Ganoderma extract activates MAP kinases and induces the neuronal differentiation of rat pheochromocytoma PC12 cells

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Abstract The pharmacology and clinical application of traditional Chinese medicine has been extensively documented. We have used an in vitro model system, PC12 cells, to demonstrate the presence of neuroactive compounds in *Ganoderma lucidum* (lingzhi). Ganoderma extract induced the neuronal differentiation of PC12 cells and prevented nerve growth factor-dependent PC12 neurons from apoptosis. Moreover, these effects of ganoderma might be mediated via the ras/extracellular signal-regulated kinase (Erk) and cAMP-response element binding protein (CREB) signaling pathways, as demonstrated by the phosphorylation of Erk1, Erk2 and CREB. Thus, our data not only present the first evidence of the presence of neuroactive compounds that mediate the neuronal differentiation and neuroprotection of the PC12 cells, but also reveal the potential signaling molecules involved in its action.

Key words: Ganoderma; Extracellular signal-regulated kinase 1/2; cAMP-response element binding protein; Apoptosis; Neuronal differentiation; MAP kinase; Chinese medicine

1. Introduction

The pharmacology and clinical application of traditional Chinese medicine (TCM) has been well-documented for centuries in China. The most ancient TCM herbals 'Shen Nong Ben Cao Jin', which was compiled in around 100 A.D., classified 365 herbs into three different categories. One important category comprises novel Chinese herbs that are effective in promoting good health and vitality, and in curing various diseases. *Ganoderma lucidum* (ganoderma or lingzhi), a medicinal Chinese mushroom belonging to this novel category, has been demonstrated to possess tumoricidal [1,2] as well as immunomodulatory activities [3,4]. From the ethnopharmacological point of view, ganoderma is as important as ginseng in China. Although the benefits of ganoderma to brain and its action in the suppression of inflammation have been noted in Chinese herbals, little is known regarding its actions and the underlying mechanisms in brain. On the contrary, increasing evidence has been accumulated on the medicinal application of ganoderma in the treatment of various diseases, such as cancers, immunological disorders and scleroderma [5,6].

The high nutritional value, wide acceptability, and potential medicinal value of ganoderma, have attracted intense interest in the search for pharmacological compounds from these edible mushrooms [5]. As a first step to examine potential neural action(s) of ganoderma, we employed an in vitro model system to examine the presence of neuroactive substances in ganoderma. Rat pheochromocytoma cell cultures, PC12 cells, have been extensively used as model systems for the study of tumorigenesis, apoptosis and neurodegenerative diseases [7–9]. These cells differentiate into dopaminergic neurons when treated with nerve growth factor (NGF) even at very low concentrations [10].

In this study, we report that ganoderma extract can reduce the cell proliferation rate of PC12 cells. Furthermore, ganoderma extract can also induce the neuronal differentiation of PC12 cells and prevent the NGF-dependent PC12 neurons from undergoing apoptosis. More importantly, our findings demonstrate that ganoderma extract can activate both the MAP kinase and cAMP-response element binding protein (CREB) signaling pathways, thus revealing the potential signaling molecules involved in its action.

2. Materials and methods

2.1. Preparation of mushroom extracts

Ganoderma was cultivated as suspension cultures in 10 ml of potato dextrose broth (Oxoid) at 25°C until complete saturation. Typically, 600–700 mg of fungal mycelia was obtained. Fungal mycelia, collected by filtration, were washed and resuspended in deionized water. Aqueous extract was obtained by autoclaving the ganoderma suspension for 20 min.

2.2. Quantitative analysis of polysaccharides in ganoderma extracts

Quantitative analysis of the polysaccharides present in the ganoderma extracts was performed by the phenol–sulfuric acid assay [11]. 1 ml of ganoderma extract was mixed with 1.6 ml 5% phenol and 7.5 ml concentrated H_2SO_4. After an incubation of 26 min, the solution was degassed and measured at 497 nm (Spectronic Genesys 5). Glucose standard solution was used as standard in the quantitative analysis.

