Synergistic protective effect of picroside II and NGF on PC12 cells against oxidative stress induced by H$_2$O$_2$

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Abstract:
Epidemiological studies suggest that nerve growth factor (NGF) is associated with a reduced risk of acute or chronic neuropathies. We studied the synergistic protective effect of picroside II and NGF against the oxidative stress in PC12 cells induced by hydrogen peroxide (H$_2$O$_2$). The fluorescent probe CDCFH was used to assess the intracellular reactive oxygen species (ROS) level, and MTT assay, morphological observation as well as LDH leakage test were conducted to measure cellular injury. The H$_2$O$_2$-induced cytotoxicity was significantly attenuated in the presence of picroside II (25 μg/ml) and NGF (2 ng/ml). Cultures with this combined treatment possessed decreased level of ROS while increased cell survival, as compared to that of picroside II or NGF alone-treated cells. Accordingly, it was concluded that their synergistic protective activities against oxidative stress in vitro were demonstrated in various aspects, including reversing morphological changes, enhancing the ability of cell proliferation and ROS scavenging. Such action supports the therapeutic potential of picroside II and NGF in treating nervous disorders based on their synergistic effect.

Key words: hydrogen peroxide, nerve growth factor, PC12 cells, picroside II, reactive oxygen species

Introduction

There are many publications about the important role of nerve growth factor (NGF) in the pathogenetic processes of acute or chronic neuropathies as well as in developmental changes in the nervous system. After various types of acute injuries, NGF and some other neurotrophic factors can be up-regulated rapidly in the nervous system, which is suggested as the response of an endogenous protective mechanism against damage. However, this response is usually too transient and moderate to protect neurons from the eventual damage [23].

Although exogenous administration of neurotrophic factors could represent an alternative strategy to treat some nervous system diseases, the neurotrophins are readily metabolized by peptidases and do not go easily through the blood-brain barrier mainly because of their large molecular weight [10]. In order to solve this problem, another useful strategy is to find some compounds that can either enhance the protection of NGF or increase the expression of NGF,
which is a very important neurotrophic factor [11, 14]. Because it has been reported that some compounds showed these abilities, there is some feasibility for this strategy. For example, various synthetic compounds such as AIT-082, SR57746 and Aroclor-1254 have been shown to enhance the action of NGF in neurite outgrowth on PC12 cells [1, 6, 17]. For natural compounds, nardosinone from *Nardostachys chinensis* potently increased NGF action in PC12D cells and scabronines stimulated the secretion of NGF from 1321NI human astrocytoma cells [8, 9].

As already reported, picroside II (h-D-glucopyranoside, 1a,1b,2,5a,6,6a-hexahydro-6-[(4-hydroxy-3-methoxybenzoyl)oxy]-1a(hydroxymethyl)oxireno[4,5]cyclopenta[1,2-c]pyran-2-yl) can function cooperatively with NGF in enhancing neurite outgrowth of PC12 cells in certain physiological conditions [5]. But there have been few reports about their cooperative effect in certain pathological conditions. Thus, the combined treatment of picroside II and NGF was investigated on PC12 cell under oxidative stress induced by H2O2, to find whether the protective effects are synergistic.

### Materials and Methods

#### Reagents

Picroside II was purchased from China Chengdu Ti- anyin Industrial Co. NGF, 6-carboxy-2',7'-dichloro- dihydrofluorescein (CDCFH) and 3-(4,5-dimethyl- thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma Chemical Corporation (St. Louis, MO, USA). All other chemicals used were of analytical and reagent grade.

#### Cells and cell culture

PC12 cells, purchased from Cell Bank of Chinese Academy of Science, were cultured on RPMI-1640 Medium (GIBCO BRL, Grand Island, NY, USA) and 10% dialyzed heat-inactivated bovine serum (GIBCO BRL, Grand Island, NY, USA) at 37°C in a humidified atmosphere of 95% air and 5% CO2.

#### Cell viability assays

Cells were seeded into 96-well plate (1 x 10^4 cells/well) with RPMI-1640 medium. After growing to confluence, cultures were treated with different concentrations of H2O2 for 3 h. Then the supernatant was discarded and cells were further incubated in RPMI-1640 medium for 12, 24, 36, or 48 h. Cell viability was determined by MTT assay. Briefly, 10 mM of MTT was added to each well for an additional 4 h. The blue MTT formazan precipitate was dissolved in 100 μl of DMSO and measured on a microplate reader model 550 (Bio-Rad, California, USA) at the absorbance wavelength of 570 nm and reference wavelength of 630 nm.

Effect of picroside II on cell viability under normal condition was also measured by MTT assay. Cells were treated for 12, 24, 36, or 48 h in the presence or in the absence of picroside II, and then cell viability was determined by MTT assay as above.

