Daucosterol protects neurons against oxygen–glucose deprivation/reperfusion-mediated injury by activating IGF1 signaling pathway

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\textbf{ABSTRACT}

We previously reported that daucosterol (a sterolin) up-regulates the expression of insulin-like growth factor I (IGF1)\textsuperscript{1} protein in neural stem cells. In this study, we investigated the effects of daucosterol on the survival of cultured cortical neurons after neurons were subjected to oxygen and glucose deprivation and simulated reperfusion (OGD/R),\textsuperscript{2} and determined the corresponding molecular mechanism. The results showed that post-treatment of daucosterol significantly reduced neuronal loss, as well as apoptotic rate and caspase-3 activity, displaying the neuroprotective activity. We also found that daucosterol increased the expression level of IGF1 protein, diminished the down-regulation of p-AKT\textsuperscript{3} and p-GSK-3\textsuperscript{b}\textsuperscript{4}, thus activating the AKT\textsuperscript{5} signal pathway. Additionally, it diminished the down-regulation of the anti-apoptotic proteins Mcl-1\textsuperscript{6} and Bcl-2\textsuperscript{7}, and decreased the expression level of the pro-apoptotic protein Bax\textsuperscript{8}, thus raising the ratio of Bcl-2/Bax. The neuroprotective effect of daucosterol was inhibited in the presence of picropodophyllin (PPP)\textsuperscript{9}, the inhibitor of insulin-like growth factor I receptors (IGF1R)\textsuperscript{10}. Our study provided information about daucosterol as an efficient and inexpensive neuroprotectants, to which the IGF1-like activity of daucosterol contributes. Daucosterol could be potentially developed as a medicine for ischemic stroke treatment.

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1. Introduction

Stroke is the third leading cause of death and disability in developed countries, and ischemic stroke accounts for approximately 85% of all strokes [1]. During ischemic brain injury, the lack of oxygen and glucose supply leads to neuronal damage. Although the cellular mechanisms of ischemic neuronal injury are not completely understood, apoptosis plays an important role in the development of ischemic neuronal cell death [2,3]. These studies suggested that reducing neuronal apoptosis is probably essential for therapeutic intervention of ischemic brain injury.

Studies have shown that insulin-like growth factor I (IGF1) reduces neuronal cell death in various injury insults [4,5] and IGF1 has a protective effect in ischemic animal models [6,7].
IGF1 displays strong anti-apoptotic activities, and its receptor is present on most cells, including neurons [8]. IGF1 suppresses apoptosis through mechanisms involving activation of multiple protein kinase pathways, which are most likely to be important early in the latent phase of evolving programmed cell death [9]. Reagents that stimulate IGF1 activity and downstream signaling, such as AKT pathway, may thus prevent apoptosis and increase neuronal survival.

We previously reported that the expression of IGF1 protein in neural stem cells was increased by treatment with daucosterol (a sterolin, chemical structure see Fig. 1), playing key role in the promotion of the proliferation of neural stem cells [10]. It is feasible that by activating IGF1 signaling pathway, daucosterol exerts the protective effects on neurons. In this study, we investigated the effects of daucosterol on the survival of cultured cortical neurons exposed to ischemic insults, and determined the corresponding molecular mechanism. To mimic an ischemic insult, the brain cortical neurons subjected to oxygen and glucose deprivation and simulated reperfusion (OGD/R) were used as an in vitro model of ischemia [11].

