

The antioxidant effects of garlic saponins protect PC12 cells from hypoxia-induced damage

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Abstract

Hypoxia frequently occurs under several different cellular circumstances. Excess reactive oxygen species that are induced by hypoxia may result in cell injury and dysfunction. Recently, garlic has been found to possess some biological and pharmacological activities. The present study examined the effects of garlic saponins (GSP) on the survival of differentiated PC12 (dPC12) cells and the oxidative–antioxidant system. dPC12 cells were exposed to 2% O₂ in order to establish a neuronal insult model. Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide reduction assay and lactate dehydrogenase (LDH) release assay. The expression of selected genes (catalase (CAT), p65 and neuron-specific class III β -tubulin) was evaluated by real-time PCR and immunoblot assays. CAT activity, malondialdehyde (MDA) and 8-hydroxy-deoxyguanosine (8-OH-dG) concentrations were also determined. The data showed that hypoxia dramatically damaged dPC12 cells, while treatment with approximately 5×10^{-2} –10 ng/ml GSP improved cell viability, decreased LDH leakage and caused the cells to maintain neuronal-like characteristics in hypoxia. The production of MDA and 8-OH-dG was attenuated by GSP. CAT activity in dPC12 cells pretreated with GSP was higher than that of the hypoxic control. Moreover, GSP up-regulated CAT expression and decreased the total protein expression as well as the nuclear expression of p65 in hypoxic cells. These data indicate that GSP has antioxidant properties that can protect dPC12 cells from hypoxia-induced damage, which may be related to the up-regulation of CAT expression and activity as well as a decrease in the expression and nucleus distribution of p65 through effects on redox-sensitive signalling pathways.

Key words: Garlic saponins: Differentiated PC12 cells: Hypoxia: Antioxidants

Hypoxia is a pervasive physiological stimulus that is encountered under various cellular conditions, such as high altitude, physical exercise, pregnancy, ageing, inflammation, cardiovascular and respiratory failures, wounds and even cancer. Moreover, an excessive load of reactive oxygen species (ROS) generated under hypoxic conditions may result in cell injury and dysfunction^(1–4).

Garlic is a common food that has been used for the treatment of many diseases, including cancer, CHD and hypercholesterolaemia^(5–7). Garlic is known for its production of steroid saponins as well as organosulphur compounds. However, organosulphur compounds are unstable and give rise to transformed products. Saponins

are more stable for cooking and storage. Moreover, garlic saponins (GSP) have been found to have some biological and pharmacological activities, including anti-fungal, anti-bacterial, anti-inflammatory and hypocholesterolaemic influences^(6,8). Previous studies have shown that garlic, garlic extract and some garlic organosulphur compounds may also have antioxidant effects^(9–12); however, no study to date has reported that GSP exhibits these effects.

Oxidative stress affects the structure and function of proteins⁽¹³⁾, nucleic acids⁽¹⁴⁾ and lipids^(15–18). Moreover, ROS may serve as messengers for the activation of adaptive responses through redox-sensitive signalling pathways, such as NF- κ B and mitogen-activated protein kinases

Abbreviations: ARE, antioxidant response element; CAT, catalase; dPC12, differentiated PC12; GSP, garlic saponin; LDH, lactate dehydrogenase; MAPK, mitogen-activated protein kinase; MDA, malondialdehyde; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NGF, nerve growth factor; Nrf2, nuclear factor E2-related factor 2; ROS, reactive oxygen species; TUJ1, neuron-specific class III β -tubulin.

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(MAPK), which regulate the expression of antioxidant genes involved in non-enzymatic and enzymatic antioxidant defences. NF- κ B is an important transcription factor that participates in the regulation of a wide range of biological processes, including cell survival and proliferation. Antioxidant enzymes provide important protective mechanisms against oxidative damage. For example, superoxide dismutase transforms superoxide radicals (O_2^-) into H_2O_2 , the enzyme catalase (CAT) removes H_2O_2 and limits the formation of free hydroxyl radicals (OH^\bullet), and glutathione *S*-transferase removes the toxic products formed by ROS damage⁽¹⁹⁾. Among these antioxidant enzymes, CAT is one of the most active catalysts produced in nature.

The PC12 cell line originates from a rat pheochromocytoma and has the ability to transform into various phenotypes based on cell culture conditions. After treatment with nerve growth factor (NGF), native PC12 cells shift their phenotype from proliferating, undifferentiated cells to post-mitotic, differentiated, neurite-bearing neurons (dPC12). Therefore, PC12 cells are widely used as a model for the *in vitro* study of neuron damage resulting from Alzheimer's disease⁽²⁰⁾, Parkinson's disease^(21,22), hypoxia and oxygen–glucose deprivation through the use of a special ischaemic device or by treatment with cobalt chloride^(23,24). The purpose of the present study was to explore the effects of GSP on the survival of NGF-dPC12 cells and the oxidative–antioxidant system in hypoxia.

Materials and methods

Preparation of garlic saponins

An improved method was used for saponin extraction based on the literature⁽²⁵⁾. GSP was extracted from dried garlic powder by soaking the powder in 65% aqueous alcohol for 72 h. The alcoholic mixture was centrifuged at 6800 rpm for 20 min at 4°C, and the alcohol in the alcoholic extract was removed using a rotary evaporator at 55°C. The remaining aqueous solution was extracted by incubating the mixture with acetoacetate followed by *n*-butanol. The *n*-butanol extract was then purified on a D101 macroporous (benzene vinyl, apolarity copolymer) resin column (Tian Jin Hai Tian Chemical Industry Company Limited, Tian Jin, China) and eluted with 70% alcohol. Finally, GSP was purified from the 70% alcohol eluate by freeze-drying the solution and storing it at –20°C.

