Original research article

Inotodiol protects PC12 cells against injury induced by oxygen and glucose deprivation/restoration through inhibiting oxidative stress and apoptosis

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\textbf{A B S T R A C T}

Ischemic stroke is a severe cause of disability and death all over the world. To search for effective therapy for ischemic stroke, PC12 cells damaged by oxygenation and glucose deprivation/restoration were employed to assess the protective effects of inotodiol. As a result, inotodiol can improve the cell viability and attenuate the leakage of lactate dehydrogenase. Meanwhile, inotodiol can prevent oxidative stress by reducing reactive oxygen species generation, decreasing the content of malonic dialdehyde, and increasing the activity of superoxide dismutase. In addition, the dysfunction of mitochondria induced by oxygenation and glucose deprivation/restoration was ameliorated through decreasing the level of intracellular calcium and increasing the mitochondrial membrane potential. At the same time, inotodiol can inhibit PC12 cells apoptosis through downregulation of Caspase-3 and Bax as well as upregulation of Bcl-2. These results reveal inotodiol can protect PC12 cells against the injury induced by oxygenation and glucose deprivation/restoration. This investigation gives promising evidences for the therapy of ischemic stroke.

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Introduction

Stroke is an increasing cause of disability and death in adults worldwide, and the burden of stroke has attracted more attention for the increasing incidence (Krishnamurthi et al., 2015). Ischemic stroke, as the main type of stroke, is caused by the occlusion of cerebral artery (Flynn et al., 2008). Poor blood flow to brain will result in the deprivation of oxygen and glucose to support cellular homeostasis, which will give rise to following cell death as well as final brain tissue injury and infarct (Doyle et al., 2008). Thereafter, reperfusion of blood flow will restore the oxygen and glucose, but the neural injury will be exacerbated (Jung et al., 2010; Li et al., 2007). Moreover, pathogenesis of cerebral ischemia has revealed the injured neurons in the ischemic penumbra around infarct core are viable and reversible due to the availability of ATP. Without effective therapeutics, they will undergo apoptosis in short time (Mehta et al., 2007). Oxidative stress plays a pivotal role in the progress of ischemic stroke due to the overproduction of reactive oxygen species (ROS) in mitochondria. Under oxygen and glucose deprivation/restoration (OGD/R), the function of mitochondria is impaired through the interruption of metabolic homeostasis. At the same time, the production of ROS exceeds the scavenging capacity of cellular antioxidant enzymes, which leads to the accumulation of ROS and accelerates the brain injury for brain is vulnerable to ROS (Chan, 2001; Jung et al., 2010; Mehta et al., 2007). The dysfunction of mitochondria also induces overload of intracellular calcium and finally triggers apoptosis through the mitochondrial pathway (Lehotský et al., 2009; Racay et al., 2009).

In clinic, few approaches have been available to attenuate the injury induced by ischemic stroke for its complicated mechanisms. In the discovery of efficient therapeutics for ischemic stroke, bioactive natural products presented promising effects such as...
aloin (Chang et al., 2016), ginsenoside Rb3 (Zhu et al., 2010), and genistein (Ma et al., 2016).

Inotodiol (Fig. 1) is a natural lanostane-type triterpenoid found in Inonotus obliquus (Nakata et al., 2007). As the major phytochemical in Inonotus obliquus, its anticancer activity was investigated formerly (Nakata et al., 2007; Nomura et al., 2008). The edible and medicinal mushroom Inonotus obliquus is mainly distributed in Europe and Asia. In Russia, it is used for the prevention of cancer, cerebrovascular diseases, diabetes, gastrointestinal diseases from the sixteenth century (Ma et al., 2013). Extract of Inonotus obliquus has shown non-toxic, antiviral, antibacterial, hepatoprotective, anti-inflammatory, antitumor, antioxidant properties (GlamoCilia et al., 2015). In our program to search bioactive phytochemicals for the treatment of ischemic stroke, we have evaluated the neuroprotective effects of inotodiol from Inonotus obliquus on PC12 cells injury induced by OGD/R. Herein we report these effects and related mechanisms.

Materials and methods

Chemicals and reagents

3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco’s modified Eagle’s medium (DMEM) was purchased from Invitrogen Gibico Co. (Grand Island, NY, USA). ROS, lactate dehydrogenase (LDH), malonic dialdehyde (MDA), superoxide dismutase (SOD) and bicinchoninic acid (BCA) assay kits were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Rhodamine-123, Fluo-3 AM and Caspase-3 assay kit as well as cleaved Caspase-3, Bcl-2, Bax and actin antibodies were purchased from Beyotime Biotechnology Institute (Nantong, China). The other reagents are analytical grade.