2. Materials and methods

2.1. Primary cortical neuron culture

Primary cultures of cortical neurons were obtained from embryonic day 18 (E18) Sprague–Dawley rat brains. The procedures have been described previously [12]. Briefly, cerebral cortices were isolated and dissociated enzymatically (0.125% glucose-free Earle’s balanced salt solution (BSS) and immediately through a nylon mesh (pore size 40 μm). Cells were plated at a density of 2 × 10^4 cells/cm² on 6-well or 96-well poly-L-lysine-coated culture cluster (Corning Incorporated, USA) and grown at 37 °C for 10 min) and mechanically and filtered through a nylon mesh (pore size 40 μm). Cells were plated at a density of 2 × 10^4 cells/cm² on 6-well or 96-well poly-L-lysine-coated culture cluster (Corning Incorporated, USA) and grown at 37 °C under a humidified atmosphere of 5% CO₂ and 95% air in neurobasal medium (Gibco, USA) supplemented with B27 (2%, Gibco, USA), glutamine (2 mM), and 1% penicillin/streptomycin. Cytosine-β-arabinofuranoside (10 μM) was added to the cultures 24 h after plating, to avoid proliferation of non-neuronal elements, and was kept for 2 days before medium replacement. More than 90% of primary cultured cortical cells were positive for neuronal marker NeuN antibody. Cells were used between 6 and 8 days in vitro [13].

2.2. Oxygen–glucose deprivation and simulated reperfusion (OGD/R) model

OGD/R model was performed as reported previously with minor modifications [11,14]. The cells were incubated with a glucose-free Earle’s balanced salt solution (BSS) and immediately transferred to a humidified anaerobic chamber for 2 h perfused with 95% N₂ and 5% CO₂ at 37 °C. To terminate OGD, the cells were cultivated in neurobasal medium and the supplements under a humidified atmosphere of 5% CO₂ and 95% air at 37 °C.

2.3. Daucosterol administration

Daucosterol (purity: 98%, provided by Department of Pharmacognosy, Nanjing University of Chinese Medicine) stock solution was prepared in dimethyl sulfoxide (DMSO) and diluted in a fresh medium to obtain a final DMSO concentration of <0.1%. For administration, daucosterol was added to neurobasal medium after termination of OGD, and was kept for 24 h.

2.4. Cell viability assay

Cell viability was analyzed using cell counting kit-8 (CCK-8) [11]. CCK-8 is a sensitive non-radioactive colorimetric assay used to determine the number of viable cells in cell proliferation and cytotoxicity assays. In the study, the neurons plated in 96-well cell culture cluster were divided into ten groups: control group, OGD/R group, and OGD/R + daucosterol treatment groups (1.25, 2.5, 5, 10, 20, 40, 80, and 100 μM). Each group was designed to establish six double-pore treatments. The cells of the nine groups were subjected to OGD/R injury except for the control group. Each daucosterol treatment group was treated with the corresponding amount of daucosterol for 24 h. CCK-8 solution (Beyotime Biotech, China) was added to the cell culture medium to a final concentration of 10 μL/100 μL, and incubated for another 4 h at 37 °C. Absorbance was measured at 450 nm to determine cell viability, using a microplate reader (ELx800, BioTek Instruments Inc., USA).

2.5. Caspase-3 activity assay

Caspase-3 has been identified as a key mediator of apoptosis in animal models of ischemic stroke [15]. In the study, caspase-3 activity was measured in lysates of neurons using the Caspase-3 Activity Assay Kit (Beyotime Biotech, Haimen, China) following the instructions of manufacturer. The colorimetric substrate N-acetyl-Asp-Glu-Val-Asp (DEVD)-p-nitroaniline (Ac-DEVD-pNA) is labeled with the chromophore p-nitroaniline that is released from the substrate upon cleavage by caspase-3. Free pNA produces a yellow color that is monitored by a spectrophotometer at 405 nm. Briefly, neurons were lysed in ice-cold lysis buffer for 20 min. After removal of cellular debris by centrifugation (10 min at 16,000 rpm at 4 °C), the supernatant was used to detect caspase-3 proteolytic activity. Samples were incubated with 200 μM caspase-3 substrate Ac-DEVD-pNA at 37 °C for 4 h and then analyzed at 405 nm in a microtiter plate reader. The protein levels in the lysates were measured with BCA protein assay kit (Pierce Biotechnology, Rockford, USA).

