Research Report

PI3K/Akt signaling pathway is required for neuroprotection of thalidomide on hypoxic–ischemic cortical neurons in vitro

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Abstract
Thalidomide, a derivative of glutamic acid, is used for immunomodulatory therapy in various diseases through inhibition of tumor necrotic factor-α (TNF-α) release. However, the effects of thalidomide in central nervous system (CNS) diseases such as stroke or hypoxic–ischemic encephalopathy (HIE) are unknown. In this study, we aimed to test whether thalidomide protects against hypoxic–ischemic neuronal damage and the possible signaling pathway involved in neuroprotection. Primary cultured cortical neurons of rats were treated with oxygen and glucose deprivation (OGD) for 3 h to mimic hypoxic–ischemic injury in vivo. Neuronal apoptosis was measured with terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining. The expression of total caspase-3 (C3), cleaved caspase-3 (CC3), Akt, phosphorylated-Akt (p-Akt) and Bcl-2 protein were detected by Western blots. We found that OGD treatment increased the expression of CC3 and induced neuronal apoptosis. Both neuronal apoptosis and CC3 expression peaked at 24 h after OGD. Furthermore, we found that thalidomide protected neurons against apoptosis by decreasing CC3 and increasing Bcl-2 expression in a dose-dependent manner. Meanwhile, we found that thalidomide induced p-Akt expression, which could be inhibited by PI3K specific inhibitor, LY294002. In addition, inhibition of PI3K increased CC3 but decreased Bcl-2 expression. In summary, thalidomide has anti-apoptotic effects on cortical neurons after OGD by modulating CC3 and Bcl-2 expression through activation of PI3K/Akt pathway.

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Abbreviations:
TNF-α, tumor necrotic factor-α; CNS, central nervous system; HIE, hypoxic–ischemic encephalopathy; OGD, oxygen and glucose deprivation; DMSO, dissolved in dimethyl sulfoxide; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling; C3, caspase-3; CC3, cleaved caspase-3; p-Akt, phosphorylated-Akt; HI, hypoxic ischemic; ERK, extracellular signal regulated kinase; DIV, day in vitro; PBS, phosphate-buffered saline

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1. Introduction

Neonatal hypoxic ischemic (HI) brain injury is a serious insult that frequently leads to high disability and mortality in neonates. One of the major mechanisms involved in brain injury after HI is neuronal loss caused by cellular apoptosis (Li et al., 2007; Qu et al., 2009). Under HI stimulation, whether neurons undergo apoptosis or survival depends on a balance between cellular apoptotic and cell survival signal transduction. Recent studies have shed light on promoting cellular survival after neonatal HI by inhibiting apoptotic neuronal loss (Lan et al., 2008; Gao et al., 2010).

Thalidomide is an important drug with a broad-spectrum of pharmacologic and immunologic effects. Over the past decades, thalidomide has been effectively and safely used in the treatment of erythema nodosum leprosum, lupus erythematosus, Crohn’s disease and rheumatoid arthritis (Raje and Anderson, 1999; Housman et al., 2003; Lamondon et al., 2007; García-Carrasco et al., 2007). Moreover, thalidomide has been proven to have anti-inflammatory and immunoregulatory effects due to its ability to inhibit tumor necrotic factor-α (TNF-α) production and enhance degradation of TNF-α mRNA both in vivo and in vitro (Klausner et al., 1996; Paul et al., 2006; Nakamura et al., 2007). Recently, some studies have been carried out to examine the neuroprotective effects of thalidomide. Lee et al. (2007) have reported that thalidomide reduces ischemic injury of the spinal cord in rabbits through reduction of TNF-α expression. In addition, Hyakkoku et al. (2009) have found that thalidomide has potential neuroprotection in focal cerebral ischemia in adult mice by inhibition of oxidative stress. We have demonstrated that increased TNF-α expression can induce neuronal apoptosis in a neonatal rat stroke model (Mao et al., 2006). Taken together, whether thalidomide can protect neurons against apoptosis after HI is becoming interesting and the mechanisms of how thalidomide exerts its neuroprotective effects need to be further investigated.

Besides regulation of TNF-α expression, thalidomide can exert its functions through modulating different signaling pathways. Previous studies have shown that thalidomide induced cell apoptosis and limb anomalies in human embryonic fibroblasts and chicken embryo by stabilization of PTEN and suppression of phosphorylated-Akt (Knobloch et al., 2008). In a monocytic leukemia cell line, thalidomide was shown to inhibit cell growth through inhibition of extracellular signal regulated kinase (ERK) 1/2 (Gockel et al., 2004). However, the possible signaling pathways involved in thalidomide function remain unclear.