2.3. Cell culture

Rat PC12 cells were cultured as previously described [7]. PC12 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; high glucose formulation) supplemented with 6% fetal bovine serum (FBS, Gibco) and 6% horse serum (HS, Gibco). As control, PC12 cells were differentiated using NGF (10 ng/ml) (Alomone Lab).

2.4. Western blot analysis and antibodies

Protein extraction and Western blot analysis were performed as

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previously described [12]. Immunoreactive bands were detected using an enhanced chemiluminescence kit (Amersham) and visualized by Fuji X-ray film (Fuji). Monoclonal antibodies detecting the proliferating cell nuclear antigen (PCNA) and β-actin, and panTrk antibodies for immunoprecipitation were obtained from Santa Cruz. Monoclonal mouse anti-sera specific for neurofilament-160 kDa phosphorylated (Zymed), and neurofilament-200 kDa (Zymed) were used as neuronal markers in the study. Rabbit polyclonal anti-sera detecting the phosphorylated and unphosphorylated forms of transcription factor CREB, p44/42 MAP kinases (extracellular signal-regulated kinases; Erk1/2), and the high affinity NGF receptor, TrkA, were obtained from New England Biolabs. Quantitation was performed using the integrated density and concentration analysis provided by the Eagle-Sight software (Stratagene).

2.5. Cell proliferation and viability of PC12 cells

The effect of ganoderma extract on cell proliferation and viability was monitored by measuring the reduction of the tetrazolium salt using the cell proliferation (MTT) kit (Roche Molecular Biochemicals). Briefly, PC12 cells (1000 cells/well) were preincubated overnight in 96-well plates. The cells were then treated with ganoderma extract at different concentrations (5–100 mg/ml) for 2 or 4 days. MTT assays were performed by incubating the ganoderma treated PC12 cells with MTT labeling solution. After 4 h of incubation, PC12 cells were lyzed and the purple formazan crystals were solubilized for detection at 570 nm. At least three independent experiments were performed for each study; representative data is presented.

2.6. Cytotoxicity assays

The cytotoxic potential of the ganoderma extract was determined by the cytotoxicity detection kit (Roche Molecular Biochemicals) which was based on the detection of lactate dehydrogenase (LDH) released from dead cells as a result of cytotoxicity. Cell-free culture supernatants from ganoderma treated PC12 cells were collected and then transferred to multi-titer plates. Substrate mixture containing tetrazolium salts were added, and then incubated for 0.5 h. The formazan dye formed was quantitated by measuring the absorbance at 500 nm.

2.7. Anti-apoptotic effects of ganoderma extract

Apoptosis of PC12 cells induced by NGF withdrawal was performed as described with minor modifications [13]. Briefly, PC12 cells were treated with NGF (50 ng/ml) for at least 7 days in 0.1% HS/FBS on collagen-coated plates. Apoptosis was induced by washing the NGF-induced PC12 neurons twice with serum-free DMEM, followed by treatment with ganoderma extracts (50 mg/ml in DMEM). In the control setup, an equal amount of water or 50 ng/ml NGF was added. PC12 neurons were then cultured for 12 h before they were air-dried and fixed in 4% paraformaldehyde (Fluka). Detection of apoptotic cell death was performed using the fluorescein-based in situ cell death detection kit (Roche Molecular Biochemicals). The assay was based upon the detection of DNA degradation using TUNEL staining. Apoptotic cells were visualized under a Zeiss Axioplot fluorescence microscope (Zeiss). The extent of apoptosis was expressed as a percentage of the total number of cells that showed positive TUNEL staining. Typically, at least 300 cells per treatment were counted in random fields. At least three independent experiments were performed for each study; representative data is presented.