Plant materials

The wild sclerotia of Inonotus obliquus were collected in Yanbian, Jilin Province, China, in December 2012 and identified by one of the authors, Dr. Chunting Zhang. The voucher specimen (No. 20121201) was deposited at School of Pharmacy, Xuzhou Medical University.

Isolation of inotodiol

The sclerotia of Inonotus obliquus (3.0 kg) were ground and extracted with petroleum ether (3.0 l × 3) under reflux. And the solvent was evaporated under reduced pressure to give the extract (210.0 g). The extract was subjected to column chromatography on silica gel (300–400 mesh) and eluted with gradient petroleum ether/ethyl acetate (from 100:0 to 30:70, v/v) to afford eight fractions. Fraction 6 was further purified by semipreparative high performance liquid chromatography equipped with a delivery system, a refractive index detector and an ODS column (1.0 × 20.0 mm) and eluted with isocratic methanol/H2O (95:5, v/v) to obtain white crystals (68.0 mg).

Cell culture and treatment

PC12 cells (rat adrenal pheochromocytoma cells) were obtained from Cell Bank of Shanghai Institute of Biological Sciences, Chinese Academy of Sciences. The cells were cultured in DMEM with 10% fetal bovine serum, 1% penicillin and streptomycin in a humid 5% CO2 and 95% air atmosphere at 37 °C. OGD/R in PC12 cells were carried out as previous description (Zhu et al., 2010) with simple modification. Briefly, the culture medium was replaced with glucose-free DMEM after washed. Then the cells were incubated under a 95% N2 and 5% CO2 condition in the presence or absence of certain inotodiol (0.1, 1 and 10 μM) for 4 h. And then the cells were cultured in DMEM with or without inotodiol under normal conditions (5% CO2 and 95% air) for 24 h. The cells incubated in DMEM at normal atmosphere were employed as control group.

PC12 cells viability assay

To evaluate the protective effect of inotodiol on PC12 cells, the MTT assay was employed to determine the cell viability. The cells were added into 96-well microplates and adjusted to 1 × 105 per well. After treated as above, MTT was added into each well to be 0.5 mg/ml and the incubation was maintained for 4 h at 37 °C. Then the medium was removed and DMSO was used to dissolve the formazan. After 10 min, the absorbance was measured at 550 nm on a microplate reader. Cell viability was expressed as relative percentage of optical density (OD) values compared with control group.

LDH release assay

To determine the activity of extracellular LDH, the LDH assay kit was employed. After treatment and incubation, 20 μl supernatant in PC12 cells culture system was sucked each time and handled according to the manufacturer’s instructions. Then the absorbance was recorded on a microplate reader at 450 nm. The activity of LDH in the supernatant can be calculated from the absorbance and expressed as U/l.

Measurement of ROS

The ROS generation was determined by the fluorescence method according to the manufacturer’s instructions. 2,7’-dichlorofluorescein diacetate (DCFH-DA) can cross cell membranes and be hydrolyzed by intracellular esterase to produce DCFH which can not be released out of the cell membranes. The ROS can make DCFH dehydrogenated to be fluorescent DCF. After treatment, the medium was removed and the cells were washed with PBS. Then the DCFH-DA in DMEM was added and the cells were incubated at 37 °C for 30 min. Then the cells were washed with PBS again and the fluorescence intensity was recorded on a spectrophorometer at 485 nm (excitation wavelength) and 520 nm (emission wavelength).

Determination of MDA level

The content of MDA was detected with the assay kit (thiobarbituric acid method) based on the manufacturer’s instructions. After treatment, 20 μl supernatant was used for the
following analysis. The reagent was added into the supernatant and the sample was boiled for 40 min. The absorbance was measured at 532 nm. The concentration of MDA in supernatant was derived from the comparison of OD value with that of standard.

**SOD activity assay**

The activity of SOD was measured by the assay kit (xanthine-oxidase method) according to the manufacturer’s instructions. The reagent was added into 20 μl supernatant and the reactive system was incubated for 20 min at 37 °C. Subsequently the absorbance was measured at 550 nm. The activity of SOD was calculated from the OD value versus the standard.