Previous studies have indicated that PI3K/Akt signaling pathway had a survival role in protecting cells from caspase-mediated apoptosis (Cantley, 2002; Ma et al., 2009). Phosphorylated-Akt (p-Akt) is proven to be necessary for neuronal survival after HI since p-Akt plays a key role in upregulation of anti-apoptotic proteins such as Bcl-2 and Bcl-xL (Hsu et al., 2010). Moreover, we recently found that PI3K/Akt pathway played important roles in neuroprotection against hypoxia-ischemia brain damage (Li et al., 2008; Zhang et al., 2009). Based on the above findings, we hypothesized that thalidomide can protect neurons from HI injury through PI3K/Akt signaling pathway. In the present study, we set up an HI model with oxygen and glucose deprivation (OGD) in cultured neurons. Using this model, PI3K specific inhibitor LY294002 was used to examine the possible signaling pathways involved in the anti-apoptotic effects of thalidomide.

2. Results

2.1. Expression of cleaved caspase-3 (CC3) and neuronal apoptosis after OGD

To study whether neuronal apoptosis is induced by OGD, TUNEL staining was used to detect apoptotic neurons. The percentage of TUNEL positive cells were calculated by selecting 5 high-power fields to count the total number of neurons and the positive staining cells. The number of positive staining cells divided by the total number of neurons was considered the percentage of positive staining. We found that the number of TUNEL positive cells in neurons at 4, 8, 24 h after OGD was around 16%, 30%, and 48%, respectively. About 7% TUNEL positive cells were found in normoxic control neurons (Fig. 1A). There was no positive cell in negative controls (data not shown).

Since caspase-3 (C3) is one of the key mediators of apoptosis upon its activation via cleavage, we detected total C3 and CC3 protein levels in both OGD treated and normoxic control neurons at 0, 2, 4, 8, 12 and 24 h after reperfusion using Western blot analysis. We found that CC3 (17 kDa) was significantly induced at 4 h, gradually increased through 12 h, and peaked at 24 h after reperfusion compared to that seen in 0 h after OGD and in normoxic cultured neurons (Fig. 1B). After normalization with β-actin, an approximate 4-fold, 6-fold, 7-fold and 10-fold increase of CC3 expression was respectively observed at 4, 8, 12 h, and 24 h after OGD compared to that at 0 h after OGD (Fig. 1C, p<0.001, n=4). There was no significant change in total C3 (35 kDa) expression between normoxic and OGD treated neurons (Fig. 1C, p>0.05, n=4).

2.2. Thalidomide decreased CC3 and increased Bcl-2 expression in a dose-dependent manner after OGD

To investigate whether thalidomide could decrease neuronal apoptosis after OGD, we examined the effect of thalidomide in the induction of anti-apoptotic proteins Bcl-2 and pro-apoptotic protein CC3. Since both CC3 expression and TUNEL positive cells peaked at 24 h after OGD (Fig. 1), we chose 24 h to perform the following experiments. Cells were pre-treated with thalidomide at 25, 50, 100 μg/mL or with dimethyl sulfoxide (DMSO) as vehicle control for 1 h before OGD. Cells then underwent OGD for 3 h with the same concentration of thalidomide or DMSO. After OGD, cells were reperfused in NB medium for 24 h. We found that thalidomide treatment reduced CC3 expression and increased Bcl-2 expression (35 kDa) in a dose-dependent manner at 24 h after OGD compared with that in DMSO treated neurons (Fig. 2A, B).