Table 1
Cytotoxic effect of ganoderma extract on PC12 cells

Treatment	Average absorbance
Ganoderma (mg/ml)	
0	0.704 ± 0.01
5	0.776 ± 0.01
10	0.791 ± 0.01
25	0.794 ± 0.01
50	0.767 ± 0.02
100	0.812 ± 0.02
Control	
Total cell lysate	2.175 ± 0.09
NGF (12.5 ng/ml)	0.774 ± 0.01

The cytotoxic effect of ganoderma extract was determined by measuring the LDH released from the cytosol of dead cells. PC12 cells were treated with ganoderma extract at working concentration as indicated for 24 h. As control, PC12 cells were treated with NGF which was capable of differentiating PC12 cells into sympathetic neurons. Cell-free supernatant was collected and the LDH released by dead cells was quantitated by adding substrate mixture containing *p*-iodonitrotetrazolium violet and sodium lactate. Water-soluble formazan dye was detected directly at 490 nm. Total cell lysate, indicating the maximum releasable LDH in PC12 cells, was prepared by incubating the PC12 cells with 2% Triton X-100 in assay medium. Experiments were performed at least three times and results indicated in the table were mean ± S.E.M. in one representative experiment; $n = 5$. Ganoderma extract alone did not give positive absorbance (data not shown).

3. Results

3.1. Ganoderma extract inhibited the proliferation of rat PC12 cells with no detectable cytotoxicity

As the first step to examine the presence of neuroactive compounds in ganoderma, we utilized an in vitro model system using rat PC12 cells. When challenged with low concentrations of NGF (< 10 ng/ml), PC12 cells undergo neuronal differentiation with extensive neurite outgrowth and expression of neuronal proteins such as neurofilaments. Aqueous extracts of ganoderma, containing 16.5 μg/μl of polysaccharides (40.6% (w/w)), were added to the PC12 cells cultured in normal medium. The effect on the cell proliferation was examined by monitoring the metabolic activities of PC12 cells after treatment with increasing concentrations of ganoderma extract for 2 or 4 days.

At day 2, treatment of PC12 cells with ganoderma extract at high concentrations (> 50 mg/ml) significantly decreased the growth rate when compared with the control cells treated with the solvent, i.e. water (Fig. 1). At day 4, ganoderma extracts significantly reduced the cell proliferation of PC12 cells in a dose-dependent manner. More than 30% decrease in cell proliferation was observed when PC12 cells were treated with 100 mg/ml ganoderma extract for 4 days.

Since the cell proliferation MTT assays measure the metabolic activities of the cells, the reduction in the absorbance could either be a consequence of cell death or the reduction in

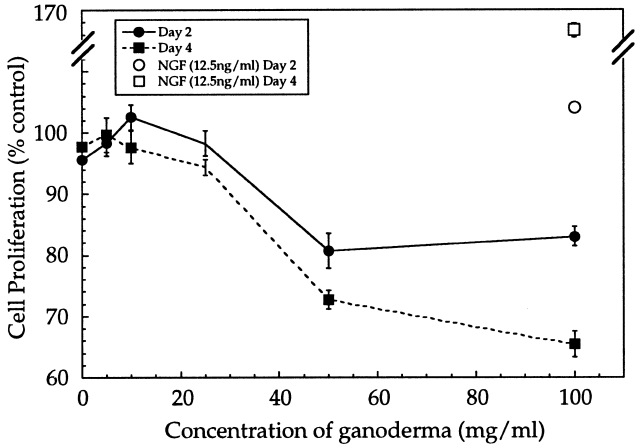

Fig. 1. Effect of ganoderma extract on the cell proliferation of PC12 cells. PC12 cells were treated with aqueous ganoderma extract at concentrations from 5 to 100 mg/ml for 2 or 4 days. NGF (12.5 ng/ml) was used as a positive control in the experiment. MTT assays were performed as described. The mean absorbance obtained using solvent control was designated 100%. Results shown represent the mean ± S.E.M. of a typical experiment; $n = 3$.