**Measurement of intracellular calcium**

The intracellular calcium was determined by Fluo-3 AM fluorescence probe according to the manufacturer’s instruction. In brief, pro-treated PC12 cells were incubated with Fluo-3 AM at 37 °C for 1 h in dark, and then were washed with PBS for three times to remove extracellular dye. The fluorescence was excited at 488 nm and emission wavelength was set at 525 nm on the spectrofluorometer.

**Determination of mitochondrial membrane potential**

Mitochondrial membrane potential (MMP) was also measured through fluorescent rhodamine-123. Rhodamine-123 can accumulate in mitochondria of normal cells, but when the MMP reduces in apoptotic cells, it will be released into cytosol and emit fluorescence. Following the treatment, rhodamine-123 (2 mM) was added into PC12 cells and incubated for 15 min. Then the fluorescence intensity was read at an excitation wavelength of 488 nm and an emission wavelength of 530 nm. MMP was expressed as the percentage of fluorescence intensity compared to control group.

**Western blot analysis for proteins related to apoptosis**

The PC12 cells were treated as above and then subjected to Western blot analysis for the expression of Caspase-3, Bcl-2, and Bax. In short, the cells were lysed with lysis buffer including 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton and 1 mM phenylmethylsulfonyl fluoride (PMSF) on ice for 30 min. Then the lysed cells were centrifuged at 12000 g and 4 °C for 15 min, and the supernatant was collected as the total protein for the analysis of Caspase-3 activity.

**Caspase-3 activity**

The activity of Caspase-3 was quantified by a colorimetric assay kit according to the manufacturer’s instructions. The PC12 cells were pretreated as above and washed with PBS. Then they were lysed and centrifuged at 16000 g and 4 °C for 10 min. The supernatant was incubated with substrate (Ac-DEVD-pNA) at 37 °C for 2 h. The OD values were measured on a microplate reader at 405 nm.

**Statistical analysis**

All results were expressed as the means ± standard deviation. GraphPad Prism was employed to analyze the results. Statistical differences between different groups were compared by one way analysis of variance (one way ANOVA) followed by Dunnett test for multiple comparisons and Student’s T-test for single comparisons. And the difference was considered to be statistically significant when p < 0.05.

**Results**

**Identification of inotodiol**

The compound isolated from *Inonotus obliquus* was analyzed on a NMR spectrometer to elucidate the structure and purity. The structure was identified as inotodiol by analysis of 13C NMR spectrum, which is in accordance with the data reported formerly (Kahlos et al., 1984). The assigned data were listed as following: 13C NMR (100 MHz, CDCl3): 6.35.6 (C-1), 28.0 (C-2), 79.0 (C-3), 38.9 (C-4), 50.4 (C-5), 18.3 (C-6), 26.5 (C-7), 134.6 (C-8), 134.2 (C-9), 37.0 (C-10), 210.0 (C-11), 273.0 (C-12), 44.9 (C-13), 49.4 (C-14), 31.0 (C-15), 31.0 (C-16), 47.3 (C-17), 15.7 (C-18), 19.1 (C-19), 41.7 (C-20), 12.6 (C-21), 73.4 (C-22), 29.1 (C-23), 121.4 (C-24), 135.2 (C-25), 26.0 (C-26), 18.0 (C-27), 15.4 (C-28), 27.9 (C-29), 24.3 (C-30). There is no peak of impurity on the spectrum.

**Effect of inotodiol on cell viability**

The effects of inotodiol on PC12 cells viability were determined by MTT assay. As shown in Fig. 2, after OGD/R, cell viability was obviously inhibited (43.05% ± 3.93%). When exposed to different concentrations of inotodiol, the cell viability was significantly increased in a dosage-dependent manner compared with the OGD/R group (50.50% ± 5.34%, 59.89% ± 7.72% and 77.92% ± 9.07%), which indicates inotodiol can promote the survival of PC12 cells induced by OGD/R.

**Effect of inotodiol on extracellular LDH**

To further confirm the effect of inotodiol on the PC12 cells viability, the activity of extracellular LDH was detected. After induction by OGD/R, the activity of LDH in the PC12 cells culture
medium was increased to 81.75 ± 7.78 U/l compared to control group (39.05 ± 3.99 U/l) (Fig. 3). But with different concentrations of inotodiol, the activity of the extracellular LDH was inhibited as 70.89 ± 3.91, 61.12 ± 5.03 and 53.33 ± 4.99 U/l. These results unveil that inotodiol reduces the release of intracellular LDH, which indicates inotodiol protects PC12 cells affected by OGD/R.