After normalization with β-actin, we found that CC3 was decreased approximately 40–50% and Bcl-2 increased approximately by 1- to 1.3-fold in thalidomide (50–100 μg/mL) treated
Fig. 1 – Expressions of neuronal apoptosis and caspase-3, CC3 after OGD. TUNEL staining showed that positive cells were increased at 4 h, 8 h, and peaked at 24 h after OGD. There was no positive cell in negative control neurons (n=4). (×400, arrows show TUNEL positive cells) (A). Western blot was used to detect caspase-3 and CC3 protein expression in cortical neurons at 0, 2, 4, 8, 12, 24 h after OGD. Equal amount of protein samples (60 μg) was analyzed with caspase-3 and CC3 antibodies. β-actin was used as a loading control. The total caspase-3 protein (C3) was not changed after OGD, but its activated form, CC3 was significantly increased at 4 h, 8 h, 12 h, and peaked at 24 h (B). The relative expression levels of proteins were calculated as relative optical density normalized to β-actin internal controls and represented as mean±SD (n=4). **p<0.001 compared to group at 0 h (C). N, normoxia.
Fig. 2 – Thalidomide regulating CC3 and Bcl-2 expression in cultured neurons after OGD. Thalidomide at 25 μg/mL, 50 μg/mL, or 100 μg/mL was added to cultured neurons 1 h before OGD. Western blot was used to detect pro-apoptotic protein CC3 and anti-apoptotic protein Bcl-2 expression. Thalidomide was found to respectively decrease CC3 and increase Bcl-2 expression in a dose-dependent manner after OGD (A). The relative expression levels of proteins were calculated as relative optical density normalized to β-actin. Data were represented as mean±SD. For each independent experiment, n=4. *p<0.05, **p<0.01 compared to DMSO control. (B). Thalidomide at 50 μg/mL reduced the number of TUNEL positive cells compared with DMSO treated neurons at 24 h after OGD by TUNEL staining (arrows show TUNEL positive cells) (C). Quantitative representation of TUNEL positive cells in DMSO or thalidomide treated neurons. *p<0.01 compared to DMSO control, n=4. (D). N, normoxia; TLD, thalidomide.
neurons compared to that seen in DMSO treated neurons (Fig. 2B, \( p<0.01, n=4 \)). Since 50 \( \mu \text{g/mL} \) of thalidomide could significantly decrease CC3 and increase Bcl-2 expression, this dosage of thalidomide was used for further studies.

To investigate the effect of thalidomide on neuronal apoptosis, apoptotic cells were detected using the TUNEL staining. We found that thalidomide of 50 \( \mu \text{g/mL} \) reduced neuronal apoptosis compared with DMSO treated neurons at 24 h after OGD (Fig. 2C). After statistical analysis, the number of TUNEL positive cells in thalidomide treated neurons was decreased approximately 34\% compared with that in DMSO control neurons (\( p<0.01, n=4 \)) (Fig. 2D).

2.3. Involvement of PI3K/Akt signaling pathway in thalidomide-induced anti-apoptosis

PI3K/Akt pathway has been proven to be necessary for neuronal survival, angiogenesis, and synaptic transmission. Akt signaling activation could antagonize the intrinsic and extrinsic apoptotic pathways in vivo and in vitro (Gong et al., 2007; Jin et al., 2007). To investigate whether thalidomide signals through the PI3K/Akt pathway to execute its anti-apoptotic effect after OGD, thalidomide, PI3K/Akt inhibitor LY294002 (20 \( \mu \text{M} \)) with or without thalidomide, or DMSO control, was added to cultured cortical neurons. Akt activity was determined by Western blot using a phosphorylated Akt (Ser473) monoclonal antibody. We found that p-Akt (60 kDa) was significantly up-regulated in thalidomide treated neurons at 24 h after OGD compared to DMSO treated neurons (Fig. 3A, TLD50). However, the upregulation of p-Akt by thalidomide could be significantly inhibited by PI3K inhibitor, LY294002 (Fig. 3A, TLD/LY). In addition, we found that LY294002 alone could completely inhibit p-Akt expression (Fig. 3A, LY). In contrast, we did not find any significant changes in total Akt expression in different agents treated neurons (Fig. 3A, B).

After normalization with \( \beta \)-actin, there was an approximately 5-fold increase of p-Akt protein in thalidomide treated neurons compared to that in DMSO treated neurons (Fig. 3B, \( p<0.001, n=4 \)). Thalidomide-induced p-Akt expression was significantly inhibited by approximately 60\% with LY294002 in both thalidomide and LY294002 treated neurons (Fig. 3B, \( p<0.001, n=4 \)).

![Fig. 3 - The effects of thalidomide on Akt pathway in cortical neurons after OGD. Thalidomide of 50 \( \mu \text{g/mL} \) and PI3K inhibitor, LY294002 of 20 \( \mu \text{M} \) were used to study the regulation of Akt signaling pathway in cultured neurons after OGD. Western blot showed that the phosphorylation of Akt (p-Akt) was significantly up-regulated in thalidomide treated neurons compared to that seen in DMSO treated neurons (A, TLD50). However, this upregulation could be significantly inhibited by PI3K inhibitor LY294002 (A, TLD/LY). In addition, we also found that LY294002 alone could completely inhibit p-Akt expression after OGD (A, LY). Total Akt was not significantly changed after OGD in thalidomide or LY294002 treated neurons compared to DMSO control neurons (A). Akt and p-Akt relative expression levels were calculated as relative optical density normalized to \( \beta \)-actin internal controls. Data were represented as mean \pm SD. For each column, \( n=4 \). *\( p<0.001 \) compared to DMSO control, \( \#p<0.001 \) compared to TLD50 (B). TLD, thalidomide; LY, LY294002.]

2.4. Thalidomide regulated CC3 and Bcl-2 protein expression through PI3K/Akt signaling pathway

To further study the effects of thalidomide on PI3K/Akt and its downstream apoptosis related proteins such as CC3 and Bcl-2, we detected the expression of CC3 and Bcl-2 after treatment with thalidomide, LY294002, or LY294002 with thalidomide using Western blot analysis. We found a significant decrease in CC3 and increase in Bcl-2 in thalidomide treated neurons compared with DMSO treated neurons at 24 h after OGD (Fig. 4A, C, TLD50). These effects of thalidomide on CC3 and Bcl-2 could be blocked by LY294002 (Fig. 4A, C, TLD/LY). In addition, we found that LY294002 alone could markedly increase CC3 and decrease Bcl-2 expression (Fig. 4A, C, LY). There was no difference in total caspase-3 expression in neurons with different treatments (\( p>0.05, n=4 \)) (Fig. 4A).

After normalization with \( \beta \)-actin, we found that CC3 was decreased approximately 45\% and Bcl-2 increased approximately 90\% in thalidomide treated neurons compared to that seen in DMSO treated neurons (Fig. 4, \( p<0.01, n=4 \)). There was an approximately 50\% increase in CC3 protein and a 35\% decrease in Bcl-2 protein in neurons treated with both thalidomide and LY294002 compared to that in neurons treated with thalidomide alone (Fig. 4, \( p<0.01, n=4 \)).

3. Discussion

In this study, we show for the first time that thalidomide protects cultured cortical neurons from hypoxic-ischemic injury by attenuating neuronal apoptosis, and this neuroprotection of thalidomide is PI3K/Akt pathway dependent.

As we know, thalidomide was widely used as a sedative and antiemetic drug in Europe and Canada in the 1960s, but was removed from the market because of its teratogenicity such as phocomelia in the infants born from pregnant women.
The mechanisms of teratogenicity caused by thalidomide have not been clarified since then. Fortunately, Ito et al. (2010) recently reported that thalidomide executes its teratogenic effects by binding to cereblon protein, which inhibits ubiquitin ligase activity leading to retardation of limb growth. These findings may contribute to the development of new thalidomide derivatives without teratogenicity by mutating the binding site of thalidomide to cereblon protein and thus may provide a new perspective for its clinical therapy.

In previous reports, thalidomide pretreatment caused significant inhibition of TNF-α production and thus played a protective role in primary rat glial cells (Gallily et al., 1999; Majumdar et al., 2002). In the present study, we focused on determining the effects of thalidomide pretreatment on neurons with OGD and tried to identify the signaling pathways involved in the protective roles of thalidomide. Our research designs are in agreement with most of the studies in detecting the role of drugs or agents including thalidomide in an in vitro pretreatment model (Majumdar et al., 2002; Hamrick et al., 2005; Schreihofer and Redmond, 2009; Yang et al., 2010). Therefore, this investigation is mainly aimed at studying the preventive function but not the treatment roles of thalidomide in neurons with OGD. More importantly, our findings that the PI3K/Akt pathway dependent neuroprotection of thalidomide in this model may help us to further explore the mechanisms of thalidomide in not only the prevention but also the treatment for some clinical diseases such as hypoxic-ischemic encephalopathy, Crohn’s disease and rheumatoid arthritis.

Thalidomide plays different roles depending on cell type through different signaling pathways. Previous studies have shown that thalidomide induced monocyte apoptosis through a mitochondrial signaling pathway (Knobloch et al., 2008). In a monocytic leukemia cell line, thalidomide was shown to inhibit cell growth through extracellular signal regulated kinase (ERK) 1/2 (Gockel et al., 2004). However, in a rabbit spinal cord ischemic model, thalidomide was proven to reduce ischemic injury via reducing TNF-α expression although the signaling pathway was not studied (Lee et al., 2007). In addition, increased TNF-α expression can induce neuronal apoptosis in a neonatal rat stroke model (Mao et al., 2006). Therefore, it is important to study whether thalidomide can protect neurons against apoptosis after HI and the possible signaling pathways involved in the neuroprotection.