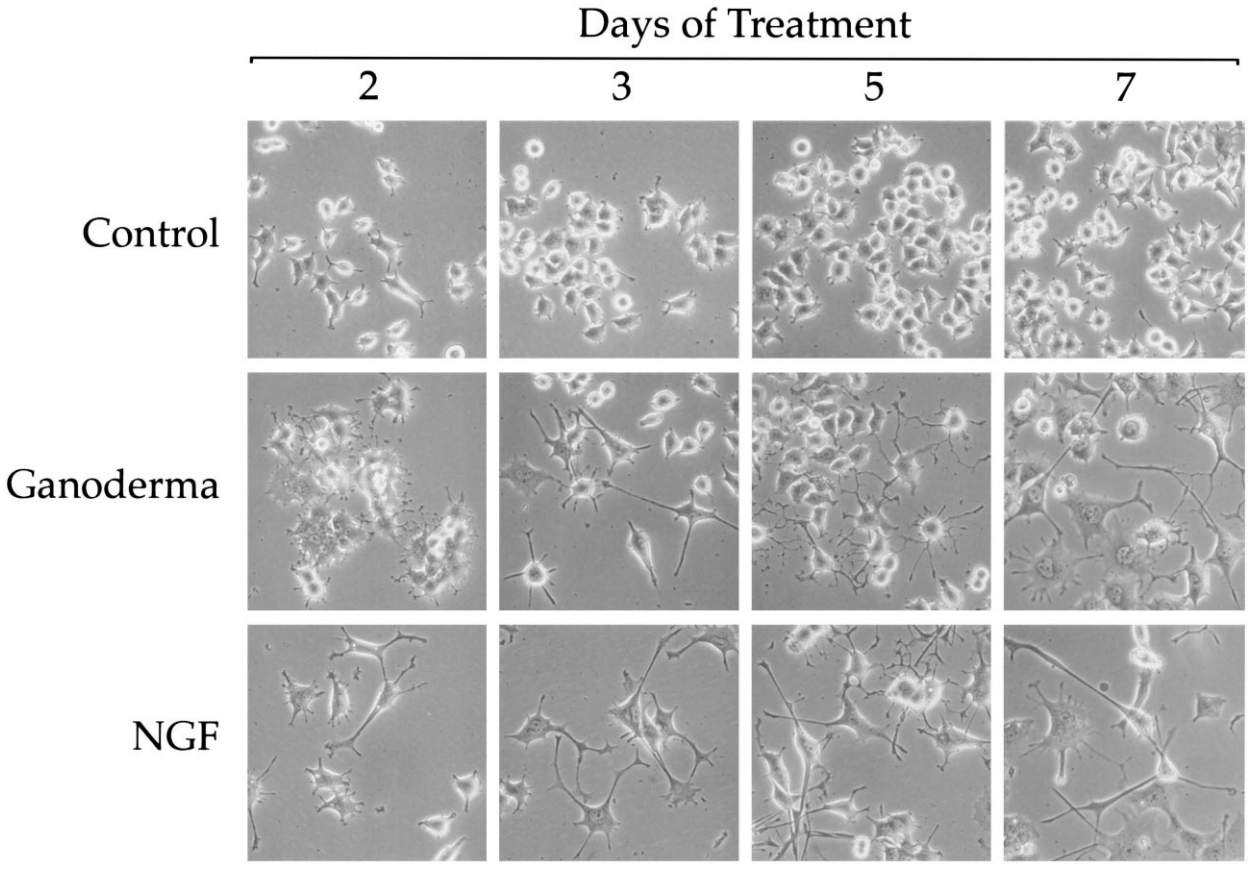

Fig. 2. Morphology of PC12 cells treated with ganoderma extracts. PC12 cells were treated with ganoderma extract (50 mg/ml) for the period indicated. PC12 cells treated with either solvent alone or NGF (10 ng/ml) were used as negative and positive control, respectively.

the cell proliferation. The cytotoxicity test was then performed to examine whether treatment of PC12 cells with the ganoderma extract could induce cell death. Increasing concentrations of ganoderma extract (5 to 100 mg/ml) was added to PC12 cells and the release of LDH was monitored after overnight incubation. It was observed that the ganoderma extracts did not induce any detectable cytotoxic effect (Table 1).

