Effects of Dexamethasone on Autophagy and Apoptosis in Acute Spinal Cord Injury

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Abstract

Objectives: To investigate the effects of dexamethasone on autophagy and apoptosis in spinal cord neurons.

Methods: We established an in vitro model of spinal cord injury and given different concentrations of dexamethasone at 24 hours after injury. Then we used western blot, MTT, flow cytometry, RT-PCR to detect the level of apoptosis. Western blotting was used to evaluate the expression of LC3. The TEM was used to observe the ultrastructure of the autophagosome. After we give the autophagy inhibitor (3-MA) and autophagy activator (Rapamycin). The autophagy and apoptosis were observed by western blot, flow cytometry, and RT-PCR

Results: The results of MTT showed that 10⁻⁶ M dexamethasone could increase the activity after cell injury. Meanwhile, flow cytometry, RT-PCR and western blot showed that 10⁻⁶ M decreased the apoptosis level. Similarly, autolysosome can be observed after treatment with dexamethasone by electron microscope. At the same time the expression of LC3 protein and beclin-1 gene expression increased. Give autophagy activator (Rapamycin) can increase the level of autophagy, reduce cell apoptosis. In contrast, the autophagy inhibitor (3-MA) inhibits autophagy and increases apoptosis.

Conclusion: In conclusion, our observations indicate that dexamethasone inhibited the level of autophagy in the injured nerve cells in a dose-dependent manner. High doses of dexamethasone protected the damaged spinal cord neurons by inhibiting apoptosis, but a protective effect from low hormone concentrations was not obvious.

Keywords: Spinal neurons; Autophagy; Apoptosis; Dexamethasone

Introduction

Spinal cord injury is caused by direct or indirect fracture or dislocation of the spine [1]. Spinal cord injuries can be classified as either primary spinal cord injuries or secondary spinal cord injuries [2]. After acute spinal cord injury, a series of cellular and molecular events occur in the central nervous system, including inflammatory reactions, necrosis and apoptosis. [3-6] In recent years, many studies have indicated that secondary injury plays an important role in acute spinal cord injury, primarily through the mechanism of apoptosis. [7,8]

Apoptosis plays a vital role in spinal cord injury. Apoptosis involves a series of proteases that induce changes in gene regulation and an orderly, energy-dependent death process with a complex molecular mechanism. Caspase-3 is the common effector of multiple downstream pathways of apoptosis and a component of a protease cascade. Therefore, the level of Caspase-3 expression can reflect the degree of apoptosis [9,10]. It is of great significance to study methods for reducing the apoptosis of injured spinal cord neurons in the repair and treatment of spinal cord injury.

In recent years, many scholars both at home and abroad have reported that autophagy occurs after spinal cord injury or brain injury [11-13]. Wang et al. used transmission electron microscopy to observe the process of autophagy associated with mechanical damage of spinal cord neurons, and they employed RNA interference technology to confirm that Beclin-1 plays an important role in autophagy in spinal cord injury [14]. However, autophagy is a double-edged sword as it can lead to cell death [15]. Work conducted by Kanno et al. confirmed that Beclin-1 levels in rats are very similar to that in humans [16]. Liu et al. as well as other researchers have reported that Beclin-1 levels in rats are very similar to that in humans [16]. However, recent studies have shown that dexamethasone inhibits autophagy and apoptosis in spinal cord injury [17].

The treatment of acute spinal cord injury has not experienced significant breakthroughs in recent decades; the current mainstream treatment is still a large dose of hormone therapy [18]. Some scholars believe that animal and clinical trials have proven that large doses of steroids have significant curative effects, supporting the administration of early high-dose glucocorticoid therapy following spinal cord injury [19-22]. Recently, Liu et al. as well as other researchers have reported that small doses of glucocorticoids induce autophagy and promote cartilage cells, but long-term large doses can promote chondrocyte apoptosis [23]. Chen et al. found that a large dose of hormone inhibited autophagy in acute spinal cord injury and reduced autophagic cell death, but the specific mechanism was not clear in vitro [24]. The present study further elaborates the mechanism of hormone therapy for treating acute spinal cord injury in relation to the
autophagy pathway and provides a new direction for the treatment of acute spinal cord injury.