GSP was validated using TLC, the Liebermann–Burchard reaction and the Molisch test. TLC was carried out on silica gel G plates, with the underlayer solution of an eluent system composed of $CHCl_3$ –methanol–water (10:7:2 by vol., 4°C). The saponins were visualised by incubation with the vanillin–sulphuric acid reagent at 100°C for 10 min. GSP contained 90% saponins after recovery on a silica gel. The purified saponins were tested positive by the Liebermann–Burchard reaction and the Molisch test of steroidal saponin. GSP was dissolved in diluted alcohol

for the cell culture experiments, and the final concentration of alcohol was less than 3:1000 (v/v).

Cell culture

Native PC12 cells (CRL-1721; ATCC, Rockville, MD, USA) were maintained in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) supplemented with 5% (v/v) fetal bovine serum (Hyclone, Logan, UT, USA), 10% (v/v) horse serum (Invitrogen), 100 units/ml penicillin and 80 μ g/ml gentamicin at 37°C in a humidified atmosphere with 5% CO_2 (v/v). All experiments were carried out with subconfluent cells plated on poly-L-lysine-coated plates. To obtain neuron-like dPC12 cells, subconfluent cells were maintained in Dulbecco's modified Eagle's medium with 2% horse serum, 1% fetal bovine serum and NGF- β (50 ng/ml) (Sigma-Aldrich, St Louis, MO, USA) for up to 8 d.

Hypoxia treatment

dPC12 cells were pretreated with GSP and cultured in Dulbecco's modified Eagle's medium containing 0.5% horse serum and 0.5% fetal bovine serum without NGF in normoxia for 3 h. The cells were then quickly placed into a multi-gas incubator (2% O_2 (v/v), 5% CO_2 (v/v) and 93% N_2 (v/v); Nuaire, Plymouth, MN, USA), which maintains a precise O_2 concentration (1–21%) by injecting N_2 and CO_2 into the incubator.

Cell viability

Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction assay and lactate dehydrogenase (LDH) release assay. PC12 cells were plated on ninety-six-well plates in 100 μ l/well media containing NGF- β at a density of 2×10^4 cells/ml and maintained for 8 d in order to induce dPC12 cells.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide reduction assay. At the end of the hypoxia exposure, the cells were incubated with MTT (0.5 mg/ml) for 4 h at 37°C. The supernatants were then carefully discarded, and 150 μ l dimethyl sulphoxide (Sigma-Aldrich) was added to each well to dissolve the MTT formazan product from the metabolically active cells. The absorbance was measured by using a microplate reader (Bio-Rad, Hercules, CA, USA) at a test wavelength of 550 nm and a reference wavelength of 630 nm.

Lactate dehydrogenase release assay. A colorimetric assay based on the measurement of LDH activity released from the cytosol of damaged cells into the supernatant was used to quantify cell death and cell lysis. The hypoxic, differentiated cells were cultured in Dulbecco's modified Eagle's medium containing 0.5% horse serum and 0.5% fetal bovine serum for the LDH assay^(26,27). At the end of

the hypoxia exposure time, 100 μ l of the cell-free media were transferred into new wells, and LDH concentration was determined using the Cytotoxicity Detection Kit (LDH; Roche Applied Science, Basel, Switzerland). The percentage of cytotoxicity was calculated using the following equation: cytotoxicity (%) = (experimental value – low control)/(high control – low control). Low control was the amount of spontaneously released LDH, and high control was the amount of maximum releasable LDH. Absorbance was measured using a microplate reader (Bio-Rad) at a test wavelength of 490 nm and a reference wavelength of 630 nm.

Immunocytochemistry and immunoblot analyses

Immunocytochemistry. To obtain dPC12 cells for immunocytochemistry, PC12 cells were seeded on a 10 mm \times 10 mm cover glass placed in a twenty-four-well plate at a concentration of 5×10^3 cells/well for 8 d and cultured in a serum-free medium. At the end of the culture time, the cells were fixed in 4% (w/v) paraformaldehyde at room temperature for 15 min, permeabilised with 0.3% (v/v) Triton X-100 at 37°C for 15 min and blocked in 2% bovine serum albumin for 10 min. Subsequently, the cells were incubated overnight with an anti-TUJ1 (neuron-specific class III β -tubulin) mAb (1:1000; Abcam, Cambridge, UK) in PBS containing 1% (v/v) bovine serum albumin at 4°C. Immunoreactivity was detected using tetramethyl rhodamine isothiocyanate-conjugated anti-mouse IgG (1:50) at room temperature for 30 min. In addition, 4'-6-diamidino-2-phenylindole (1 μ g/ml) was added for 5 min to stain the cell nucleus. The samples treated with the antibody dilution instead of the primary antibody were used as negative controls for anti-TUJ1 immunostaining. Fluorescence was visualised under a confocal laser scanning microscope (Leica TCS SP5, Wetzlar, Germany).