2.6. Cell apoptosis analysis

Cell apoptosis was analyzed by use of flow cytometry and double staining cells with Annexin V and propidium iodide (PI) [16]. After treatment with daucosterol (10 and 20 μM) for 24 h, the neurons plated in 6-well cell culture cluster were washed twice with PBS and subjected to Annexin V-FITC/PI double staining at room temperature in the dark as described in the manufacturer’s instructions (Annexin V-FITC Apoptosis Detection Kit (BD Biosciences, San Jose, CA)). After 15 min of staining, the neurons were

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**Fig. 1.** Chemical structure of daucosterol. Molecular weight: 576.85.
analyzed by flow cytometry (BD Bioscience, San Jose, CA) and the apoptotic rate was determined.

2.7. IGF1 protein quantitation

IGF1 protein was quantified by ELISA. The cells were treated as described above and the supernatant was collected and processed using the QuantiKine Rat IGF1 ELISA kit (R&D Systems, Inc., USA), following the instructions provided by the manufacturer. The absorbance of each well was determined using a microplate reader (ELX800, BioTek instruments, Inc., USA) set at 450 nm.

2.8. Western blot analysis

The cells were treated as described above, and then schizolysed in RIPA buffer containing protease inhibitors and phosphatase inhibitors. Protein concentration was determined colorimetrically by BCA assay. Protein lysates were separated by 12% SDS-PAGE electrophoresis and were transferred onto polyvinylidene difluoride (PVDF) membranes. After blocking with 5% BSA for 1 h, the membranes were incubated with Phospho-AKT (Ser 473, Cell Signaling Technology, 1:200), AKT (Cell Signaling Technology, 1:200), phospho-GSK-3β (Ser 9, Cell Signaling Technology, 1:200), GSK-3β (Cell Signaling Technology, 1:200), Bcl-2 (Cell Signaling Technology, 1:200), Bax (Cell Signaling Technology, 1:200), Mcl-1 (Santa Cruz, 1:100), GAPDH (Invitrogen, 1:1000) antibodies at 4°C overnight, followed by incubation with horseradish peroxidase-conjugated secondary antibodies for 1 h. Then, the blots were visualized using ChemiDoc™ XRS+ System with Image LabTM Software (Bio-Rad Laboratories, Inc., USA). Experiments were performed 3 times.

2.9. PPP inhibition test

To investigate the role of increased IGF1 in the neuroprotective effects of daucosterol, picropodophyllin (PPP) inhibition test was performed. Among the various IGF1R chemical inhibitors that are currently available, we decided to focus on the cycloolignans family member PPP, because of previous reports showing specific inhibition of IGF1R tyrosine phosphorylation and activity, without effects on other relevant tyrosine kinase receptors [17]. In particular, cycloolignans can specifically and efficiently reduce the auto-phosphorylation, activity and hence downstream signaling of IGF1R in different cell lines. Furthermore, PPP has been shown to be virtually non-toxic in animal models [17,18]. In this study, if the neuroprotective effects induced by daucosterol were blocked up by PPP, it can demonstrate that the neuroprotective activity depends on the increased IGF1 protein.

In the study, PPP (Tocris Bioscience, Bristol, UK) stock solution was prepared in DMSO and stored at 4°C. Before each experiment was performed, the solution was diluted in a fresh medium to obtain a final DMSO concentration of <0.1%. The cells, which were cultured in 96-well culture cluster at a cell density of 1 × 10^4 cells per well, were divided into control group, OGD/R group, OGD/R + daucosterol 10 μM group, OGD/R + daucosterol 20 μM group, OGD/R + PPP group, OGD/R + PPP + daucosterol 10 μM group, and OGD/R + PPP + daucosterol 20 μM group (with PPP dose of 200 nM, a nontoxic and effective dose [19]). Each group was designed to establish six double-pore experiments. After exposure to OGD/R injury, the PPP-daucosterol treated cells were treated with PPP for 1 h ahead of daucosterol administration. The daucosterol-treated cells were treated with daucosterol for 24 h. Then cell viability was analyzed using CCK-8 assay. Absorbance was measured at 450 nm to determine cell viability.