3.2. *Ganoderma extracts induced the neuronal differentiation of PC12 cells*

Following treatment with ganoderma extract, PC12 cells showed neuronal phenotypes, including compaction of cell bodies and the extension of neurites (Fig. 2). Although not all PC12 cells differentiated into neuron-like cells even at day 14 of the ganoderma treatment, the first occurrence of neuron-like cells was comparable to that observed with NGF treatment. Total proteins were prepared from PC12 cells treated with ganoderma extract for 3 to 14 days as well as from PC12 cells treated with NGF (10 ng/ml). Immunoblotting was performed to examine the expression of neuronal markers in the PC12 cells treated with ganoderma extract. It was observed that the ganoderma extracts induced the expression of neuronal markers including phosphorylated forms of the 200 kDa neurofilament (NF-H-P), and 160 kDa neurofilament (NF-M-P) (Fig. 3). Since the neurofilaments are the major proteins of the neuronal cytoskeleton, our findings suggested that the ganoderma extract contained neuroactive compounds that induced the neuronal differentiation of PC12 cells.

3.3. *Ganoderma extract rescued the PC12 neurons from apoptosis induced by NGF withdrawal*

Following NGF treatment, PC12 cells differentiate into neurons which will undergo apoptosis upon NGF withdrawal

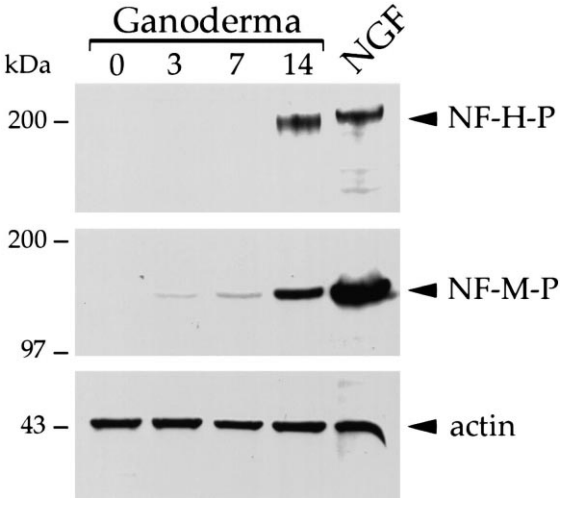

Fig. 3. Expression of neurofilament proteins following treatment of PC12 cells with ganoderma extract. PC12 cells were treated with aqueous ganoderma extract at concentrations of 50 mg/ml for 3 to 14 days. NGF (10 ng/ml) was used as the positive control. Total proteins were prepared and Western blot analysis was performed using antibodies specific for NF-H-P, NF-M-P and actin as indicated on the right. Positions of the protein size markers are as indicated.

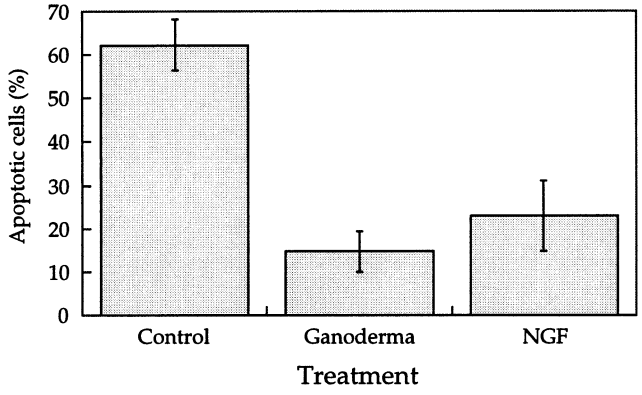

Fig. 4. Ganoderma extract protected PC12 neurons from NGF withdrawal apoptosis. PC12 cells were cultured in normal medium supplemented with 50 ng/ml for 8 days. After two washes with NGF-free normal medium, the neurons were cultured in normal medium supplemented with water, 50 mg/ml ganoderma extract, or 50 ng/ml NGF for 12 h. TUNEL staining was performed as described. Apoptotic cells with positive fluorescent staining were scored and expressed as the percentage of total number of cells. Results shown are the mean ± S.E.M. of a typical experiment; $n = 3$.