Immunoblot analyses. For immunoblots, the cells were washed with PBS and lysed with lysis buffer (50 mM-Tris, 150 mM-NaCl, 1 mM-EDTA, 50 mM-NaF, 1 mM-phenylmethylsulfonyl fluoride and 1% (v/v) Triton X-100, pH 7.5) containing one tablet of the complete protease inhibitor cocktail (Roche Applied Science) per 10 ml of the buffer. The extraction of nuclear and cytoplasmic protein was performed using an extraction kit (Beyotime Institute of Biotechnology, Shanghai, China) according to the manufacturer's instructions as described previously⁽²⁸⁾. Protein concentration was determined using the Bradford assay. Proteins were separated by SDS-PAGE on a 12% acrylamide–bisacrylamide separation gel and 5% acrylamide–bisacrylamide stacking gel, and electrotransferred onto nitrocellulose membranes. After blocking with 2% bovine serum albumin, the membranes were subjected to immunoblot analysis by incubating the membrane overnight at 4°C with the following primary antibodies: rat anti-TUJ1 monoclonal antibody (1:800; Abcam, Cambridge, UK); rabbit anti-CAT polyclonal antibody

(1:300; USCN Life Science & Technology Company, Missouri, TX, USA); rabbit anti-p65 monoclonal antibody (1:800; Signalway Antibody Company Limited, Pearland, TX, USA); anti- β -actin monoclonal antibody (1:2000; ProMab Biotechnologies, Inc., Richmond, CA, USA). Peroxidase-conjugated AffiniPure rabbit anti-goat IgG (H + L) and peroxidase-conjugated AffiniPure goat anti-mouse IgG (H + L) were used as the secondary antibodies (1:5000; ZSGB-BIO, Inc., Beijing, China). Protein bands were visualised by using enhanced chemiluminescence, as described previously⁽²⁹⁾, using an enhanced chemiluminescence Western blot analysis system kit (Amersham Biosciences Corporation, Piscataway, NJ, USA). Image densitometric analyses of the immunoreactive protein bands were performed using Gel photodensitometry analysis software (Bio-Rad).

Real-time PCR

Total RNA extraction. Total RNA was isolated from PC12 cells by extraction with Trizol (Invitrogen) according to the manufacturer's instructions. The RNA samples were quantified by measuring A_{260} on a spectrophotometer. The $A_{260}:A_{280}$ ratio ranged from 1.95 to 2.0, and the samples were checked for integrity on a formaldehyde agarose gel. Reverse transcription of 5 μ g RNA was performed with SuperScript III RT (Invitrogen) according to the manufacturer's instructions.

Quantitative real-time PCR of the catalase and p65 genes. Quantitative real-time PCR was performed using the SYBR Green Kit (Invitrogen) on a Bio-Rad IQ5 (Bio-Rad). The PCR mixtures contained 25 ng complementary DNA, 0.2 μ mol/l of each specific primer and the SYBR Green reaction mix (Invitrogen). The mixtures were incubated at 95°C for 2 s, and then forty cycles at 95°C for 10 min, 60°C for 20 min and 72°C for 45 min were conducted. This step was followed by a melting curve analysis performed between 72 and 95°C, holding 45 s on the first step and 5 s on the next steps. The primers used were as follows: CAT forward, 5'-AAC AGC TTC AGC GCA CCA GA-3'; CAT reverse, 5'-TTC AGG TGG TTG GCA ATG TTC-3'; p65 forward, 5'-GCC TTG CTT GGC AAC AAC AC-3'; p65 reverse, 5'-ATC CTG TCA CCA GGC GAG TT-3'; β -actin forward, 5'-AGT GTG ACG TTG ACA TCC GTA-3'; β -actin reverse, 5'-GCC AGA GCA GTA ATC TCC TTC T-3'. Independent real-time PCR experiments were performed using the same complementary DNA for both the target gene and reference gene. At the end of the PCR, the amplified product was resolved on a 2% agarose gel to determine whether a single product was amplified. Data were analysed using Bio-Rad IQ5 operating software to determine the threshold cycle (C_T) above the background for each reaction. The relative transcript amount of the target gene was normalised to that of β -actin using the $2^{-\Delta\Delta C_T}$ method based on a previous study⁽³⁰⁾.

Malondialdehyde content determination

Cells were collected after being exposed to hypoxia for 24h. The cells were carefully rinsed with cold PBS and homogenised with cold 0.86% isotonic NaCl. MDA content was then determined according to the method described previously^(31,32) and according to the manufacturer's instructions (Nanjing Jiancheng Company, Nanjing, China).

8-Hydroxy-deoxyguanosine ELISA assay

Cells were pretreated with GSP (10 ng/ml), exposed to hypoxia for 24h, and then the supernatant was immediately collected for an ELISA. The supernatant was centrifuged (1000g) for 20min to remove particulates before the assay was performed. The concentration of 8-hydroxy-deoxyguanosine in the samples was determined using the ELISA method⁽³³⁾ according to the instructions from the ELISA kit (USCN Life Science & Technology Company).

Antioxidant activity determination

For the antioxidant assay, the cells were treated with the same conditions as those used in the MDA assay. CAT activity was determined with UV spectrophotometry

according to the instructions from the assay kit (Nanjing Jiancheng Company)⁽³⁴⁾. Protein content was then determined using the Bradford assay.

