with one coverslip in each well). The immunocytochemistry steps were performed as previously recommended with minor modifications (5, 13). In brief, the cells were placed in a 4% paraformaldehyde solution for 20 minutes. Following washing with phosphate buffer, the cells were immersed in 0.3% Triton X-100 for 15 minutes. After rinsing with phosphate buffer, the cells were exposed to the primary antibody for 24 hours at 4°C. The primary anti-CD31 antibodies (Abcam, ab24590), anti-CD34 (Abcam, ab81289), anti-CD106 (Abcam, ab134047) and anti-Nestin (Abcam, ab6142) were used. The cells were washed using phosphate buffer and then exposed to the appropriate FITC-conjugated secondary antibodies (immunoreactive markers shown green) for 2 hours at room temperature. The total number of cells was assessed using propidium iodide (PI), which turns the cell nucleus red while cells positive for a specific marker were counted using a fluorescence microscope.

Viability test

Prior to the experiments, the viability test was conducted applying trypan blue on the cells to evaluate the toxicity and protective effects of SIM. Thus, 1,000 cells were dispersed into the wells of a 96-well plate. The survival rate of NSCs was measured at different doses of SIM (0, 1, 2, 4 and 8 μM) for 48 hours. The dose of SIM with minimal lethal concentration for NSCs was selected to continue the study. To monitor the cell viability, a volume of cell suspension and an equal volume of trypan blue were mixed, and the cells were counted using a Neubauer slide under a microscope. In this method, the dye penetrates the dead cells which become blue while unstained cells represent the living cells. The percentage of cells viability was obtained by counting the total number of all cells and stained cells. The counting under microscope was done three times for each cell group.

Protective effect of SIM on NSCs against H₂O₂-induced oxidative stress

The viability test was conducted applying trypan blue on NSCs to evaluate the H₂O₂ toxicity and protective effects of SIM. Thus, 1,000 cells were dispersed into the wells of a 96-well plate. In order to investigate the toxicity of H₂O₂, the survival rate of NSCs was measured at a dose of 100 μM of H₂O₂ for 48 hours. The concentration of hydrogen peroxide was selected according to a previous study (14). The NSCs were divided into three groups. The first group (BMSCs-derived NSCs) was not treated and was considered the control group (N group). The second group (BMSCs-derived NSCs + H₂O₂) contained cells exposed to 100 μM of H₂O₂ for 48 hours (NH group). The third group (BMSCs-derived NSCs + SIM + H₂O₂) was treated with 2 μM of SIM for two hours before exposure to the medium containing 100 μM of H₂O₂ to monitor the protective effect of SIM as the treatment continued up to 48 hours (NISH group).

Nrf2 immunocytochemistry

To evaluate the effect of SIM on the anti-oxidant activity of Nrf2 signaling pathway. The localization of Nrf2 in the NSCs was detected using an anti-Nrf2 monoclonal antibody (Abcam, ab89443). Its nuclear translocation was evaluated using immunocytochemistry in all groups according to the recommended protocol by the recent study (15).

Western blotting analysis

The anti-LC3 (ab192890), anti-GAPDH (ab181602), anti-SQSTM1 / p62 (ab56416), anti-Bcl-2 (ab59348) and anti-Bax (ab32503) primary antibodies (all provided from Abcam Company) were used for detecting the protein in the blots as previously reported with minor modifications (16). In brief, the cell culture media were suctioned off and the cell surfaces washed three times for 5 minutes with pre-cooled phosphate-buffered saline. The extraction of protein from the experimental groups was performed on ice with a lysis buffer (Proteo Jet Mammalian Cell Lysis Reagent, Fermentas), and a protease inhibitor cocktail (Fermentas) was added immediately to the samples. The protein concentrations were assessed using Bradford assay. Equivalent amounts of each cell lysate were diluted in the sample buffer, simmered at 95°C for 10 min and then subjected to SDS-PAGE using two separate gels with the gradient running (from 4 to 12% gel). The gels were transferred onto the nitrocellulose membrane (Millipore) by semi-dry transferring method (BioRad), and the membrane was then blocked for 1 hour at 37°C in the Tris-Buffered Saline-
Tween 20 (TBST) containing 5 % blocking solution (Amersham). The nitrocellulose membrane (Millipore) was blocked for 1 h at 37 °C in TBST containing 5 % blocking solution (Amersham). Next, the membrane was incubated for 1 hour at room temperature in TBST containing 5 % blocking solution and recommended dilution of the primary monoclonal antibody, and then washed three times for 15 minutes, using TBST buffer. It was then incubated for 1 hour in TBST containing 5% blocking solution and 1:10,000 dilution of horseradish peroxidase-conjugated secondary antibody and after washing three times for 15 min with TBST buffer, the membrane was immersed in a mixture of equal volumes of ECL (enhanced chemiluminescence) detection solutions A and B (Amersham) and exposed to X-ray film in a dark room. The exposed films were developed by specific solutions and then digitally photographed.