Effect of inotodiol on ROS generation

ROS is produced in mitochondria and involved in the dysfunction of mitochondria and oxidative stress. OGD/R can elevate the level of ROS in PC12 cells (141.50 ± 3.69) by comparison of fluorescence intensity with control group (40.72 ± 3.15) (Fig. 4). But the fluorescence intensity was decreased to 115.10 ± 11.80, 84.39 ± 10.11 and 64.66 ± 10.43 following the treatment with inotodiol (0.1, 1 and 10 μM). These results give the evidence for that inotodiol can reduce the ROS generation.

Effect of inotodiol on MDA level

The level of MDA is related to cellular oxidative stress. Treated with OGD/R, the content of MDA was increased as 0.73 ± 0.06 μM compared with the control group (0.40 ± 0.06 μM). While treated with inotodiol, the content of MDA was reduced with different degrees (0.63 ± 0.06, 0.53 ± 0.10 and 0.46 ± 0.04 μM) (Fig. 5). These results further clarify the inhibitory effect of inotodiol on the injury induced by oxidative stress.

Effect of inotodiol on MMP

The MMP was determined through the fluorescence intensity of rhodamine-123 to evaluate the depolarization of mitochondria for...
rhodamine-123 is proportionally specific to MMP. As shown in Fig. 8, with the OGD/R, the MMP in PC12 cells was obviously reduced to 31.05% ± 2.94%. On the contrary, when inotodiol at 0.1, 1 and 10 μM appeared, the MMP recovered with different extents as 39.00% ± 5.67%, 56.25% ± 7.02% and 72.22% ± 4.71%. These results present the fact that inotodiol can attenuate the dysfunction of mitochondria in PC12 cells damaged by OGD/R.

Effect of inotodiol on Caspase-3, Bcl-2 and Bax

The loss of MMP has been observed in PC12 cells after OGD/R, which usually appeared at the early stage of apoptosis. Then Western blot was employed to analyze the expression of Caspase-3, Bcl-2 and Bax and assess the anti-apoptotic effect of inotodiol. As a result, the expression of cleaved Caspase-3 and Bax was upregulated after OGD/R, while Bcl-2 was downregulated. Moreover, with the different concentrations of inotodiol in the culture system, the expression of cleaved Caspase-3 and Bax was downregulated as well as Bcl-2 was upregulated with different extents (Fig. 9a). The relative amounts of Bcl-2 and Bax derived from the densitometric analysis further confirmed the result that inotodiol can enhance the expression of Bcl-2 and inhibit expression of Bax (Fig. 9b). The ratio of Bcl-2 to Bax also presents the result directly (Fig. 9c). The colorimetric assay further validated the effect of inotodiol on the activity of Caspase-3. From Fig. 9d, the absorbance in OGD/R group (0.87 ± 0.05) was larger than that of control group (0.32 ± 0.07). With the addition of inotodiol, the OD

![Fig. 7. Inotodiol reduces intracellular calcium. n = 6, ***p < 0.001 vs. control group, *p < 0.05, **p < 0.01 vs. OGD/R group.](image)

![Fig. 8. Inotodiol attenuates the loss of MMP in PC12 cell treated by OGD/R. n = 6, ***p < 0.001 vs. control group, *p < 0.05, **p < 0.01 vs. OGD/R group.](image)

![Fig. 9. Inotodiol inhibits the apoptosis in PC12 cells induced by OGD/R. (a) Western blot analysis of the expression of cleaved Caspase-3, Bcl-2 and Bax; (b) Densitometric analysis of Bcl-2 and Bax derived from the Western blot; (c) The Blc-2/Bax ratio; (d) The activity of Caspase-3 in PC12 cells. n = 6, ***p < 0.001 vs. control group, *p < 0.05, **p < 0.01, ***p < 0.001 vs. OGD/R group.](image)
values were significantly reduced as 0.79 ± 0.06, 0.69 ± 0.09 and 0.56 ± 0.06, respectively. The inhibitory effect of inotodiol on the activity of Caspase-3 was presented by these results.