Statistical analysis

Data are presented as means with their standard errors. ANOVA followed by Fisher's least significant difference test or Dunnett's multiple comparison test was used to assess differences between groups. A *P* value less than 0.05 was considered to be statistically significant. The relative changes of mRNA levels are presented as percentages of the control samples and assumed to be 100%.

Results

Garlic saponins protect cell viability and decrease the lactate dehydrogenase release rate of hypoxic differentiated PC12 cells

When dPC12 cells were cultured under hypoxic conditions for 36h, a decrease in cell viability was observed. However, cells treated with GSP under hypoxic conditions showed an increase in cell viability by 51.5, 80.8 and 53.6% when the cells were treated with 5×10^{-2} , 0.5 and 5 ng/ml of GSP, respectively, compared with the control. The 0.5 ng/ml dose of GSP was found to be most effective

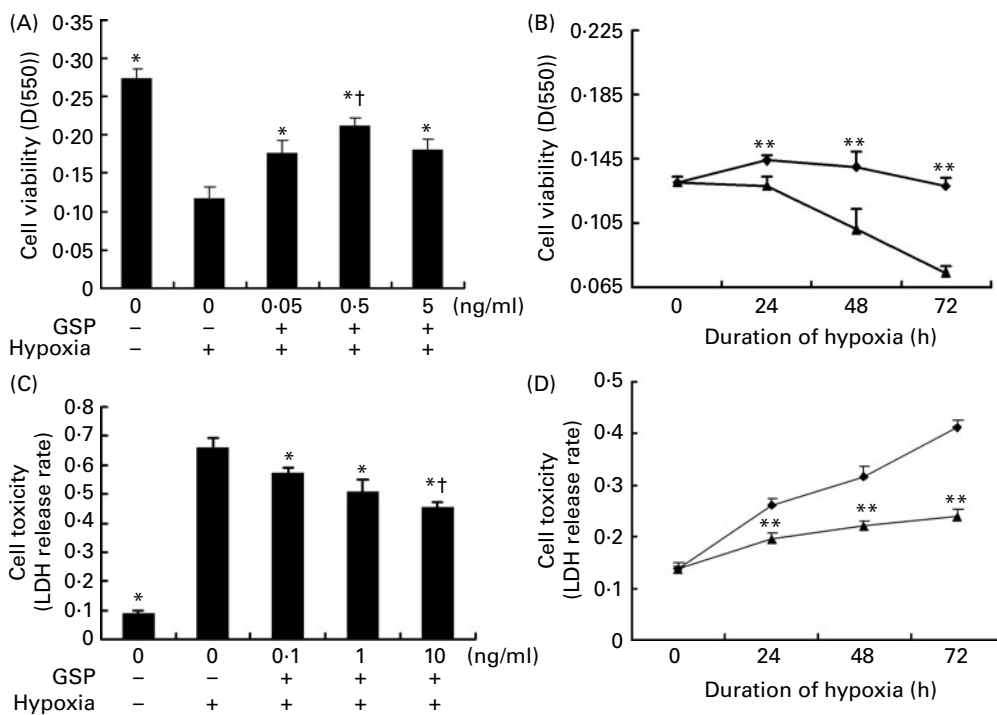


Fig. 1. Protective effects of garlic saponins (GSP) on hypoxia-induced toxicity in differentiated PC12 cells. Cells treated with GSP were incubated in normoxia for 3h before exposure to hypoxia. Cell viability was assessed by measuring 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide reduction ((A) and (B)). Cell toxicity was assessed by the lactate dehydrogenase (LDH) release rate ((C) and (D)). (A) The viability of cells treated with 5×10^{-2} –5 ng/ml GSP with 36h of hypoxic exposure. (B) The viability of cells incubated in hypoxia for 0, 24, 48 or 72h. (C) LDH release rate of cells treated with 0.1–10 ng/ml GSP with 48h of hypoxic exposure. (D) LDH release rate of cells incubated in hypoxia for 0, 24, 48 or 72h. Values are means, with their standard errors represented by vertical bars (*n* 5); * *P* < 0.01 v. hypoxia control; † *P* < 0.05 v. the other GSP groups. ** *P* < 0.05 v. the control at the same time point. (B) —◆—, GSP (10 ng/ml); —▲—, control; (D) —◆—, control; —▲—, GSP (10 ng/ml).

at increasing cell viability (Fig. 1(A)). The hypoxic dPC12 cells showed decreasing cell viability at 24, 48 and 72 h, while the cells treated with GSP showed an increase in the rate of cell viability of 12.5, 38.6 and 73%, respectively, compared with the control (Fig. 1(B)).

Treatment with GSP (0.1–10 ng/ml) attenuated LDH leakage of hypoxic dPC12 cells, which was increased by hypoxia due to cell damage (Fig. 1(C)). Moreover, the LDH release rate of cells treated with GSP decreased 25.4, 30.6 and 41.6% at 24, 48 and 72 h, respectively, compared with the control (Fig. 1(D)).

Hypoxia caused dramatic changes in the neuronal morphology of dPC12 cells, and many of them lost the characteristic irregular shape with neurite outgrowth and acquired a round appearance with shorter neurites that were reduced or absent. However, cells treated with GSP

maintained a differentiated state and had longer neurites compared with the hypoxic control (Fig. 2(A) and (B)). Moreover, the expression of TUJ1, a neuronal marker protein, was increased in cells treated with GSP compared with the hypoxic control (Fig. 2(C)).

