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(11–4).

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(15–16). Moreover, ROS may serve as messengers for the activation of adaptive responses through redox-sensitive signalling pathways, such as NF-κB and mitogen-activated protein kinases.
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Materials and methods
Preparation of garlic saponins

An improved method was used for saponin extraction based on the literature. GSP was extracted from dried garlic powder by soaking the powder in 65 % aqueous alcohol for 72h. 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 acetocetate 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 CHCl₃–methanol–water (10:7:2 by vol., 4°C). The saponins were visualised by incubation of the plate with 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 % fetal bovine serum (HyClone, Logan, UT, USA), 10 % horse serum (Invitrogen), 100 units/ml penicillin and 80 µg/ml gentamicin at 37°C in a humidified atmosphere with 5 % CO₂ (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-B (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 3h. The cells were then quickly placed into a multi-gas incubator (2 % O₂ (v/v), 5 % CO₂ (v/v) and 93 % N₂ (v/v); Nuaire, Plymouth, MN, USA), which maintains a precise O₂ concentration (1–21 %) by injecting N₂ and CO₂ 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 µl/well media containing NGF-B at a density of 2 × 10⁴ 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 µl 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 650 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. At the end of...
the hypoxia exposure time, 100 μl 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 × 10 mm cover glass placed in a twenty-four-well plate at a concentration of 5 × 10^5 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 μg/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 method based on a previous study (30).

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 μg 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 μmol/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 CGC GAG TT-3′; β-actin forward, 5′-AGT GTG AGC TTG ACA TCC GTA-3′; β-actin reverse, 5′-GCC AGA GCA GFA 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^-ΔΔC_T method based on a previous study (30).
Malondialdehyde content determination

Cells were collected after being exposed to hypoxia for 24 h. 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 24 h, and then the supernatant was immediately collected for an ELISA. The supernatant was centrifuged (1000 g) for 20 min 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 36 h, 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.

![Graphs showing the protective effects of garlic saponins (GSP) on hypoxia-induced toxicity in differentiated PC12 cells.](image-url)

**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 36 h of hypoxic exposure. (B) The viability of cells incubated in hypoxia for 0, 24, 48 or 72 h. (C) LDH release rate of cells treated with 0·1–10 ng/ml GSP with 48 h of hypoxic exposure. (D) LDH release rate of cells incubated in hypoxia for 0, 24, 48 or 72 h. 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) – control, GSP (10 ng/ml); – control, GSP (10 ng/ml).
at increasing cell viability (Fig. 1(A)). The hypoxic dPC12 cells showed decreasing cell viability at 24, 48 and 72h, 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 72h, 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](https://www.cambridge.org/core/terms.https://doi.org/10.1017/S0007114510004939)
GSP-treated cells were significantly higher than in the corresponding control \((P < 0.01; \text{Fig. 4})\). 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 \((P < 0.05; \text{Fig. 4})\). 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 \((P > 0.05; \text{data not shown})\).

**Discussion**

Hypoxia can cause cell injury and dysfunction\(^{(3)}\), 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% \(\text{O}_2\) 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% \(\text{O}_2\) 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...
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. 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 H2O2, which not only oxidises proteins, nucleic acids and lipids, but also modulates several proteins including the NF-κB activation. Moreover, it has been shown to prevent the H2O2-induced activation of MAPK and other MAPK, such as extracellular signal-regulated kinase 1/2, which activates transcription factors including p38 MAPK and other MAPK, such as extracellular signal-regulated kinase 1/2, which activates transcription factors including NF-κB. The present study showed that hypoxia can activate NF-κB and increase its nuclear translocation in PC12 cells, which is consistent with previous findings in other cell types. Moreover, p38 or extracellular signal-regulated kinase 1/2 MAPK was activated in hypoxic PC12 or other cell types. Therefore, ROS may be an important signalling pathway for NF-κB activation in hypoxic PC12 cells. Recently, Oliver et al. has shown that both continuous and intermittent hypoxia activate NF-κB through the p65 signalling pathway, rather than through the non-canonical pathway. On the other hand, pretreatment with classical antioxidants has been shown to decrease the H2O2-induced activation of MAPK. Overexpression of the CAT gene also causes a decrease in intracellular H2O2 levels and downregulates NF-κB activation. In the present study, we also found that the total and nuclear expression of NF-κB p65 in hypoxic cells was decreased by the treatment with GSP. NF-κB is composed of five homo- and heterodimers of proteins containing a Rel homology domain in mammalian cells. p65 (RelA), one of the predominant NF-κB isoforms, has a transactivation domain at the C-terminus. Since p65 is responsible for the transcriptional activity of NF-κB, it has been extensively studied. In unstimulated cells, the NF-κB dimers are sequestered in the cytoplasm by IκB. When IκB becomes phosphorylated, NF-κB 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-κB 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-κB 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-κB pathway. Prolyl hydroxylases appear to repress the p65 pathway through mechanisms that may include the direct hydroxylation of IκB kinase β. Furthermore, the asparaginyl hydroxylase factor inhibiting the hypoxia-inducible factor has been shown to hydroxylate IκBα. 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
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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 \(\text{H}_2\text{O}_2\) 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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References