**Reactive oxygen species (ROS) assay**

The ROS level was measured by the ROS-sensitive fluorescence reporter, dichloro-dihydro-fluorescein diacetate (DCFH-DA). In order to investigate the activity of ROS scavenging inside the BMSCs-derived NSCs, 10,000 cells per well were seeded onto a 96-well plate. After 24 hours of adhesion of cells to the plate floor, the cells belonging to the different groups were treated with SIM and hydrogen peroxide according to the aforementioned protocol for experimental groups. The cells were then subjected to DCFH-DA (20 μM) for 30 min, and the fluorescence intensity was measured using a fluorescence microplate at the wavelengths of 504 nm of excitation and 529 nm of emission. Each test was repeated three times for consistency of response.

**Statistical analysis**

All values are given in Mean ± SEM. The cell viability and cell count data were analyzed using Student’s t-test, one-way ANOVA and Tukey’s tests at the statistical significance level of 0.05.

**Results**

**Studying the BMSCs and their differentiation into NSCs**

Forty-eight hours after separation, the BMSCs with spherical appearance adhered to the flask bottom. The floating cells were discarded by washing the plate with phosphate buffer saline. After the third passage, the BMSCs were evaluated for their stemness and mesenchymal origin by using immunocytochemistry of cell surface markers. The cells were negative for CD31 (marker for endothelial cells) and CD34 (marker for hematopoietic stem cell), but positive for CD106 (marker for BMSCs) (Fig. 1). The expression level of CD31, CD34, CD106 and nestin protein markers in BMSCs was 3 %, 4 %, 95 % and 8 %, respectively. The mesenchymal origin of the isolated cells was proven by high positive percentage of CD106 expression and low levels for CD31, CD34 and nestin (Fig. 2). After the differentiation of BMSCs into NSCNs (BMSCs-derived NSCs), the expression level of nestin protein as a marker for NSCs showed a significant increase (p < 0.05) as compared with BMSCs, while the expression level was as high as 90 % (Fig. 1). Moreover, after the differentiation, the low expression levels of CD31, CD34, and CD106 protein markers by 1 %, 2 %, and 9 %, respectively, indicated the differentiation of BMSCs into NSCs (Fig. 2).

**Viability test to obtain a non-toxic dose of the SIM**

In the viability test, the cells were treated with different doses of SIM for 48 hours to assess the lethal concentration 50 (LC50) of SIM in the NSCs medium culture. The NSCs without SIM were treated with different concentrations of SIM including 0 (control), 1, 2, 4, and 8 μM. The survival percentages were 97 %, 98 %,
81% and 78% in NSCs receiving 1, 2, 4, and 8 μM of SIM after 48 hours, respectively. The highest mortality rate was observed in the cells receiving 4 and 8 μM of SIM and there was a significant difference (p < 0.05) in comparison with the control group (Fig. 3). However, the dose of 2 μM is considered as the optimal concentration due to lower mortality rates.

Viability test to evaluate the SIM dose effective on the cell groups

To evaluate the protective effects of 2 μM of SIM on NSCs exposed to 100 μM of hydrogen peroxide, the cells were divided into three groups. In the first group, the BMSCs-derived NSCs were considered as the control (N group). In the second group, the BMSCs-derived NSCs were treated with 100 μM of H₂O₂ (NSCs + H₂O₂, NH group). In the third group, the BMSCs-derived NSCs were pretreated with 2 μM of SIM for 2 hours prior to 100 μM of H₂O₂ exposure (BMSCs-derived NSCs + SIM pretreatment + H₂O₂, NSH group). In all groups, the duration of treatments was 48 hours. The mortality rates in both NH and NSH groups were higher than in the control group; however, the results showed that SIM pretreatment increases cell viability. The cell viability in the medium containing 2 μM of SIM and 100 μM of hydrogen peroxide was 76% (Fig. 4) whereas in the NH group not pretreated with SIM in the cell culture medium, the mortality rates of cells increased significantly (p < 0.05) and only 40% of the cells survived. Comparing the H₂O₂-exposed cells treated and not treated with SIM showed that SIM significantly increased cell viability and decreased cell death due to hydrogen peroxide (Fig. 4).