Garlic saponins protect differentiated PC12 cells from oxidative damage induced by hypoxia

Hypoxia treatment for 24 h induced oxidative damage of NGF-deprived dPC12 cells. We observed an increase in intracellular MDA and 8-hydroxy-deoxyguanosine concentrations in the medium due to hypoxia. However, the production of MDA and 8-hydroxy-deoxyguanosine was significantly attenuated by treatment with GSP ($P < 0.01$; Fig. 3). Moreover, the activity and abundance of CAT in

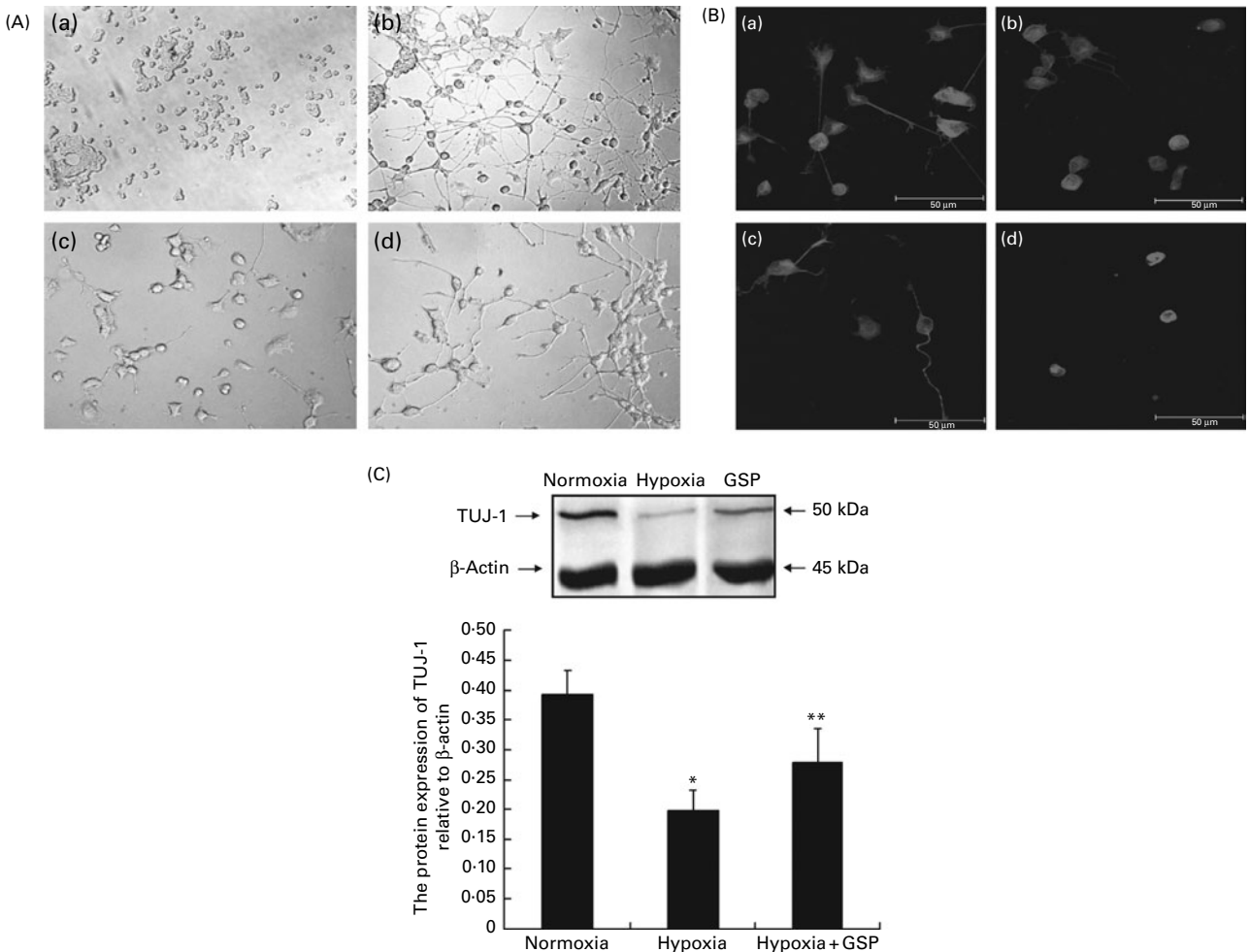


Fig. 2. Effects of garlic saponins (GSP) on the neuronal morphology of hypoxic differentiated PC12 (dPC12) cells. A number of hypoxic cells tended to lose their characteristic shape, acquiring a round appearance and showing shorter, few or even no neurites. However, hypoxic cells treated with GSP maintained the number and length of the neurites. (A) PC12 cell photomicrographs using an Olympus microscope (10 × 32). (a) Native PC12 cells, (b) dPC12 cells, (c) dPC12 cells with 36 h of hypoxic exposure, (d) dPC12 cells treated with GSP (10 ng/ml) and 36 h of hypoxic exposure. (B) Immunofluorescence of neuron-specific class III β-tubulin (TUJ1) in cells using a confocal laser scanning microscope. Cells were stained with an anti-TUJ1 antibody (red fluorescence) for TUJ1 localisation and 4'-6-diamidino-2-phenylindole (blue fluorescence) for nuclear visualisation. The neuronal morphology of hypoxic dPC12 cells was protected, and the expression of TUJ1 was up-regulated by GSP. (a) dPC12 cells treated with GSP (10 ng/ml, 24 h hypoxia), (b) dPC12 cells (24 h hypoxia), (c) dPC12 cells treated with GSP (10 ng/ml, 72 h hypoxia), (d) dPC12 cells (72 h hypoxia). (C) Detection of TUJ1 protein by Western blotting with cell lysate from dPC12 cells under hypoxic conditions for 24 h. Values are means, with their standard errors represented by vertical bars ($n = 4$). * $P < 0.01$ v. normoxia; ** $P < 0.05$ v. normoxia and hypoxia, respectively.

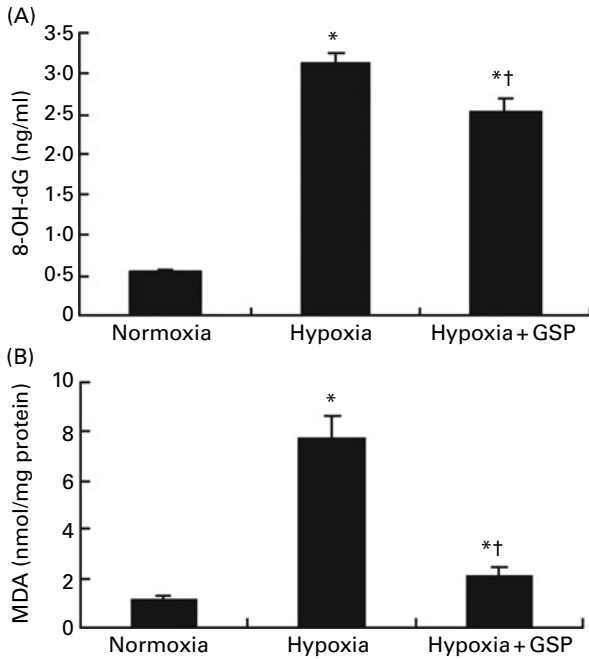


Fig. 3. Protective effects of garlic saponins (GSP, 10 ng/ml) on the oxidative damage of differentiated PC12 cells induced by hypoxia (24 h). (A) Malondialdehyde (MDA) content and (B) 8-hydroxy-deoxyguanosine (8-OH-dG) content. Values are means, with their standard errors represented by vertical bars (n 5). * $P < 0.05$ v. normoxia; † $P < 0.01$ v. hypoxia.

GSP-treated cells were significantly higher than in the corresponding control ($P < 0.01$; Fig. 4). The CAT activity in GSP-treated cells was 5.5 times higher than in the hypoxic control cells. Moreover, treatment with GSP up-regulated the mRNA and protein expression of CAT, which was significantly decreased in the hypoxic control cells ($P < 0.05$; Fig. 4). After exposure to hypoxia for 24 h, the total protein expression of p65 in GSP-treated cells was lower than that in the hypoxic control cells. Moreover, the protein expression of nuclear p65 was also decreased (Fig. 5). GSP had no significant effect on the mRNA expression of p65 in dPC12 cells ($P > 0.05$; data not shown).

Discussion

Hypoxia can cause cell injury and dysfunction⁽¹⁾, which are in part due to the fact that oxidative stress affects the structure and function of proteins⁽¹³⁾, nucleic acids⁽¹⁴⁾ and lipids^(15,17,18). dPC12 cells exposed to 2% O_2 for 24–72 h were dramatically damaged and showed a significant decrease in cell number and viability. In the present study, we demonstrated that GSP had a protective effect against cell injury and death caused by hypoxia. Using MTT and LDH assays, we demonstrated that GSP protected cell viability under hypoxic conditions. In addition, hypoxia has been shown to decrease the expression of neuronal marker genes in PC12 cells⁽³⁵⁾. Here, we also found that 2% O_2 decreased the protein expression of the neuronal cell marker TUJ1; however, GSP attenuated this decrease.

These results indicated that GSP has protective effects against hypoxia-induced damage of dPC12 cells.

Excessive ROS induced by hypoxia are extremely reactive and can cause molecular oxidative damage and subsequent cell death. MDA, an oxidative degradation product of cell membrane lipids, is generally considered as an indicator of lipid peroxidation^(36–38). The oxidised

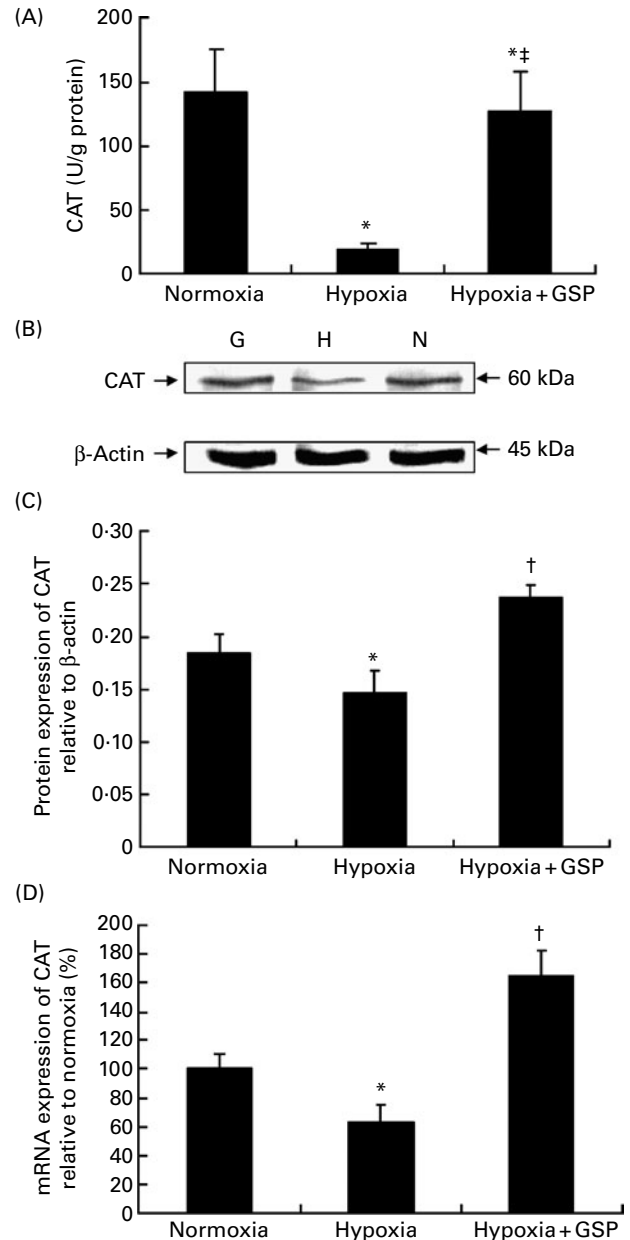


Fig. 4. Garlic saponins (GSP, 10 ng/ml) up-regulate the expression and activity of catalase (CAT) in hypoxic differentiated PC12 cells (24 h hypoxia). Hypoxia decreased the expression and activity of CAT, but GSP treatment markedly ameliorated this effect. (A) CAT activity. (B) Detection of CAT protein by Western blot. (C) The mRNA expression of CAT analysed by real-time RT-PCR. $2^{-\Delta\Delta C_T}$ was calculated to represent the relative value of mRNA, and normoxia was used as the control and assumed to be 100%. Values are means, with their standard errors represented by vertical bars (n 4). * $P < 0.05$ v. normoxia; † $P < 0.01$ v. normoxia or hypoxia; ‡ $P < 0.01$ v. hypoxia.

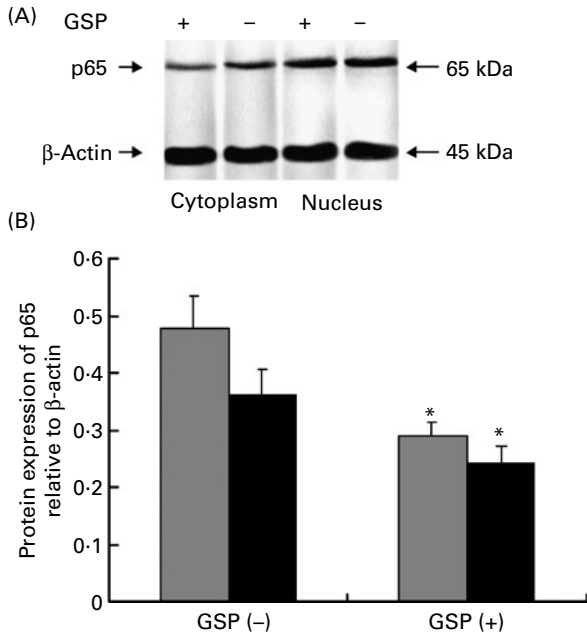


Fig. 5. Garlic saponins (GSP) down-regulate the total and nuclear expression of p65 in hypoxic differentiated PC12 (dPC12) cells. dPC12 cells were pretreated with GSP (10 ng/ml) and exposed to hypoxia for 24 h. (A) Detection of p65 by Western blot. Nuclear and cytoplasmic proteins were extracted from cells after exposure to hypoxia for 24 h. (B) Protein expression of p65 relative to β -actin. The total expression of p65 in hypoxic cells was significantly decreased by GSP, and the expression of nuclear p65 decreased. Values are means with their standard errors represented by vertical bars (n 4). * P <0.01 v. the corresponding GSP (-) group. ■, Cytoplasm; ■, nucleus.

nucleoside 8-OHdG is a frequently detected and extensively studied factor of oxidative DNA damage, since it is the major oxidative lesion that occurs in nuclear and mitochondria DNA and can be detected by a variety of assays^(39–42). In the present study, the production of MDA, LDH leakage and 8-hydroxy-deoxyguanosine was significantly decreased by pretreatment with GSP before hypoxic exposure. These results indicated that the oxidative damage of membrane lipids, membrane structure and DNA of hypoxic cells was inhibited by GSP treatment.