[14]. The ability of the ganoderma extract to protect these neurons from undergoing apoptosis was examined. NGF-dependent neurons were prepared by differentiating the PC12 cells in 50 ng/ml NGF for 8 days, and apoptosis was induced by the removal of NGF for 12 h. During the NGF withdrawal

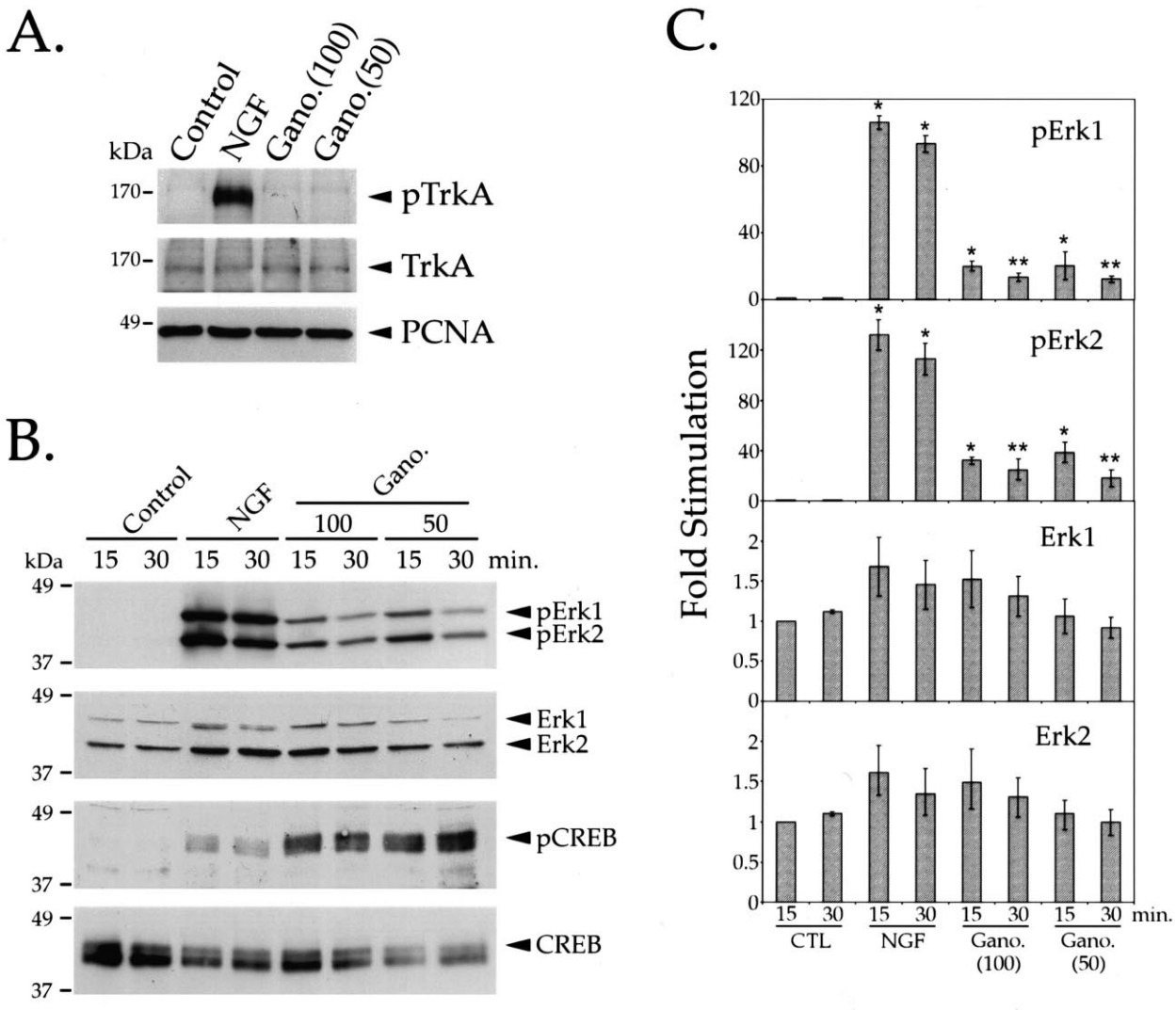

Fig. 5. Ganoderma extract activated the MAP kinases and CREB but not the TrkA proteins. PC12 cells were serum-starved for 5 h, and then treated with aqueous ganoderma extract (50 and 100 mg/ml) for various times as indicated. NGF (10 ng/ml) was used as the positive control. A: Total cell lysate was immunoprecipitated with panTrk antibodies in total alkaline RIPA at 4°C. Precipitated proteins were subjected to SDS–PAGE, electrotransferred to nitrocellulose and immunoblotted with phospho-TrkA (pTrkA) antibodies. The membrane was subsequently stripped and reblotted with anti-TrkA antibodies as indicated. Total lysates were immunoblotted with PCNA to demonstrate equal quantities of protein employed for the immunoprecipitation. B: Total lysates were immunoblotted with antibodies specific for the phosphorylated proteins (pErk1/2 and pCREB), or the phosphorylation-independent state of the corresponding proteins (TrkA, Erk1/2 and CREB), as indicated on the right. Positions of the protein size markers are as indicated. C: Quantitative analysis of pErk1/2 and Erk1/2 expression was performed as described in Section 2. The quantitative data were normalized relative to control and expressed as fold stimulation. Results from three separate experiments are expressed as mean ± S.E.M. $^{*}P < 0.001$ and $^{**}P < 0.01$, unpaired t-test, compared with control.