Materials and Methods

Primary neuron culture and injury model of the spinal cord

Briefly, spinal cord neuron cells were prepared from the spinal cords of rat embryos (gestational day 16). Embryo isolation was performed under sterile conditions as follows. The ridge was removed, and the tissue was carefully minced on ice before digestion with 0.25% trypsin at 37°C for 3 minutes. Cultivation medium (DMEM) containing 20% FBS and 1% penicillin/streptomycin was used to terminate digestion. After filtration through a 400-µm mesh, the samples were centrifuged at 1000 rpm for 5 minutes, and the suspension was plated in 6-well plates. After 6 hours, serum-free Neurobasal medium containing 2% B27, 1% glutamine and 1% penicillin streptomycin was added. The medium was changed every 3 days. After 6 days, sterile needles were employed to generate cuts at the bottom of the culture plate with a spacing of 4 mm to effectively simulate spinal cord injury \textit{in vitro}.

Immunofluorescence

Cells were washed twice with PBS and fixed with 4% formaldehyde for 20 minutes at room temperature. The samples were then placed on ice, and Triton-X-100 (0.1%) was added to permeabilize the membranes. Then, the cells were blocked for 2 hours in PBS containing 5% BSA to prevent non-specific binding. Anti-NSE and anti-TUBB3 antibodies were diluted to a working concentration (1:150), followed by overnight incubation at 4°C. Then, the cells were incubated with FITC-conjugated secondary antibodies in 1% BSA for 2 hours at room temperature in the dark. The nuclei were subsequently stained with DAPI for 2 minutes. Images were captured under a fluorescence microscope after incubation with a quenching agent.

Western blot analysis

The samples were first added to 5X SDS loading buffer and heated to 100°C for 5 minutes. Samples of equal volume with an equal protein concentration were subsequently separated via sodium dodecyl sulfate gel electrophoresis and transferred to a PVDF membrane (Millipore, Billerica, MA, USA). The membranes were then blocked for 2 hours at room temperature with 5% non-fat dry milk and incubated with the primary antibodies overnight at 4°C. Then, the membranes were washed with TBST three times for 10 minutes each. Next, the membranes were incubated with an HRP-conjugated secondary antibody in 1% BSA for 2 hours at room temperature and subsequently with ECL in a dark room. The densities of the resultant protein bands were detected with X-ray film and quantified using ImageJ software.

Electron microscopy

Cells were fixed with 4% paraformaldehyde and 2.5% glutaraldehyde in PBS. After dehydration, Acetone EPON812 was added, and the cells were embedded for 4 minutes. Ultrathin (50 nm) sections were subsequently prepared on an ultramicrotome (LKB-V, Sweden) with a diamond knife and were stained with uranyl acetate and lead citrate before viewing with an electron microscope (PHILIPS TECNAI-10, The Netherlands).

Flow cytometry

Cells were cultured in 6-well plates and treated, followed by washing, centrifugation, the addition of 50 µL binding buffer in 5 L of 7-AAD solution, and mixing. After the addition of 450 µL of binding buffer and 1 L of AnnexinV-PE, the reaction was allowed to proceed for 5–15 minutes prior to detection via flow cytometry.

Real-time-PCR detection

The expression of the autophagy- and apoptosis-related mRNAs was detected through PCR. The oligonucleotide primers used were as follows:

- caspase-3: forward–primer-5’-CGAACAATCTCTTCATCATTCCAGGC-3’;
- reverse–primer-5’-TAGAGTAAGCATACGGAAGTCGG-3’;
- Beclin-1: forward–primer-5’-GCTCAGTACCAGCGAGAATA-3’;
- reverse–primer-5’-GTCAGGGACTCCAGATACGA-3’;
- β-actin: forward – primer-5’-CCATCTATGAGGGTACGC-3’;
- reverse – primer-5’-TTAATGTGCAGCGACGATTTC-3’.

Total RNA was isolated from cells using RNAzol®. cDNAs from different groups were prepared by Beijing DingGuo Changsheng Biotechnology. A cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA) was employed according to the manufacturer’s instructions. Quantitative RT-PCR was performed using SYBR Green PCR master mix (Thermo Scientific, Waltham, MA, USA) according to the manufacturers’ instructions. Amplification of the housekeeping gene β-actin was used as an endogenous control. Normalized values were employed for plotting bar graphs.

Statistical analyses

Results are expressed as the mean ± SD. Statistical comparisons were performed using one-way ANOVA, and statistical significance was set at P<0.05.