Effects of picroside II and NGF on cell viability under oxidative stress were examined as follows. Cells were seeded into 96-well plate with or without 150 μM H2O2 for 3 h. Then the supernatant was discarded and cells were further incubated in the medium with or without picroside II and NGF for 48 h. Cell viability was determined by MTT assay.

#### Intracellular ROS level assays

Assay of intracellular ROS level was carried out in the same way as done for cell viability assay. Cells were rinsed three times with phosphate-buffered saline (PBS) (pH 7.4) and further incubated with 10 μM CDCFH solution for 4 h. Membrane-impermeable CDCFH was absorbed by the cells and oxidized into highly fluorescent CDCFH by ROS. The supernatant containing CDCFH was discarded, and 100 μl PBS (pH 7.4) was added to the 96-well plate wells. The fluorescence intensity was measured by a fluorescence microplate reader CytoFluor2350 (Millipore, Bedford, MA, USA) at excitation wavelength of 485 nm and emission wavelength of 538 nm.

#### LDH activity assays

In addition, LDH activity in the medium was measured as an index of cellular injury. The enzymatic test kit for LDH (Kyokuto Pharmaceutical Co. Ltd, Tokyo, Japan) was used. Cells were centrifuged (1,000
rpm for 5 min), the supernatants were collected and transferred to the plates. Nitrozolium blue mixture in 100 mM Tris-buffer (pH 7.5) was added to each plate. After 30 min of incubation at 37°C, 0.1 ml of 1 M HCl solution was added to stop the reaction. The absorbance at 560 nm was measured. Results were expressed as a percentage of LDH activity.

**Morphological observations in vitro experiments**

Cells were observed with an inverted phase-contrast microscope (IMT-2, Olympus, Tokyo). This process was recorded by a video tape via a color video camera (CCD-IRIS, Sony Co., Tokyo, Japan), and digitized data of each picture were stored in the computer at a regular sampling time for later analysis.

**Statistical analysis**

The synergistic effects of picroside II and NGF were calculated using the formula AB/A·B, where A and B are the effects of each individual agent on the cell viability and AB is the effect of the combination. When the ratio (combination index) is greater than or less than 1, the effect is considered synergistic or additive, respectively [16, 19].

Results are presented as the mean values ± standard deviation (SD) of at least three different experiments. Statistical significance was evaluated by Student’s t-test. Probability values (p) less than 0.05 were considered significant. The SPSS statistical software package was used.

**Results**

**Effect of picroside II or H$_2$O$_2$ on the viability of PC12 cells**

Effect of picroside II on the cell viability is presented in Figure 1A. The data indicated that during the treatment time, picroside II was not cytotoxic to PC12 cells. Moreover, various concentrations of picroside II did not promote the proliferation of PC12 cells. Figure 1B showed that H$_2$O$_2$ inhibited the proliferation of PC12 cells and caused the oxidative stress in a dose-dependent way. The IC$_{50}$ (50% inhibitory concentration) value of H$_2$O$_2$ was 146 ± 10.2 μM, and the cell viability at 150 μM H$_2$O$_2$ was 54.2 ± 4.1%. Therefore, picroside II (0–100 μg/ml) was used to examine its protective effect on PC12 cells against oxidative stress induced by 150 μM H$_2$O$_2$.

**Effect of picroside II and NGF alone on the viability of PC12 cells under the oxidative stress**

Figure 2 showed the concentration-dependent protective effect of picroside II and NGF alone on PC12 cells. When picroside II concentration was ≤ 5 μg/ml, or NGF concentration was ≤ 2 ng/ml, they exhibited generally no significant protective effect. As the concentration increased, the positive effect of picroside II
or NGF alone was enhanced gradually, and the cell viability reached 76.2% at 100 μg/ml of picroside II, or 94.9% at 50 ng/ml of NGF, respectively, compared to 51.2% in the control group with 150 μM H₂O₂.

**Synergistic effect of picroside II and NGF on the viability of PC12 cells**

Next, we investigated the synergistic protective effect of picroside II and NGF (2 ng/ml) on PC12 cells against oxidative stress induced by 150 μM H₂O₂. PC12 cells were treated with NGF (2 ng/ml) combined with various concentrations of picroside II. The protective effect of picroside II on the survival of PC12 cells was significantly enhanced when it was given in combination with NGF (Fig. 3A). Analysis of the combination index (Fig. 3B) showed that the effect of picroside II at the dose of 25 μg/ml and NGF at the dose of 2 ng/ml was the most significant. Moreover, we further inspected the synergistic effect of picroside II (25 μg/ml) and NGF (2 ng/ml) on PC12 cell proliferation by LDH activity assays and morphological observations.