period, PC12 cells were either treated with ganoderma extract (50 mg/ml) or NGF (50 ng/ml). TUNEL staining was performed to detect the apoptotic cells and the TUNEL stained PC12 cells were scored. While NGF withdrawal induced 60% of the PC12 neurons to undergo apoptosis, treatment with ganoderma extract (50 mg/ml) reduced the extent of apoptosis to 15% (Fig. 4).

3.4. *The MAP kinases, but not TrkA, were activated during the neuronal differentiation of PC12 cells induced by ganoderma extracts*

In order to examine whether the neuroactivity of ganoderma was mediated by NGF-like activities, we investigated the ability of ganoderma extract to activate TrkA. Total protein was collected from PC12 cells that were treated with ganoderma extracts for 15 min. Immunoblotting was performed using specific antibodies that detected the phosphorylated form of TrkA. It was observed that the ganoderma extracts neither activated any detectable phosphorylation of TrkA nor increased the expression of TrkA protein (Fig. 5A). Thus, the ability of ganoderma extract to induce neuronal differentiation of PC12 cells was mediated by a signaling pathway that did not involve TrkA activation.

Phosphorylation assays were performed to examine the possible involvement of the ras/Erk pathway and the CREB pathway. When PC12 cells were treated with ganoderma extract (50 or 100 mg/ml), both Erk1 and Erk2 were activated (Fig. 5B, top panels) for at least 30 min. The quantitative analysis from three independent experiments is depicted in Fig. 5C. In addition to Erk activation, the ganoderma extract could also activate CREB via the phosphorylation at the serine-133 (Fig. 5B, lower panels).