Discussion

Ischemic stroke results from the OGD/R caused by the occlusion of cerebral artery, which can induce neuronal apoptosis (Mehta et al., 2007). PC12 cells treated by OGD/R can mimic the ischemic stroke in vitro and be used to evaluate the potential effects of bioactive substances on that injury (Chang et al., 2016; Zhu et al., 2010). Herein we investigated the protective effects of inotodiol on PC12 cells damaged by OGD/R.

After exposed to OGD/R, the survival of PC12 cells was decreased, which indicates the cells were injured. But with inotodiol, the cells viability was increased and the injury was attenuated. LDH is a stable enzyme in cytoplasm for energy metabolism. It will be released into the culture medium when the cells die and subsequently the cytoplasm membrane is destroyed (Zhu et al., 2010). The activity of LDH in the culture medium indicates the cells viability. In this investigation, inotodiol can block the leakage of LDH in extracellular matrix induced by OGD/R and promote the survival of PC12 cells.

Mitochondrion is an important organelle affording energy metabolism and ROS generation in living cells. Dysfunction of mitochondria is an early event of ischemic stroke in nervous system (Zhu et al., 2014). Ischemia/reperfusion can interrupt the homeostasis between the ROS generation and the detoxification and scavenging capacity of cellular antioxidant enzymes, which will lead to the oxidative stress in mitochondria and exacerbate the ischemia insult (Doyle et al., 2008; Mehta et al., 2007). As the final product of lipid peroxidation, the content of MDA reflects the response to oxidative stress and disposition to free radicals in organism. Brain is specifically susceptible to insult mediated by free radicals due to its high lipid content. As one of the anti-oxidative enzymes, the activity of SOD presents the capacity of free radical scavenging (Mehta et al., 2007). In our investigation, inotodiol can mitigate the injury caused by oxidative stress in PC12 cells through inhibiting the production of MDA, increasing the activity of SOD and preventing the generation of ROS.

Calcium plays a crucial role in signal transduction as a messenger and affords neurotransmitter release, modulation of membrane excitability and regulation of synaptic plasticity in the nervous system. The concentration of intracellular calcium is maintained through NMDA receptors and ion exchangers (Iijim et al., 2008). High levels of calcium cause mitochondria to produce detrimental ROS, and eventually lead to neuronal apoptosis (Doyle et al., 2008; Zhu et al., 2014). In addition, the overproduction of ROS can destroy the mitochondrial membrane through oxidative stress and result in the collapse of MMP (Ji et al., 2014). MMP is a sensitive indicator of mitochondrial function. As one of the earliest intracellular events, the MMP will break down following the apoptosis (Zhu et al., 2010). Our results have shown the dysfunction of mitochondria in PC12 cells happened after OGD/R through increasing intracellular calcium level and decreasing MMP. While treated with inotodiol, the function of mitochondria was significantly improved.

ROS-associated OGD/R can induce apoptosis through the mitochondrial pathway (Mehta et al., 2007). Caspase-3 is a member of the cysteine-dependent aspartate proteases family and takes part in cell apoptosis (Budihardjo et al., 1999). In ischemic stroke, Caspase-3 is activated through the cleavage as the effector enzyme (Galluzzi et al., 2009; Ji et al., 2014). Bcl-2 and Bax are members of the Bcl-2 protein family and play a pivotal role in the mitochondrion-driven apoptosis. Bcl-2 can prevent apoptosis and attenuate the activation of Caspase-3 while Bax promotes cell apoptosis (Pilchova et al., 2015; Youle and Strasser, 2008). In present studies, the expression of cleaved Caspase-3 and Bax in PC12 cells was upregulated and Bcl-2 was downregulated after OGD/R, which implied the apoptosis occurred. Oppositely, in the presence of inotodiol, the downregulated Caspase-3 and Bax together with upregulated Bcl-2 were detected. The increased activity of Caspase-3 affected by OGD/R was inhibited after the treatment with inotodiol. In addition, the densitometric analysis for Bcl-2 and Bax also enhanced the inhibitory effect of inotodiol on the cell apoptosis induced by OGD/R.

Conclusion

In summary, the protective effects of inotodiol on the PC12 cells injury induced by OGD/R were investigated in vitro. Inotodiol can attenuate the PC12 cells injury through preventing oxidative stress and inhibiting ROS-mediated mitochondrial apoptosis. This investigation gives promising therapeutic approaches for ischemic stroke.

Conflict of interests

The authors declare there is no conflict of interest.

Acknowledgements

The authors Yan Li and Wenting Zhang contributed equally to this work.

References