2.10. Statistical analyses

Statistical analyses were performed by use of SPSS version 16.0 software. Multiple comparisons were made using one-way ANOVA, followed by the Bonferroni post-test. All data were presented as mean ± S.D., and statistical significance was accepted at the 5% level.

3. Result

3.1. Daucosterol significantly ameliorated the cell viability

Cell viability assay was performed using CCK-8. Cell viability was notably aggravated when neurons were subjected to OGD/R injury. However, the cell viability was significantly ameliorated after post-treatment with daucosterol at concentrations of 20 μM (p < 0.01) and 10 μM (p < 0.05, Fig. 2), indicating that daucosterol reduced neuronal loss.

3.2. Daucosterol significantly suppressed caspase-3 activity

Caspase-3 activity was notably enhanced when the neurons were subjected to the OGD/R insult, which was significantly suppressed after the insulted neurons were post-treated with daucosterol at concentrations of 20 μM (p < 0.01) and 10 μM (p < 0.05, Fig. 3).

3.3. Daucosterol significantly reduced cell apoptosis

Cell apoptosis was measured by flow cytometry using Annexin V–FITC and PI double staining. The apoptotic rate was determined by ELISA. The cells were treated as described above and the supernatant was collected and processed using the Quantikine Rat IGF1 ELISA kit (R&D Systems, Inc., USA), following the instructions provided by the manufacturer. The absorbance of each well was determined using a microplate reader (ELX800, BioTek instruments, Inc., USA) set at 450 nm.

3.4. Daucosterol significantly upregulated the expression of IGF1 protein

IGF1 protein quantitation was performed by ELISA. IGF1 levels were markedly up-regulated after the insulted neurons were post-treated with daucosterol at concentrations of 20 and 10 μM (p < 0.001, Fig. 4).

3.5. Daucosterol significantly activated the AKT signal pathway, and exerted the effects on the expression levels of apoptosis-related proteins

Western blot analysis showed that in OGD/R-treated cells, daucosterol remarkably diminished the down-regulation of p-AKT and p-GSK-3β (p < 0.01, Fig. 6), activating the AKT signal pathway. In addition, daucosterol remarkably diminished the down-regulation of the anti-apoptotic proteins Mcl-1 (p < 0.01, Fig. 7) and Bcl-2 (p < 0.001, Fig. 8B), but decreased the expression level of the pro-apoptotic protein Bax (p < 0.01, Fig. 8B), thus raising the ratio of Bcl-2/Bax (p < 0.001, Fig. 8C). In a word, daucosterol post-treatment ameliorated the serious consequence induced by OGD/R.

3.6. The neuroprotective effect of daucosterol was inhibited in the presence of PPP

PPP inhibition test was performed by the assay of cell viability using CCK-8. The cell viability was significantly ameliorated after...
the cells were post-treated with daucosterol at concentrations of 20 \mu M (p < 0.01) and 10 \mu M (p < 0.05). However, the amelioration of cell viability was ceased to exist in the presence of PPP, the inhibitor of IGF1R, indicating that when the function of IGF1 was blocked, the neuroprotective effect of daucosterol was inhibited (Fig. 9).

In summary, the results showed that daucosterol significantly reduced neuronal loss and apoptotic rate, displaying the neuroprotective activity. The results also showed that daucosterol increased the expression level of IGF1 protein, activated the AKT signal pathway, diminished the down-regulation of the anti-apoptotic proteins Mcl-1 and Bcl-2, and decreased the expression level of pro-apoptotic protein Bax raising the ratio of Bcl-2/Bax. The neuroprotective activity of daucosterol was inhibited in the presence of PPP, the inhibitor of IGF1R.