**Synergistic effect of picroside II and NGF on LDH leakage from PC12 cells**

The release of LDH, a marker for the increased membrane permeability and lethal cell injury, was measured to evaluate the degree of traumatic injury to PC12 cells. When picroside II (25 μg/ml) or NGF (2 ng/ml) alone was used, the activity of LDH did not decrease...
markedly (Fig. 4). However, the combined treatment elicited a considerable reduction of the LDH activity, which implied that cells under the oxidative stress were greatly rehabilitated.

**Synergistic effect of picroside II and NGF on the morphology of PC12 cells**

As shown in Figure 5, normal PC12 cells were of fibrous shape, bearing neurites. The refrangibility and the adherence rate were also very good. Under oxidative stress induced by 150 μM H₂O₂, cells became detached and rounded or erosion associated with the retractive neuritis worsened refrangibility. Treatment with picroside II (25 μg/ml) or NGF (2 ng/ml) alone did not reveal a remarkable protective effect. After cells were treated with picroside II and NGF in combination, the morphology of PC12 cells was obviously recovered.

**Effect on scavenging of intracellular ROS**

CDCFH, quantified as a redox indicator by fluorimetry, was used to reflect ROS level. In Figure 6, picroside II was able to scavenge intracellular ROS in a dose dependent way. With treatment of picroside II and NGF (2 ng/ml) in combination, the ROS level was effectively decreased in a synergistic manner and the intracellular ROS was nearly down to normal value. From our findings, it appears that the synergis-
tic effect of picroside II and NGF on increasing the viability of PC12 cells might be due to their cooperative ability based on antioxidant properties.

Discussion

Oxidative stress is a ubiquitous phenomenon in all cell types, and it primarily occurs in mitochondria which is essential for multicellular organisms. Antioxidant systems have evolved to help to defend the body against free radicals, but free radicals might become overwhelming during periods of chronic oxidative stress, and have been found to increase with age in a variety of species [12]. It was pointed out that the crucial balance between ROS generation and antioxidant defense is a key factor in disease prevention [2]. It becomes apparent that an imbalance between protection against the ROS and their generation is definitely associated with the pathogenesis of a wide variety of chronic diseases.

H$_2$O$_2$, a major source of ROS, may perturb the natural cellular antioxidant defense systems, resulting in damage to the major classes of biological macromolecules, which have been implicated in several pathological processes including Alzheimer’s disease and Parkinson’s disease [4, 13]. High levels of H$_2$O$_2$ have been shown to be toxic to neurons [22], and the 150 μM H$_2$O$_2$ used in our experiment resulted in substantial cell loss. With the increase in concentration, such H$_2$O$_2$-induced cytotoxicity was reversed and the corresponding intracellular ROS levels decreased concurrently by the treatment. What is more, picroside II protective effect was not due to a direct promotion of the cell proliferation.

It has been reported that nerve growth factor NGF stimulates the outgrowth of neurites in neuronal cells and plays an important role in the survival and maintenance of neurons in the central nervous system [7, 15]. Many neurons require the presence of neurotrophic factors such as NGF or brain-derived neurotrophic factor for their survival and neuronal sprouting. The neuronal loss is observed in neurodegenerative disorders such as Alzheimer’s disease [3]. Thus, NGF may have therapeutic efficacy in the treatment of neurodegenerative diseases, including Alzheimer’s disease and cerebrovasculopathies. However, many of them including NGF are polypeptides of large molecular weight, do not cross the blood-brain barrier and are metabolized by peptidases when administered peripherally. A useful strategy of addressing the drug delivery problem is to administer drugs that either enhance the action of or increase the expression of neurotogenic substances such as NGF in the central nervous system [20]. In our study, the cell viability was considerably improved by the treatment with picroside II and NGF in combination, compared to the treatment alone.

As we know, picroside II has protective effects in various disorders in vitro and in vivo, by modulation of free radical-induced oxidative stress and lipid peroxidation [18, 21]. NGF induced endogenous cellular antioxidants, glutathione peroxidase or catalase in PC12 cells, and the induction of antioxidants by NGF may be one of the reasons why the cells were rescued from hyperoxia-induced apoptosis. Maybe the synergistic effect in certain pathological conditions can also be attributed to some signaling pathways or gene expression, which might have relation to ROS level. So further research is still required to study the mechanism operating under oxidative stress. In addition, if we can confirm the synergistic effect in animal models, it will be more encouraging for the application research of picroside II to neurodegenerative diseases.

In conclusion, our results showed that picroside II and NGF could scavenge ROS cooperatively, leading to the synergistic protective effect on PC12 cells against oxidative stress induced by H$_2$O$_2$.

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References:


