SPANDIDOS PUBLICATIONS

Cytoprotective effects of *Lycium barbarum* against reducing stress on endoplasmic reticulum

MAN-SHAN YU[1], YUEN-SHAN HO[1], KWOK-FAI SO[1-3], WAI-HUNG YUEN[4] and RAYMOND CHUEN-CHUNG CHANG[1-3]

[1]Laboratory of Neurodegenerative Diseases, Department of Anatomy, [2]Research Centre of Heart, Brain, Hormone and Healthy Aging, Faculty of Medicine, [3]Central Laboratory of the Institute of Molecular Technology for Drug Discovery and Synthesis, [4]Department of Chemistry and Open Laboratory of Chemical Biology of the Institute of Molecular Technology for Drug Discovery and Synthesis, The University of Hong Kong, Hong Kong, SAR

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Abstract. Chinese medicinal herbs have been consumed for thousands of years for the purpose of healthy aging. *Lycium barbarum* is valued in Chinese culture for its benefits to anti-aging, vision, kidney and liver. Recent studies showed that extracts from *L. barbarum* possess biological activities including anti-aging, anti-tumor, immune-stimulatory and cytoprotection. Most of these studies emphasized that the protective function of *L. barbarum* is due to its anti-oxidative effects. We have previously demonstrated that extract from *L. barbarum* can protect neurons against ß-amyloid (Aß) peptide-induced apoptosis. Since Aß toxicity may be mediated via oxidative stress, it is still unclear whether the extract from *L. barbarum* is a simple anti-oxidant exhibiting cytoprotective effects. We hypothesized that extract from *L. barbarum* is not simply an anti-oxidant in order to function as a neuro-protective agent. The aim of this study is to investigate whether the extract from *L. barbarum* (LBG) protect neurons via mechanisms independent of anti-oxidative effects. Using a reducing agent, dithiothreitol (DTT), we found that LBG exhibits cytoprotective effects against reducing stress by lowering the DTT-induced LDH release and caspase-3 activity. DTT can trigger endoplasmic reticulum (ER) stress leading to PKR-like ER kinase (PERK) activation. We also showed that LBG attenuates DTT-induced PERK phosphorylation. The extract from *L. barbarum* is not simply an anti-oxidant; it can also exhibit cytoprotective effects against reducing stress by DTT.

Correspondence to: Dr Raymond Chuen-Chung Chang, Department of Anatomy, Faculty of Medicine, The University of Hong Kong, 21 Sassoon Road, Hong Kong, SAR
E-mail: rccchang@hkucc.hku.hk

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Introduction

The fruits of *Lycium barbarum* (also called *Fructus Lycii*, Wolfberry or Gouqizi), the small red berries, have been used for thousands of years in traditional Chinese medicine and cuisine since *L. barbarum* is believed to be beneficial to the eyes, liver and kidney. Different biological activities of *L. barbarum* have been demonstrated, including anti-aging, anti-tumor, immune-stimulatory and cytoprotection (1-3). Many studies suggested that the protective effect of *L. barbarum* mainly depends on its anti-oxidative action. For example, *L. barbarum* polysaccharides can capture free radicals and restrain the DNA damage of testicle cells caused by the oxidative stress, H_2O_2 (4). Another report showed that *Fructus Lycii* polysaccharides protect cultured seminiferous epithelium against hyperthermia-induced damage via an anti-oxidant mechanism (5). We have previously proved that the aqueous extract of *L. barbarum* exhibits neuroprotective effects against ß-amyloid (Aß) peptide-induced apoptosis in cultured neurons (6). Generation of reactive oxygen species (ROS) may be one of the mechanisms caused by Aß to trigger neuronal cell death (7). Thus, we cannot exclude the possibility that the neuro-protective effects of the aqueous extract of *L. barbarum* against Aß toxicity are mediated through anti-oxidation. It is an interesting question to ask whether the extract from *L. barbarum* is simply an anti-oxidant. We hypothesized that anti-oxidative effects are not the sole neuroprotective effects exhibited by *L. barbarum*. The aim of this study is to investigate whether the extract from *L. barbarum* (LBG) protects neurons via mechanisms independent of anti-oxidation. Dithiothreitol (DTT), an ER stress inducer, was used to trigger reducing stress in neurons by inhibiting the formation of disulfide bonds in the ER (8,9). We found that LBG exhibited cytoprotective effects against this reducing stress by lowering the DTT-induced LDH and caspase-3 activity. LBG also attenuated DTT-induced PERK phosphorylation. Therefore, the extract from *L. barbarum* is not simply an anti-oxidant; it can also exhibit cytoprotective effects against reducing stress on the ER.

Materials and methods

Materials. *Lycium barbarum* extract (LBG) from The Hong Kong Institute of Biotechnology (Shatin, Hong Kong) was used in this study. LBG was standardized by their chemical composition using thin layer chromatography to ensure batch-to-batch consistency. Materials used for neuronal cell culture were purchased from Gibco BRL (Burlington, Ontario, Canada). 1,4-Dithiothreitol (DTT), all-*trans* retinoic acid (RA), protease inhibitor cocktail, phosphatase inhibitor cocktail and anti-ß-actin monoclonal antibody were obtained from Sigma-Aldrich, Inc. (St. Louis, MO, USA); the LDH cytotoxicity assay kit was from Roche Diagnostics (Mannheim, Germany); colorimetric caspase-3 substrate (Ac-DEVD-pNA) was from Calbiochem, Inc. (Darmstadt, Germany); rabbit polyclonal antibodies for phospho-PERK were from Cell Signaling Technology (Beverly, MA, USA); horseradish peroxidase (HRP)-conjugated goat anti-rabbit and goat anti-mouse antibodies were from Dako (Glostrup, Denmark); PVDF membrane was from Bio-Rad (Richmond, CA, USA); Biomax X-way film was from Kodak (Tokyo, Japan); the enhanced chemiluminescence (ECL) detection kit was from Amersham (Buckinghamshire, UK); and the Re-Blot Western blot recycling kit was from Chemicon International, Inc. (Temecula, CA, USA).

Primary neuronal cultures and human neuroblastoma cell line. Primary neuronal cultures were prepared from embryonic day 17 Sprague-Drawley rats (Laboratory Animal Unit, The University of Hong Kong) according to our previous published methods (6,10-15). Cortical neurons were cultured for 7 days with minimum essential medium (MEM) supplemented with 5% fetal bovine serum and antibiotics prior to the treatment. Human neuroblastoma SH-SY5Y cells were obtained from American Tissue Type Collection (ATTC, Rockville, MD, USA). SH-SY5Y cells were cultured with MEM supplemented with 10% fetal bovine serum, 1% glutamine, penicillin (50 U/ml) and streptomycin (50 μg/ml). The cells were differentiated with medium containing 3% fetal bovine serum and 10 μM all-*trans* retinoic acid for 7 days before the treatment.

Treatment of neurons. In order to investigate whether LBG exerts neuroprotective effects, the cells were treated with LBG at concentrations ranging from 10 μg/ml to 500 μg/ml for 1 h. Then, cultured neurons were exposed to DTT (1 mM) for 24 h as our previous experiments showed that this treatment condition could induce ER stress (15). The culture medium and cells were harvested for different biological assays.

Measurement of general cytotoxicity. Culture medium was collected for LDH activity assay to determine the level of general cytotoxity. The procedures were carried out in accordance with the manufacturer's instructions and our previous published method (6,11,12). In brief, the culture medium was incubated with assay reagents in the dark for 30 min. The absorbance at 492 nm was measured to determine the level of LDH released. The results were expressed as the fold of control.

Caspase-3-like activity assay. Neurons were harvested for protein extraction, protein assay and caspase-3-like activity assay as described in our previous studies (6,10-17). In principle, colorimetric caspase-3 substrate (Ac-DEVD-pNA) was cleaved to form a yellow product (pNA). The absorbance of the yellow product at 405 nm was measured to determine the caspase-3-like activity. The values of specific activity (s.a., unit = pmol/min/μg) were calculated from the absorbance reading. Results were expressed as the fold of control.

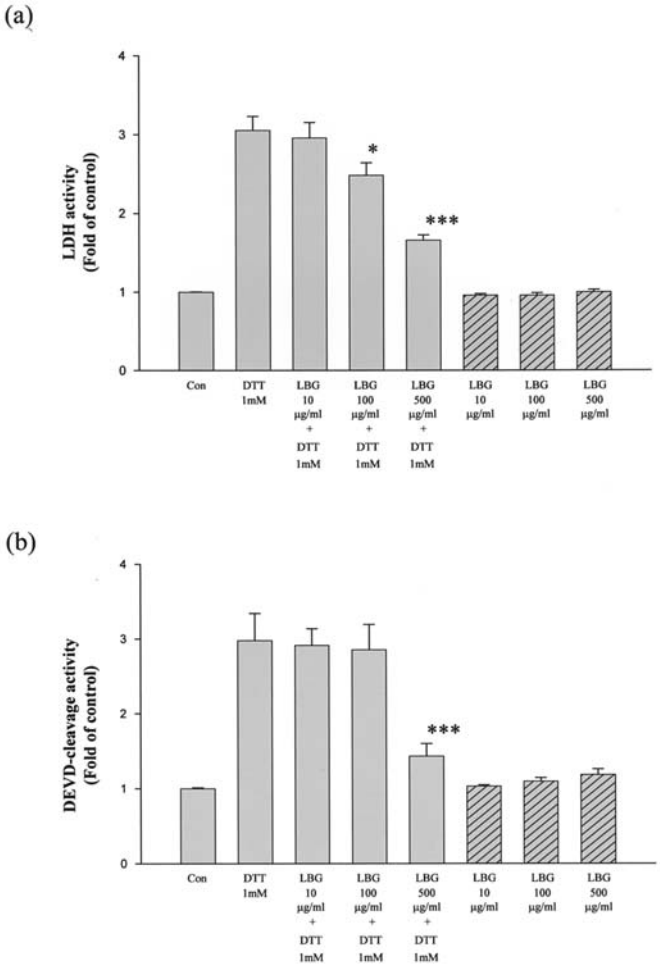

Figure 1. Neuroprotective effects of the LBG extract on DTT-induced cytotoxicity in cultured rat cortical neurons. Cortical neurons prepared from embryonic SD rats were treated with different concentrations of LBG for 1 h, followed by a 24-h incubation with or without DTT (1 mM). (a) LDH activity assay was performed to measure the level of cytotoxicity. LDH activity was expressed as fold of control. (b) Caspase-3 activity assay was performed to determine the extent of neuronal apoptosis. DEVD-cleavage activity was expressed as fold of control. Results are expressed as mean ± SE from at least three independent experiments. $^{***}p<0.001$ and $^{*}p<0.05$ vs. the group treated with DTT only by one-way ANOVA for multiple comparison and Student-Newman-Keuls test as post-hoc test.

Western blotting. To examine the level of phospho-PERK, primary cultured neurons after different treatments were harvested for protein extraction and protein assay as described in our previous studies (6,10-17). Fifty micrograms of protein per lane were separated in sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were then transferred onto a polyvinylidene difluoride (PVDF) membrane. The membrane was incubated with anti-phospho-PERK antibody (1:1000) for 2 h at room temperature, followed by incubation with goat anti-rabbit HRP-conjugated

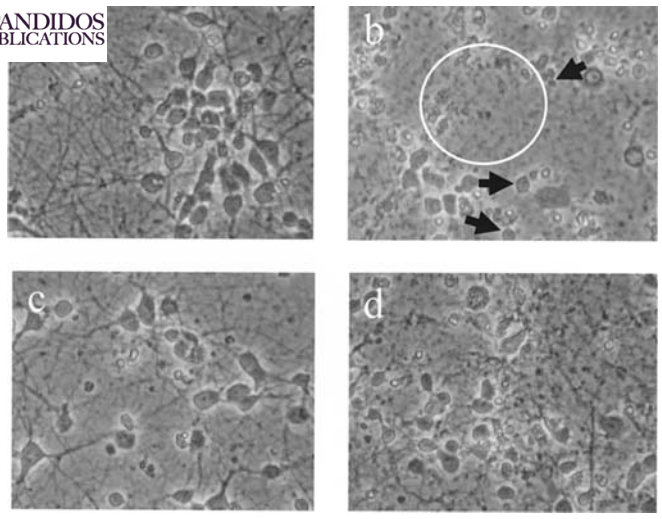

Figure 2. Morphology of cultured neurons after different treatments. Neurons were pretreated with LBG for 1 h, followed by a 24-h exposure to DTT. (a) Control, (b) DTT (1 mM) (arrow indicates shrunk cell body and the circled area indicates the destructed neurite network), (c) LBG (500 μg/ml) and (d) LBG (500 μg/ml) + DTT (1 mM).

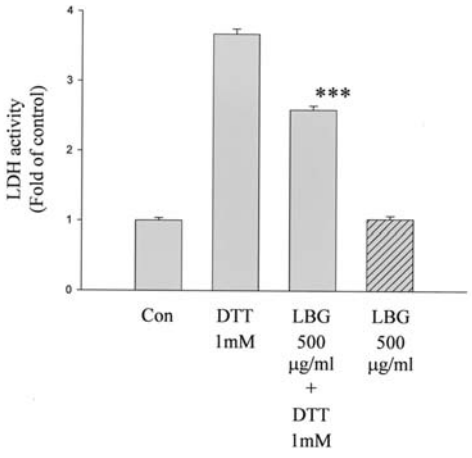

Figure 3. Cytoprotective effects of the LBG extract on DTT-induced cytotoxicity in SH-SY5Y cells. SH-SY5Y cells were treated with LBG (500 μg/ml) for 1 h, followed by 24-h incubation with or without DTT (1 mM). LDH activity assay was performed to measure the level of general cytotoxicity. Results are expressed as mean ± SE from at least three independent experiments. $^{***}p<0.001$ vs. the group treated with DTT only by one-way ANOVA for multiple comparison and Student-Newman-Keuls test as post-hoc test.

secondary antibodies (1:2000) for 1 h at room temperature. Bands on X-ray film were developed by using a chemiluminescent ECL detection kit. Membranes were then stripped using the Re-Blot Western blot recycling kit and re-probed with anti-ß-actin antibody (1:5000) as primary antibody, and goat anti-mouse-HRP as secondary antibody.

Statistical analysis. For quantitative data, the results are expressed as mean ± SE from at least three independent experiments. One-way ANOVA was used to determine the significance of differences among different groups, followed by Student-Newman-Keuls test as post-hoc test. A value of $p<0.001$ or $p<0.05$ was considered statistically significant in all experiments.

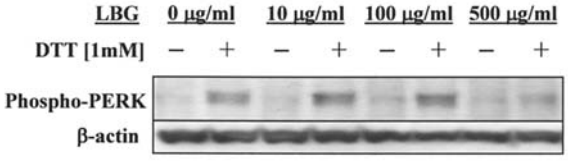

Figure 4. Western blot analysis of phospho-PERK in cultured cortical neurons treated with LBG and DTT. Neurons were treated with different concentrations of LBG for 1 h, followed by 24-h incubation with or without DTT (1 mM). Proteins extracted were subjected to Western blot analysis to detect the level of phospho-PERK. Membranes were stripped to re-probe for ß-actin, which was used as the internal control. The representative blots are from at least three independent experiments.

Results

Neuroprotective effects of the LBG extract in cultured rat cortical neurons. To examine the neuroprotective effects of the LBG extract on DTT-induced cell death, cortical neurons prepared from embryonic SD rats were pre-treated with LBG at 10, 100 or 500 μg/ml for 1 h, followed by incubation with or without the reducing stress DTT (1 mM) for 24 h. After treatment, the extent of general cytotoxicity and apoptosis was determined by LDH and caspase-3 activity assays, respectively. Fig. 1a shows the LDH activity of various treatment groups. For the control group, LDH activity was calculated as 1.00±0.00-fold of control, neurons treated only with DTT showed a significant increase in LDH activity to 3.05±0.18-fold of control. Pretreatment with LBG at 100 and 500 μg/ml markedly reduced the LDH release triggered by DTT (2.48±0.16 and 1.66±0.07-fold of control). To examine the effects of LBG on DTT-activated caspase-3 activity, the colorimetric caspase-3-like activity assay was performed. In Fig. 1b, the DEVD-cleavage was 2.98±0.36-fold of control of the group treated with DTT only. LBG at 500 μg/ml showed the best neuroprotective effect since it lowered the DEVD-cleavage to 1.43±0.17-fold of control when compared with the DTT-treated group. The results showed that LBG exerted neuroprotective effects against the reducing stress DTT. The morphology of neurons after different treatments is shown in Fig. 2. In the control group (Fig. 2a), neurons were healthy, with fine neurite network and round cell bodies. Neurons treated with DTT only were dying (Fig. 2b), as revealed by the destructive neuronal network and shrinkage of cell bodies. LBG alone did not alter the morphology of the neurons (Fig. 2c). Pretreatment with LBG protected neurons against DTT toxicity, as shown by the fine morphology in Fig. 2d.

Cytoprotective effects of the LBG extract in SH-SY5Y cells. The cytoprotective effects of LBG were re-confirmed using the human neuroblastoma cell-line, SH-SY5Y. DTT markedly increased the LDH activity to 3.67±0.07-fold of control in SH-SY5Y cells (Fig. 3). Pretreatment with LBG at 500 μg/ml for 1 h significantly reduced the LDH activity to 2.59±0.06-fold of control when compared with the group treated with DTT only. The results further proved that LBG exerts cyto-protective effects against the reducing stress DTT in neurons.

Western blot analysis of phospho-PERK in neurons treated with LBG. DTT is one of the ER-stress inducers which can activate the phosphorylation of PERK (18). In order to investigate whether the neuroprotective effects of LBG are related to ER-stress signaling pathways, proteins extracted after treatment were subjected to Western blotting to detect the level of phospho-PERK. Fig. 4 shows that DTT induced the phosphorylated form of PERK. Pretreatment with LBG at 500 μg/ml significantly attenuated the level of phospho-PERK. The protein level of ß-actin was unchanged after all treatment.

Discussion

This is so far the first report showing that the extract from *Lycium barbarum* can protect neurons from cell death induced by the reducing agent, DTT. We have demonstrated that the extract from *L. barbarum*, LBG, exhibits cytoprotective effects against the reducing stress in primary cultured cortical neurons by attenuating the LDH activity and the caspase-3-like activity triggered by DTT. Pretreatment with LBG in the SH-SY5Y cell line has a similar cytoprotective effect, as shown by the LDH activity assay. DTT is an ER stress inducer which can activate PERK (18). LBG can markedly attenuate the phosphorylation of PERK induced by DTT. Our results suggest that the extract from *L. barbarum* has novel protective effects in neurons against reducing stress in the ER.

Extracts isolated from *L. barbarum* have been investigated extensively in the past 10 years. The biological effects of *L. barbarum* include anti-aging, anti-oxidation, anti-tumor and immuno-stimulating (1-5). The anti-oxidative property of *L. barbarum* should be attributed to its rich anti-oxidants such as carotenoids, riboflavin, ascorbic acid, thiamine and nicotinic acid (3). In our previous study (6), we have shown that an aqueous extract from *L. barbarum* (LBA, mainly consists of polysaccharides) protects neurons from Aß peptide-induced apoptosis by interrupting the c-Jun N-terminal kinase (JNK)-c-Jun signaling pathway. Since the Aß peptide can also induce oxidative stress, it is also possible that LBA exerts neuroprotective effects via anti-oxidation. The present study provides new information about the biological activity of the extract from *L. barbarum* by proving that LBG exhibits cytoprotective effects against the reducing stress triggered by DTT.

In order to investigate the cytoprotective effects of *L. barbarum* against non-oxidative stress, we chose a commonly used reducing agent, DTT, to trigger neuronal cell death. Owing to its reducing capability, DTT is a well-known ER stress inducer (8). The ER oxido-reductive potential is disturbed by DTT, affecting disulfide bond formation, which can result in improper protein folding. Prolonged disruption of protein folding may lead to cell death. A recent study demonstrated that DTT treatment effectively triggered Eca109 cell apoptosis by activating p38 MAP kinase (19). Another report also shows that DTT can induce the PERK-dependent activation of both JNK and p38 MAP kinase (20). Our data show that pre-treatment with LBG significantly attenuates DTT-induced PERK phosphorylation. Therefore, it is of interest to investigate whether the signaling pathways of JNK and p38 MAPK are involved in the protective mechanisms of LBG against DTT toxicity in future study.

Collectively, our results suggest that extract from *L. barbarum* possesses cytoprotective effects against reducing stress on the ER. Further studies are needed to elucidate the major active components in *L. barbarum* responsible for this anti-reducing property.

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