### 4. Discussion

Several studies [20,21] suggested that IGF1 levels may be associated with neurological recovery and functional outcome. The higher IGF1 levels observed in patients with better outcomes suggest a possible neuroprotective role of IGF1. Circulating IGF1 may predict functional performance during rehabilitation and ischemic stroke outcome. The ectogenic IGF1 protein has been shown to be a potent neuroprotective compound in rodent models of ischemic stroke [6]. However, ectogenic IGF1 is pricey and chemically unstable, limiting its clinical application. The present study revealed that daucosterol, an inexpensive and stable sterolin, remarkably up-regulated the expression level of endogenous IGF1, exhibiting IGF1-like activity and thus displaying the neuroprotective functions. As the majority of cultured cells were neurons, which are reported to express IGF1 mRNA [22], it is likely that most of IGF1 were released from neurons and some remaining astrocytes may be the additional sources of IGF1. However, the receptors which could be stimulated by daucosterol remain to be identified in further studies.

Early studies revealed that daucosterol exhibits anti-inflammatory and immunomodulating activities [23–26], which help to treat ischemic stroke [27]. In addition, there are reports that daucosterol has effects on the respiratory activities and generation of reactive oxygen species (ROS) in brain mitochondria and daucosterol is neurotoxic to motor neurons [28,29]. However, the cultured cell (NSC-34), the duration of action (3 days), and the test assay (trypsin blue exclusion test) in previous studies were largely different from our present study. Moreover, we validated the doses of 10–20 \mu M to be neuroprotective, which was much lower than the venomous doses in previous studies. According to our findings, short term (24 h) and low doses (10–20 \mu M) treatment with daucosterol can exert the protective effects on OGD/R-treated primary cortical neurons. It remains to determine whether the corresponding low doses of daucosterol are neuroprotective in vivo in the future study.

In the present study, neuroprotective action of daucosterol resulted in reduced cell apoptosis by diminishing caspase-3 activation in the OGD/R-treated cells. The neuroprotective function was inhibited when the cells were exposed to PPP, the inhibitor of IGF1R, demonstrating that the neuroprotective effects of daucosterol depends on its IGF1-like activity.
IGF1 signalling is mediated by IGF1R, activating two main downstream signalling pathways, namely the RAF-MEK-ERK phosphorylation cascade and the phosphatidylinositol 3 kinase-AKT (PI3K-AKT) pathway [30–32]. AKT is activated by binding of phosphatidylinositol 3-phosphate, which promotes its recruitment to the plasma membrane and sequential phosphorylation at threonine (Thr308) and serine (Ser473) residues by the phosphoinositide dependent kinases [33]. The PI3K-AKT pathway is a critical survival mediator in the signal transduction pathways after cerebral ischemia and the activation of PI3K-AKT pathway is a therapeutic target for stroke [34,35]. In the present study, daucosterol remarkably increased the phosphorylation level of AKT, indicating that AKT pathway was activated and exerted protective effect on OGD/R-treated neurons, as the result of up-regulation of the expression level of IGF1 protein. A previous study has shown that activation of AKT has a number of positive effects on ischemic organs [36]. Our findings provided evidence to support that activated AKT is important to ischemia treatment because its actions allow cells to escape apoptosis.

One of the downstream target inactivated by AKT is GSK-3β [37]. GSK-3β is a serine/threonine protein kinase that becomes inactivated upon phosphorylation at serine residue 9 (Ser 9), unlike most other protein kinases that are constantly active. Interestingly, inactivation of GSK-3β via phosphorylation at Ser 9 increases stabilization and accumulation of Mcl-1 protein and thus prevents apoptosis [38]. Mcl-1 is an important anti-apoptotic protein that mainly distributes in mitochondria and cytoplasm. Mcl-1 exerts anti-apoptotic effects by interacting with pro-apoptotic proteins such as Bim Noxa, Bak and Bax. Also, Mcl-1 may function by facilitating normal mitochondrial fusion, ATP production and respiration [39]. Unlike the other members of the Bcl-2 family, Mcl-1 has a very short half-life, which allows for either the rapid induction or elimination in response to cell survival or cell death events [40]. It has been reported that the activity of GSK-3β was required for Mcl-1 degradation, which is an essential mechanism for GSK-3β-induced apoptosis [41,42]. The Mcl-1 protein level in the OGD/R-treated neurons was up-regulated after treatment with daucosterol, as the result of increased phosphorylation level of GSK-3β, the downstream target inactivated by phospho-AKT.