4. Discussion

In the present study, we utilized a model system, PC12 cells, to demonstrate the presence of neuroactive compounds in the Chinese mushroom, *G. lucidum*. Incubation with ganoderma extract results in a reduction of cell proliferation, as well as induction of neuronal differentiation of PC12 cells. More importantly, the ganoderma extract also protects the neurons from apoptosis induced by NGF withdrawal. Furthermore, the ability of ganoderma extract to induce the phosphorylation of both Erk1/2 and CREB proteins in PC12 cells suggests that the action of ganoderma may be mediated via both the ras/Erk and CREB signaling pathways. Our findings not only demonstrate that *G. lucidum* comprises a rich source of neuroactive compounds, but also reveals the potential signaling molecules involved in its action.

The anti-tumor effect of ganoderma has been suggested to result from immunosuppressive protein [15,16], inhibition of DNA polymerase by cerebrosides [17] or inhibition of post-translational modification of oncoproteins [18]. Polysaccharides containing anti-tumor activities, with molecular weights ranging from 4×10^5 to 1×10^6, have also been extracted from several species of ganoderma [2,19]. Recent findings suggest that the anti-tumor effect of ganoderma mediated by the polysaccharides is a consequence of the potentiation of cytokine production by activated macrophages and T lymphocytes [20,21]. In light of the high percentage (40% (w/w)) of polysaccharides present in our ganoderma extract, polysaccharides are likely candidates for mediation of the neuroactivity observed in this study. During the mycelial growth, the polysaccharides can be secreted to the culture media [22]. This is consistent with our recent findings that the ganoderma culture filtrate can reduce the cell proliferation of PC12 cells to the same extent as the autoclaved aqueous extract. Furthermore, similar Erks activation can also be demonstrated using a commercially available preparation of ganoderma that is enriched in polysaccharides (data not shown). Work is in progress in our laboratory to determine the precise identity of the neuroactive compounds in the ganoderma extract.

Since very little is known regarding the molecular mechanism underlying the action of ganoderma, our findings present the first evidence on the potential signaling molecules involved, such as the ras/Erk signaling pathway. This pathway involves protein kinases that are important in regulating the growth and differentiation of PC12 cells [23]. Although ganoderma extract does not directly activate TrkA, its action may be mediated by other types of receptors, the stimulation of which leads to activation of the downstream ras/Erk signaling cascade. It has been reported that NGF deprivation inhibits the phosphorylation of the Erks but activated the c-Jun NH_2-terminal protein kinase and p38 kinase [24]. Thus, the neuroprotective effect of the ganoderma extract may be explained by the activation of Erks that maintain the survival of the NGF-dependent neurons. Furthermore, our finding on the activation of CREB upon treatment with ganoderma raises the intriguing possibility that ganoderma may mediate neuronal functions by modulating the activities of different signaling pathways. For example, recent studies demonstrate the importance of CREB signaling on learning and memory [25] and hyperphosphorylation of CREB plays an important role in the long-term potentiation of the hippocampus [26]. It will be of interest to examine the potential use of ganoderma extract in the enhancement of memory.

The involvement of abnormal apoptosis, which results in excessive loss of neurons, has been demonstrated in various neurodegenerative diseases [27]. We report here that the ganoderma extract contains neuroactive compounds which can prevent cell death in NGF deprived PC12 neurons. Taken together with the data on the low cytotoxicity and high nutritive value of ganoderma, our finding reveals the potential use of ganoderma extract in protecting the neurons from undergoing apoptosis, such as that in disease states. Although pharmacological agents such as calpain and caspase inhibitors possess remarkable neuroprotective effects, they show distinctive modes of action [28]. It will be of interest to examine whether the ganoderma extract can have additive or synergistic effects with these agents in the prevention of neuronal apoptosis.

While the present study reports the first evidence on the neuroactive actions of ganoderma polysaccharides, the bioactive compounds that are responsible for the neuroactivity remain to be determined. It is noteworthy that a common and interesting feature of TCM is the presence of multiple compounds which could act either independently or synergistically to elicit their pharmacological effects. Our assay systems presented in this report may serve as a good bioassay system for such systematic analysis.

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