Activity of Nrf2 and its nuclear translocation

The immunofluorescence test showed that SIM increased the Nrf2 expression. It also increased the Nrf2 nuclear localization and decreased the cytosolic Nrf2 homing. As compared to other groups, SIM significantly increased the Nrf2 phosphorylation and its cytosol to nuclear translocation (Fig. 5). These results demonstrated that via over-activating the Nrf2 pathway, SIM protects BMSCs-derived NSCs and prevents oxidative stress-induced damage to the cells.

Examining autophagy and apoptosis

The expression of autophagic (p62, LC3I, LC3II) and apoptotic (Bax and Bcl-2) proteins was investigated using Western blotting in the three experimental groups (Fig. 6A). Then, the results of the Western blotting quality band for each protein (after three replications) were quantitated using Image J software (Fig. 6B). In the autophagy study, the expression level of p62 in NH and NSH groups was significantly lower than in the N (control) group (Fig. 6). The expression ratio of LC3II/LC3I in the NSH group increased significantly as compared with the other two groups (Fig. 6B). The expression of the anti-apoptotic protein, Bcl-2, showed that its expression rate was the highest in the control group, but it decreased significantly after exposing the cells to hydrogen peroxide. SIM partially prevented the Bcl-2 expression reduction in
Fig. 5. Nrf2 protein level assessment using immunocytochemistry. SIM pretreatment increased the Nrf2 activation in the NSH group (BMSCs-derived NSCs + SIM pretreatment + H2O2). The BMSCs-derived NSCs were pretreated with 2 μM of SIM for 2 hours and were further treated with 100 μM of H2O2 for another 48 hours. (A) Nrf2 protein was analyzed by immunocytochemistry in the N group. (B) Immunocytochemistry of total, nuclear and cytoplasmic fractions of Nrf2 in the NH group. (C) Immunocytochemistry of total, nuclear and cytoplasmic fractions of Nrf2 in the NSH group. (D) One-way ANOVA followed by Tukey’s multiple comparison tests was used for statistical analysis. * p < 0.05.

Fig. 6. Western blotting analyses of apoptosis (Bcl-2 and Bax) and autophagy (LC3I, LC3II, p62) proteins. Representative Western blots (A) and quantification of autophagic and apoptotic mediators (B). The expression of LC3I, LC3II, p62, Bcl-2 and Bax assays in N, NH and NSH groups. GAPDH was considered as the internal control. As a result, SIM induced NSCs autophagy via the conversion of LC3I to LC3II and by reducing p62 levels. H2O2 induced overexpression of Bax and overexpression and downregulation of Bcl-2 protein, however, SIM reduced the Bax levels and increased the Bcl-2 protein expressions. (B) Quantification of Western blot. The results were all normalized to GAPDH expression. Each set of the results was quantified upon three independent experiments. All data are presented as mean ± SD. a, b, c, d, e, p < 0.05 versus LC3I, LC3II, p62, Bcl-2 and Bax in each protein group using one-way ANOVA with the post hoc statistical test.
Recent investigations (24–26) are in accord with those of our study in demonstrating that SIM actually possesses pleiotropic effects. Moreover, according to several studies, SIM acts as a neuroprotective agent (27, 28) in neuronal lesions by increasing neurotrophic levels such as BDNF (brain-derived neurotrophic factor) and GDNF (glial cell-derived neurotrophic factor) and thus it can augment neurogenesis and synaptogenesis in the damaged brain (28). Augmentation of autophagy by using micromolecules is a novel and attractive strategy in the treatment of neurodegenerative disorders, as two main characteristics of these diseases are oxidative stress and protein misfolding and aggregating in cells and extracellular matrix as it occurs in Parkinson’s and Alzheimer’s diseases. The statins prevent the progression of Parkinson’s disease and have beneficial effects in the early stages of multiple sclerosis and Alzheimer’s disease (12). It is well known that \( \text{H}_2\text{O}_2 \) causes Keap1 to oxidize in its cysteines, thus Nrf2 stabilizes and accumulates in the nucleus (29). Also, Chartoumpekis et al. showed that through the pathway of phosphatidylinositol-4, 5-bisphosphate 3-kinase/protein kinase B (Akt), the statins activate Nrf2 in embryonic fibroblasts (30). Notably, the neuroprotective effects of statins have been shown (31) and it is interesting to attenuate oxidative stress using statins. On the other hand, it is well accepted that the main pathway for removing the aggregated and misfolded proteins is the autophagy pathway (10, 32). Our current study investigates the possibility of real interaction between autophagy and SIM in NSCs. Researchers use p62 and the ratio of LC3II/LC3I as markers of autophagy induction to assess autophagic activities (33, 34). In addition to the fact that our study showed that SIM increased the levels of p62, it also showed that the response to the LC3II/LC3I ratio was dose-dependent, thus indicating autophagy activation by SIM. In autophagy, p62 acts as a link between LC3 and ubiquitinated substrates, which in turn is degraded in the autolysosome (33). The decrease in p62 and increase in the LC3II/LC3I ratio indicate an increase in autophagy induction in the NSH group compared with two other groups. In accordance with our observation, recent studies reported that via inhibition of mTOR pathway, SIM led to autophagy