In the present study, we investigated the activity and expression of CAT in dPC12 cells. CAT activity in dPC12 cells decreased due to hypoxia-induced oxidative stress. We demonstrated that treatment with GSP significantly increased the activity and expression of CAT in hypoxic cells. It is known that CAT may decompose toxic H_2O_2 , which not only oxidises proteins, nucleic acids and lipids, but also modulates several proteins including the NF- κ B p65 signalling pathway. Antioxidant enzymes provide important protective mechanisms against oxidative damage. CAT is one of the most active catalysts produced in nature. At high substrate concentrations, CAT decomposes toxic H_2O_2 at an extremely rapid rate in a catalytic reaction, whereby H_2O_2 acts as both an acceptor and donor of hydrogen molecules in the equation $2H_2O_2 \rightarrow 2H_2O + O_2$. Moreover, CAT is unique among H_2O_2 scavenger enzymes, in that it degrades H_2O_2 without consuming

cellular reducing equivalents. It has been proposed that CAT may be uniquely suited to regulate the homeostasis of H_2O_2 in the cell.

It has been previously shown that oxidants can induce NF- κ B activation and concomitantly increase the translocation of p65 to the nucleus in PC12 cells, where intracellular ROS accumulates and oxidative damage subsequently occurs⁽⁴³⁾. ROS, primarily H_2O_2 , can alter the protein redox status and modulate several signalling pathways. H_2O_2 activates p38MAPK and other MAPK, such as extracellular signal-regulated kinase 1/2, which activates transcription factors including NF- κ B. The present study showed that hypoxia can activate NF- κ B and increase its nuclear translocation in PC12 cells, which is consistent with previous findings in other cell types^(44–48). Moreover, p38 or extracellular signal-regulated kinase 1/2 MAPK was activated in hypoxic PC12 or other cell types^(49,50). Therefore, ROS may be an important signalling pathway for NF- κ B activation in hypoxic PC12 cells. Recently, Oliver *et al.*⁽⁵¹⁾ has shown that both continuous and intermittent hypoxia activate NF- κ B through the p65 signalling pathway, rather than through the non-canonical pathway. On the other hand, pretreatment with classical antioxidants has been shown to prevent the H_2O_2 -induced activation of MAPK^(52–54). Overexpression of the CAT gene also causes a decrease in intracellular H_2O_2 levels and down-regulates NF- κ B activation⁽⁵⁵⁾. In the present study, we also found that the total and nuclear expression of NF- κ B p65 in hypoxic cells was decreased by the treatment with GSP. NF- κ B is composed of five homo- and heterodimers of proteins containing a Rel homology domain in mammalian cells. p65 (RelA), one of the predominant NF- κ B isoforms, has a transactivation domain at the C-terminus. Since p65 is responsible for the transcriptional activity of NF- κ B, it has been extensively studied. In unstimulated cells, the NF- κ B dimers are sequestered in the cytoplasm by I κ B⁽⁵⁶⁾. When I κ B becomes phosphorylated, NF- κ B is activated and the p65 subunit is then free to enter the nucleus, where it acts as a transcription factor and regulates the expression of specific genes. In mammalian systems, NF- κ B is not only involved in the regulation of the oxidative stress response, but also plays a central role in regulating normal cellular growth and metabolism. Moreover, NF- κ B activation can lead to apoptosis or necrosis of cells under oxidative stress conditions.

In addition, the hydroxylases that confer oxygen sensitivity to the hypoxia-inducible factor pathway may also play a role in oxygen sensing in the NF- κ B pathway. Prolyl hydroxylases appear to repress the p65 pathway through mechanisms that may include the direct hydroxylation of I κ B kinase β ⁽⁵⁷⁾. Furthermore, the asparaginyl hydroxylase factor inhibiting the hypoxia-inducible factor has been shown to hydroxylate I κ B α ⁽⁵⁸⁾. Hydroxylase activity is attenuated by an increase in ROS, which is generated during the hypoxic stress response. Therefore, we hypothesise that a decrease in the inhibition of

hydroxylase activity induced by hypoxia may be another mechanism for the GSP-mediated effects on the p65 signalling pathway.

The expression of most antioxidant enzymes, including CAT, is tightly controlled by the antioxidant response element (ARE) and is activated by nuclear factor E2-related factor 2 (Nrf2)⁽⁵⁹⁾. ARE is present in the promoter region of mammalian glutathione S-transferase, metallothionein-I, MnSod and CAT genes, and causes the induction of these genes in response to oxidants. Nrf2 is the transcription factor that binds to the ARE. The protective effects of Nrf2 activation in reducing oxidative stress in both *in vitro* and *in vivo* models of neuron damage have been described in several studies⁽⁵⁹⁾. Other studies have reported that the oxidative damage induced by H₂O₂ or mitochondrial toxins can be protected by Nrf2/ARE activation in PC12 cells^(60,61). Therefore, we hypothesise that Nrf2/ARE activation may be an important pathway for the induction of CAT expression that is induced by GSP treatment. GSP may not only act as an antioxidant for the direct elimination of ROS, but also may act as a signalling molecule for the activation of Nrf2/ARE. Further study will be needed to explore the mechanisms of the protective effects of GSP in hypoxic dPC12 cells.

The present study suggests that GSP may act as an antioxidant to protect dPC12 cells from the direct damage of hypoxia-induced ROS or may mediate protective effects through redox-sensitive signalling pathways mediated by ROS. Moreover, the GSP-mediated increase in CAT activity, which may act by decreasing the production of ROS or indirectly regulating the expression of p65, may be an important mechanism for the protective effects of GSP on hypoxic dPC12 cells. In conclusion, GSP can protect dPC12 cells against hypoxia through an antioxidant effect, which is in part due to an up-regulation of the activity and expression of CAT and a decrease in the expression and nuclear distribution of p65.

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