Fig. 4. The effect of daucosterol on the apoptotic rate of the OGD/R-treated neurons. A: Annexin V-FITC/PI double-staining cells were examined by flow cytometry. B: Analysis of apoptotic rate. The neurons were treated with daucosterol after they were subjected to OGD/R injury. Cell apoptosis was analyzed by use of flow cytometry and double staining cells with Annexin V-FITC and propidium iodide (PI). Data are presented as mean ± S.D. (n = 6). ###p < 0.001, compared with Control. ***p < 0.001, compared with OGD/R. The apoptotic rate significantly decreased after post-treatment with daucosterol at concentrations of 20 and 10 μM (p < 0.001).

Fig. 5. The effect of daucosterol on the expression levels of IGF1 protein. The neurons were treated with daucosterol after they were subjected to OGD/R injury. IGF1 protein was quantified by ELISA. Data are presented as mean ± S.D. (n = 6). ***p < 0.001, compared with OGD/R. The IGF1 levels were markedly up-regulated after post-treatment with daucosterol at concentrations of 20 and 10 μM (p < 0.001).
In addition, GSK-3\(\beta\) exerts its pro-apoptotic effects in neurons by regulating the mitochondrial localization of Bax a key component of the intrinsic apoptotic cascade [43]. Neuronal apoptosis often occurs via an intrinsic apoptotic cascade triggered by the translocation of Bax a pro-apoptotic Bcl-2 family member, to mitochondria. During apoptosis, cytochrome c is released from mitochondria through the permeability transition pore, which is regulated by the interactions of Bcl-2 family proteins [44]. After releasing from the mitochondrial inter-membrane space, cytochrome c forms the apoptosome together with apoptosis-activating factor Apaf-1 and procaspase-9, leading to the activation of initiator caspase-9 [45–48]. Subsequent activation of downstream members of the caspase family, including effector caspase-3, leads to apoptosis [49]. Caspase-3 cleaves many substrate proteins, including poly (ADP-ribose) polymerase (PARP), which leads to DNA injury and subsequently to apoptotic cell death. It is known that the Bcl-2 protein family tightly regulates cytochrome c release from the mitochondria into the cytosol [50]. Cells are protected when Bcl-2 is in excess and Bcl-2 homodimers predominate, whereas cells are vulnerable to apoptosis when Bax is in excess and Bax homodimers predominate. Therefore, the ratio of Bcl-2/Bax is regarded as a factor that determines a cell’s fate in response to apoptotic stimuli [51]. Bcl-2 protein is a cell survival-promoting factor located within the mitochondria, endoplasmic reticulum, and interconnected paranuclear membrane [52–54]. A previous report indicated that the PI3K/AKT signaling pathway could directly induce up-regulation of the anti-apoptotic protein Bcl-2 [55], facilitating the increase of the ratio of Bcl-2/Bax. In the present study, the up-regulated expression level of Mcl-1 and the increased ratio of Bcl-2/Bax and the decreased caspase-3 activity induced by daucosterol inhibited apoptosis and favored cell survival in the OGD/R-treated neurons.

The present study revealed the neuroprotective effects of daucosterol on OGD/R-treated neurons, and demonstrated that the IGF1-like activity of daucosterol contributed to its neuroprotective
effects. Daucosterol could be potentially developed to treat ischemic stroke.

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