PI3K/Akt signaling pathway plays a survival role in protecting cells from caspase-mediated apoptosis (Cantley, 2002; Ma et al., 2009). We recently found that PI3K/Akt pathway is involved in the upregulation of HIF-1α which is shown to play important roles in neuroprotection after HI (Li et al., 2008; Zhang et al., 2009). Therefore, it is important to study the role of PI3K/Akt pathway in thalidomide mediated anti-apoptosis in cultured cortical neurons after HI.

In this study, we found that both TUNEL and CC3 positive neurons peaked at 24 h after OGD, which indicates that neuronal apoptosis occurred evidently at 24 h after exposure to OGD. This is in agreement with previous in vivo findings that neuronal apoptosis is mostly detected at 24 h after HI (Li et al., 2007). In our preliminary study, neuronal apoptosis can be detected at 24 h, 48 h, and 72 h after OGD with a peak at 24 h. Apoptotic peak was maintained at 48 h, but started to decline at 72 h. Therefore, 24 h time point was used to study the neuroprotection of thalidomide. Bcl-2 is a potential inhibitor of apoptosis in response to a variety of cytotoxic stimuli (Henshall et al., 2002; Creson et al., 2009). Bcl-2
prevents disruption of the mitochondria and the subsequent release of cytochrome c and caspase activation (Akhtar et al., 2004; Kuhn et al., 2005; Shacka and Roth, 2005). Therefore, Bcl-2 is a classical anti-apoptotic protein and was used as a marker to determine the role of thalidomide in this study. We found that thalidomide enhanced the expression of neuronal Bcl-2 and decreased CC3 and TUNEL positive cells after OGD, suggesting that thalidomide has anti-apoptotic function. Our findings are consistent with recent studies that thalidomide plays an anti-apoptotic role in adult mice focal ischemic model (Hyakkoku et al., 2009).

In embryonic fibroblasts, thalidomide was reported to induce apoptosis through inhibiting PI3K/Akt-mediated survival signaling (Knobloch et al., 2008). In our study, we found that thalidomide increased the phosphorylation of Akt (p-Akt), and this phosphorylation could be significantly inhibited by PI3K inhibitor, suggesting that PI3K/Akt pathway was activated by thalidomide in this neuronal OGD model. Our findings suggest that the functions of thalidomide were neuron dependent. In this study, the expression of CC3 was reduced by thalidomide treatment but was increased in the presence of PI3K inhibitor, suggesting that CC3 was the downstream mediators of the PI3K/Akt pathway in OGD neurons. On the contrary, Bcl-2 was increased when PI3K/Akt pathway was activated by thalidomide, suggesting an anti-apoptotic role of thalidomide in neuroprotection. Our findings are in agreement with previous studies showing that thalidomide reduced cellular apoptosis in an in vivo mouse model of focal cerebral ischemia (Hyakkoku et al., 2009).

In conclusion, we found that thalidomide can protect neurons from apoptosis after OGD through activation of PI3K/Akt signaling pathway. The neuroprotection of thalidomide is related to the down-regulation of pro-apoptotic protein CC3 and an up-regulation of anti-apoptotic protein Bcl-2. Our findings suggest a new mechanism of thalidomide in neuroprotection, and may provide an investigative tool to study signaling transduction in HI. Although we have shown that thalidomide has anti-apoptotic effects on cortical neurons after OGD by modulating CC3 and Bcl-2 expression through activation of PI3K/Akt pathway, additional potential mechanisms for the protective function of thalidomide may exist and need to be further investigated.

4. Experimental procedures

4.1. Cortical neuron culture

All animal research was approved by Sichuan University Committee on Animal Research. Cell culture was performed as previously described (Zhang et al., 2009). Primary neuronal cultures were prepared from the cerebral cortices of Sprague-Dawley rat embryos at 16–18 days of gestation. The whole cerebral cortex was isolated from the fetuses and cells were dissociated in a trypsin solution (1.25 mg/mL in Hank’s Buffer salt solution) for 10 min at 37 °C. The cortex cell suspension was centrifuged and resuspended, then seeded in 6-well plates precoated with poly-D-lysine (Sigma), and grown in neurobasal medium (Gibco) with 2% B27 supplement (Gibco) (NB) and 500 μM glutamine (Gibco) in a humidified incubator with 5% CO2 at 37 °C. To change the cell culture medium, half volume of the medium was aspirated and replaced with the same volume of fresh medium on day in vitro (DIV) 3. The following experiments were performed at DIV5.