### Discussion

Oxidative stress damages the nerve cells in many neurodegenerative and other diseases (17–20). Attenuation of oxidative stress increases the survival of NSCs after transplantation into the affected area of the central nervous system. Statins are well-known for their efficacious actions in reducing the cholesterol level and as such they are used in the treatment of cardiovascular disease. Recently, their protective effects against neuroinflammation of the nervous system have been considered (21). There are many natural and synthetic lipid-lowering drugs that may act in a pleiotropic way, other than by reducing the lipid synthesis to combat disease progression (22, 23). Although the antioxidant role of the statins is evident, there is a controversy on their function in inducing or inhibiting the apoptosis. As a matter of fact, SIM acts like a double-edged sword, as it induces autophagy at low doses whereas at higher doses, it activates the apoptotic pathway. In this study, we showed that SIM at its effective doses activates the master antioxidant transcription factor, Nrf2, and its cytosol to nuclear translocation, reduces ROS-induced autophagy (decreased expression of p62 protein and increased LC3II/LC3I ratio) and inhibits apoptosis (downregulation of Bax and overexpression of Bcl-2 proteins) in NSCs under condition of hydrogen peroxide-induced oxidative stress. The results of recent investigations (24–26) are in accord with those of our study in demonstrating that SIM actually possesses pleiotropic effects.

**Fig. 7.** The effect of SIM on the reduction of ROS generation in BM-SCs-derived NSCs. The BMSCs-derived NSCs were pretreated with SIM (2 μM) for 48 hours, and the ROS level was determined using the DCFH-DA dye. (A) Relative ROS level for N, NH and NSH, indicating BMSCs-derived NSCs as a control group, BMSCs-derived NSCs with 100 μM of H_2O_2 (untreated), and BMSCs-derived NSCs treated with 2 μM of SIM and 100 μM of H_2O_2, respectively. ROS production induced by H_2O_2 (100 μM) in BMSCS-derived NSCs. The pretreatment of BMSCs-derived NSCs with 2 μM of SIM protects them against apoptosis increased by H_2O_2. (B) Data represent the mean ± SEM for fold changes in comparison with the control (N) group. One-way ANOVA followed by Tukey’s multiple comparison tests was used for statistical analysis. *p < 0.05.
induction (35). Thus, SIM counteracts the two main characteristics of neurodegenerative pathological states, namely oxidative stress and protein aggregation, and as shown by the expression of two main apoptotic markers Bcl-2 and Bax, it increases the cell survival. Previous studies have demonstrated that statins at high concentrations cause induction of apoptosis in cancer cells. Nevertheless, the findings of the present study support its protective roles in NSCs at low concentrations (2 μM). Further, in vivo experiments may open the way to the possible usage of statins in therapy combined with NSCs in the treatment of neurological diseases in the near future.

Conclusions

Altogether, the results suggested that SIM has the ability to protect NSCs by activating antioxidant cell defense and autophagy system as well as by inhibiting apoptosis. Thus, SIM actually protects NSCs after H2O2-induced apoptosis in a pleiotropic manner.

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