Results

Cultivation and identification of primary spinal cord neurons

The images of neurons presented in Figure 1 represent cultures at different stages \textit{in vitro}. DAPI-labeled nuclei emitted blue fluorescence, TUBB3 and NSE are two neuron-specific markers distributed on neurons, axons and dendrites. Neurons showed cytoplasmic staining, which was absent from nuclei, and exhibited long processes, granular cytoplasm and a healthy morphology. An overlay of the three markers showed that the expression levels of the labels were high within the same cells (Figure 1A).
Figure 1: Figure A shows the neurons were observed via fluorescence labeling, and nuclei were stained blue. Green represents staining with one neuron-specific antibody (TUBB3), and red represents staining with another (NSE); Figure B shows the neurons after mechanical injury under treatment with different concentrations of dexamethasone. Neuron viability was assessed with MTT; Figure C shows Caspase-3 mRNA expression in neurons after mechanical injury under treatment with different concentrations of dexamethasone. Figures D and E shows Caspase-3 protein expression in neurons after mechanical injury under treatment with different concentrations of dexamethasone. Figures F and G shows the results of flow cytometry detection of apoptosis(*: Mean compared with the control group P<0.05; #: Mean compared with the injury group P<0.05).

Different concentrations of dexamethasone induced apoptosis in neuronal cells

First, the results of the MTT assay showed that the injury group presented significantly decreased cell viability compared with that of the normal group, whereas the cell viability of the hormone-treated (10^{-7} M) injury group was increased, though the increase was not significant (P>0.05). The cell activity of the 10^{-6} M hormone injury group and control group increased significantly, while the cell viability of the 10^{-5} M hormone group was significantly decreased (P<0.05). Furthermore, we assessed the levels of the apoptosis factor Caspase-3 via qRT-PCR and western blot. The experimental results showed that compared with the expression in the normal group, the expression of the LC3 II and Beclin-1 proteins was increased significantly in the injury group. LC3 II and Beclin-1 protein expression gradually decreased with increasing concentrations of dexamethasone in a dose-dependent manner (P<0.05). In the 10^{-7} M group, LC3 II and Beclin-1 protein expression was decreased, but this difference was not significant (P>0.05). The immunoblotting results regarding Caspase-3 protein expression agreed with the qRT-PCR results (Figures 1B-1E).

The flow cytometry experimental results showed that 24 hours after being subjected to damage, the apoptosis rate in the injury group was increased significantly compared with that of the normal group. The level of apoptosis in the 10^{-7} M hormone group was decreased, but the difference was not statistically significant (P>0.05). In the 10^{-5} M and 10^{-6} M hormone groups, the apoptosis rate was decreased (P<0.05); however, when the 10^{-5} M group was compared with the 10^{-6} M group, the apoptosis level was found to be increased, but not significantly so (P>0.05) (Figures 1F-1G).

Different concentrations of dexamethasone induced mechanical damage in neurons through autophagy

TEM observations of autophagosome ultrastructure were used to assess autophagy, and we observed normal cell structural integrity in normal cells, rarely detecting autophagic vacuole structures. Under injury conditions, double-membrane autophagosomes were typically found to contain organelles and cytoplasm to be degraded (Figure 2).

Figure 2: The ultrastructure of cells was observed under treatment with different concentrations of dexamethasone after mechanical injury of neurons, and the structure of the double membrane was observed via transmission electron microscopy.

The experimental results showed that compared with the expression in the normal group, the expression of the LC3 II and Beclin-1 proteins was increased significantly in the injury group. LC3 II and Beclin-1 protein expression gradually decreased with increasing concentrations of dexamethasone in a dose-dependent manner (P<0.05). In the 10^{-7} M group, LC3 II and Beclin-1 protein expression was decreased, but this difference was not statistically significant (P>0.05) (Figure 3A). The results regarding protein expression determined through
immunoblotting were consistent with the observed mRNA levels (Figure 3B).

**Figure 3:** Figure A shows LC3 II and Beclin-1 protein expression in neurons after mechanical injury under treatment with different concentrations of dexamethasone. Figure B shows Beclin-1 mRNA expression in neurons after mechanical injury under treatment with different concentrations of dexamethasone; (*: Mean compared with the control group P<0.05, #: Mean compared with the injury group P<0.05).

**Effects of dexamethasone on the mechanical injury of spinal cord neurons**

For further investigation of the regulation of autophagy after dexamethasone treatment in the presence of mechanical injury, neuronal apoptosis was assessed after 24 hours. The expression level of Caspase-3 in the 3-MA group was significantly lower than in the other two dexamethasone treatment groups and the Rap group (p<0.05). The level of apoptosis in the 3-MA group was significantly lower than in the other two groups, and the level of apoptosis in the Rap group was highest (P<0.05) (Figures 4A-4E).