4.2. OGD treatment

Cultured cortical neurons were exposed to OGD for 3 h followed by reoxygenation. To induce OGD, cultured cells were gently washed twice with phosphate buffered saline (PBS, pH7.4), and then placed in DMEM without glucose. Cells were placed in an incubator attached to a hypoxia chamber that could maintain a humidified atmosphere with 1% oxygen at 37 °C for 3 h. After 3 h of OGD treatment, the cell culture medium was changed back to NB medium and then returned to 5% CO2, 37 °C incubator. To elucidate the effects of thalidomide on cortical neurons with OGD, cells were pretreated with thalidomide (Sigma) dissolved in DMSO (Sigma) at different concentrations of 25 μg/mL, 50 μg/mL or 100 μg/mL for 1 h before OGD treatment. PI3K inhibitor, LY294002 of 20 μM was used for 1 h before OGD treatment. Control neurons were treated with the equivalent amount of DMSO solution.

4.3. Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining

For TUNEL assay, cells were stained using In Situ Cell Death Detection Kit (Roche). Cultured cells were washed three times in 0.1 M PBS, fixed with 4% paraformaldehyde, and treated with 0.1 M citrate solution and 3% hydrogen peroxide at room temperature for 10 min. Cells were washed in PBS and incubated with biotinylated nucleotide and the Terminal Deoxynucleotidyl Transferase, Recombinant (rTdT) enzyme at 37 °C for 1 h. After washes, cultures were incubated with Streptavidin HRP solution in a humidified chamber for 30 min, and then developed using DAB and counterstained with hematoxylin. As negative controls, alternate cultures were processed in parallel without rTdT enzyme. After washing, cultures were mounted on gelatin-coated slides, dried, and coverslipped. Images were observed using microscope (Leica, CM 2000). To quantify the number of TUNEL positive cells (%) in cultured neurons, we selected 5 high-power fields to count the total number of neurons and positive cells. The number of positive cells divided by the total number of neurons was considered the percentage of positive staining.

4.4. Western blot

Cells were lysed in CHAP lysis buffer, and then collected by centrifugating at 14,000 rpm for 30 min at 4 °C as previously described (Zhang et al., 2009). The protein concentrations in the supernatant were measured using the BCA protein assay kit (Pierce). Equal amounts of proteins (60 μg) were electrophoresed through standard 12% SDS-PAGE in Tris-glycine electrophoresis buffer [25 mM Tris, 192 mM glycine (pH 8.3) and 0.1% SDS]. Recombinant protein molecular weight marker (Fermentas) was used as a size reference. Proteins were transferred to PVDF membranes at 320 mA for 1 h. The membranes were then incubated in blocking buffer (5% nonfat dry milk and 0.1% Tween-20 in 20 mM Tris–HCl buffer, pH 7.6,
containing 137 mM sodium chloride), at room temperature for 1 h with rotation. The membranes were then separately incubated with rabbit caspase-3 antibody or cleaved caspase-3 antibody (Cell Signaling, diluted 1:200 in blocking solution), rabbit Akt polyclonal antibody, mouse phospho-Akt (Ser473) monoclonal antibody (Cell Signaling, diluted 1:200), or rabbit Bcl-2 antibody (Abcam, diluted 1:200), overnight at 4 °C. Mouse β-actin monoclonal antibody (Santa Cruz Biotechnology, diluted 1:1000) was detected as the internal control. The membranes were washed three times with Tris-buffered saline containing 0.1% Tween-20, and then incubated with horseradish peroxidase conjugated secondary antibody, goat anti-mouse or goat anti-rabbit IgG (Santa Cruz Biotechnology, 1:3000) in blocking buffer at room temperature for 1 h. The bands were developed using enhanced chemiluminescence (Millipore Corporation). NIH image was used to measure the densities of the protein signals.

4.5. Statistics

Data are represented as mean±standard deviation (SD). One-way ANOVA with Bonferroni/Dunnett post-hoc tests was performed for multiple comparisons. Statistical differences between two groups were compared using t tests. p<0.05 was considered statistically significant.

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