The experimental results showed that the levels of LC3 II and Beclin 1 in the 24-hour group were significantly lower those in the control group after 3-MA injury, whereas LC3 II and Beclin 1 levels were significantly higher in the Rap group than in the control group (P<0.05). In terms of mRNA expression, Beclin-1 levels were significantly lower in the 3-MA group than in the control group 24 hours after injury. The ratio of Beclin-1 mRNA in the Rap group was significantly higher than in the control group (P<0.05) (Figures 4F-4I).

**Discussion**

The treatment of acute spinal cord injury is still dominated by drug treatment, but the administration of high-dose methylprednisolone pulse treatment has been increasingly questioned in recent years. Based on analyses at the protein level and transmission electron microscopy observations of autophagosomes, along with other research methods.

The authors have hypothesized that a large hormone dose reduces spinal cord injury by inhibiting lipid peroxidation and inflammatory reactions, but these results may also be due to inhibition of autophagy in neurons and, thus, spinal cord cell death. Borsello et al. also suggested that autophagy exacerbates neuronal injury [25]. The results of hormone intervention using different concentrations in damaged spinal cord neurons showed that the applied hormone could inhibit the expression of LC3 and Beclin 1. Regarding apoptosis, our results demonstrated that different hormone concentrations produced vastly different effects on the apoptosis of spinal cord neurons. A low hormone concentration showed no obvious effects following spinal cord injury. The level of Caspase-3 was decreased compared with that in the injury group, but this difference was not statistically significant. In contrast, a high hormone concentration exerted an obvious protective effect, significantly reducing the level of apoptosis of spinal cord neurons. Thus, we boldly suggest that the protective mechanism of the hormone associated with spinal cord injury involves inhibition of autophagy and apoptosis. Interestingly, high concentrations of the hormone further reduced the level of apoptosis of spinal cord neurons, according to flow cytometry results, but increased Caspase-3 expression, possibly due to the high concentrations of hormone tested. Our flow cytometry results also showed that certain hormone
concentrations exerted a protective effect on injured spinal cord neurons. The high hormone concentration tested decreased the protective effect on damaged spinal cord neurons. MTT assays showed that the high concentration of hormone significantly reduced spinal cord neuron activity, while the intermediate concentration exerted a protective effect on injured spinal cord neurons.

Kanno and others have confirmed that autophagy is activated following spinal cord injury and is associated with the occurrence of autophagy-induced cell death [17]. Research on the role of autophagy in the promotion of cell necrosis has mainly focused on the role of Beclin-1-dependent autophagy in cell death. This study evaluated the inhibition of autophagy in spinal cord neurons using 10^-6 M dexamethasone after damage via 3-MA promoter application. We found that the expression of LC3 and Beclin 1 was significantly inhibited at the same time, and Caspase-3 levels also decreased. In addition, to assess the relationship between autophagy and apoptosis in spinal cord neurons, we also used the autophagy agonist rapamycin to further elucidate the effects of hormone application. The results showed that LC3 and Beclin 1 expression levels (used as an autophagy index) were significantly increased, and the levels of Caspase-3 (as an apoptosis index) were also increased. These results suggest that the protective effect of hormones on spinal cord neurons may occur through inhibition of autophagy in spinal cord neurons, thereby reducing autophagic cell death. Seo et al. suggested that the treatment of spinal cord injury using a high dose of hormone could reduce the level of autophagy and apoptosis in injured spinal cord neurons to reduce spinal cord injury [26].

Because the mechanism of action of high-dose hormone therapy for acute spinal cord injury is highly complex and, in recent years, an increasing number of voices have questioned the role of hormones in treating spinal cord injury, there is still no clear explanation. Therefore, many scholars have proposed that the use of a variety of drugs as a combined treatment for spinal cord injury may achieve good results. One study showed that the administration of hormone combined with aminoguanidine (AG) could significantly improve the function of the hind limbs of rats with acute spinal cord injury compared with rats administered single-use hormone therapy for acute spinal cord injury [27]. Genovese et al. found that the application of hormone combined with etanercept for the treatment of acute spinal cord injury resulted in better spinal cord injury recovery than dexamethasone treatment alone [28]. Xu et al.'s research suggests that the application of AG combined with dexamethasone can significantly improve the obtained anti-inflammatory effect in rats with acute spinal cord injury [29]. Therefore, the treatment of acute spinal cord injury must be approached using a variety of drugs and multidisciplinary treatment to achieve more favorable results.

Conclusion

Our observations indicate that dexamethasone inhibited the level of autophagy in the injured nerve cells in a dose-dependent manner. High doses of dexamethasone protected the damaged spinal cord neurons by inhibiting apoptosis, but a protective effect from low hormone concentrations was not obvious.

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References


