Original Article

Protective role and related mechanism of Gnaq in neural cells damaged by oxidative stress

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Received 24 October 2016; Editorial Decision 20 February 2017

Abstract

Gnaq is a member of G protein family and is rich in brain tissue. It has attracted the attention of many researchers in melanoma due to its high ratio of mutation. We have previously reported that the expression level of Gnaq in the mouse forebrain cortex was significantly decreased with age. Oxidative stress (OS) is the main cause leading to brain aging and related diseases. The roles and mechanisms of Gnaq in antioxidation in the brain have not been fully explored. In the present study, gene recombinant technique and lentivirus transfection technique were used to generate a Gnaq-overexpression cell model (Gnaq-SY5Y) coupled with H2O2 to build an OS model. The viability of cells, concentration of reactive oxygen species (ROS), apoptosis-related proteins (Bcl-2 and Bax), and signal pathways (NF-κB and Erk1/2) were compared between model cells and control cells. Results showed that the antioxidative ability of Gnaq-SY5Y cells was significantly improved. Concomitantly, the ROS level in Gnaq-SY5Y cells was significantly decreased whether the cells were subject to or not to H2O2 treatment. Anti-apoptotic protein Bcl-2 was up-regulated and apoptosis-promoting protein Bax was down-regulated in Gnaq-SY5Y cells after treatment with H2O2. NF-κB and phosphorylated Erk1/2 (p-Erk1/2) were significantly down-regulated in Gnaq-SY5Y cells. H2O2 treatment decreased Gnaq expression but increased NF-κB and p-Erk1/2 expressions in Gnaq-SY5Y cells. It is therefore concluded that Gnaq plays a pivotal role in antioxidation in neural cells. A possible mechanism for this would be that the overexpressed Gnaq inhibits the cellular damaging effect mediated by NF-κB and Erk1/2 signal pathways.

Key words: Gnaq, overexpression, SY5Y cell line, oxidative stress, antioxidation

Introduction

Population aging is becoming a more and more serious society problem; indeed, age-related diseases have been the focus of many studies in recent years. It is well recognized that among the common neurodegenerative diseases, Alzheimer’s disease and Parkinson’s disease are closely related to aging [1]. One of the mechanisms of aging is the well-known Oxidative Radical Imbalance Theory. Under normal circumstances, the production and removal of the reactive oxygen species (ROS) are well maintained and in balance. Once the balance is broken, too much ROS is generated or ROS can not be removed in time, oxidative stress (OS) will occur in organisms, which would result in irreversible damage [2]. It has been reported that most
brain-aging-related neurodegenerative diseases are due to OS damage [3].

ROS includes singlet oxygen, O$_3^-$, -OH, H$_2$O$_2$, NO, and O$_2^-$, most of which are toxic to cells. In OS state, ROS would damage cellular membrane, reduce the activity of proteinase, and lead to mutations of DNA. Furthermore, lipid peroxidation produced by reaction between oxygen radicals and phospholipids of cellular membranes would affect all the cells throughout the body, which would accelerate the processes of aging and related geriatric diseases [4]. Many cellular factors and signal proteins are involved in the regulation of ROS metabolism, among which the nuclear transcription factor kappa B (NF-κB) [5–7] and extracellular signal regulated kinase1/2 (Erk1/2) [8] play important roles. NF-κB belongs to the Rel protein family. It is a dipolymer composed by two subunits p50 and p65. NF-κB p50/p65 locates in the cytoplasm in an inactive tri-polymer format by combining with IκB. Once the cell is stimulated by a proper signal, IκB degrades after phosphorylation so that NF-κB p50/p65 is activated. The activated p50/p65 is immediately translocated into the nucleus and combines to DNA to modulate the target gene [9]. Erk1/2 is a member of the mitogen activated protein kinase (MAPK) family. Erk1/2 is ubiquitous in various tissues and cells and plays important roles in many cell processes, such as proliferation, survival, migration, and apoptosis [10,11]. Previous studies have shown that NF-κB and Erk1/2 signal pathways are activated in OS and that both pathways are involved in mediating neuronal injury caused by OS [12,13].

Gnaq is a member of the guanine nucleotide binding protein (G protein) α subunits and has been reported to be rich in the brain tissue. Mice whose Gnaq gene was knocked out showed serious dysfunctions affecting the nervous system and endocrine system [14]. Very interestingly, 50% mutation of Gnaq gene was detected in uveal melanoma [15]. Mutation of Gnaq results in the defect of its GTPase activity, so the G protein is maintained in a continuously activated state, and through the cascade reactions mediated by protein kinase C (PKC), the MAPK signal pathway is constantly initiated, leading to abnormal proliferation of normal cells and enhanced capacity of growth and antiapoptosis of cancer cells [16]. We previously reported that the expression level of Gnaq in SAMP8 mouse forebrain cortex was significantly decreased with age, alluding to the possibility that Gnaq expression may be closely associated with brain aging [17]. However, the antioxidative function and corresponding mechanisms of Gnaq in the brain still remain elusive. In the present study, gene recombinant technique and lentivirus transfection technique were used to construct a Gnaq-overexpression cell model using SY5Y cells. Furthermore, SY5Y cells were directly treated with H$_2$O$_2$ to build an OS model. Subsequently, the viability of cells, concentration of ROS, and related signaling pathways (NF-κB, Erk1/2) were detected and analyzed to determine the role and mechanism of Gnaq in antioxidation in neurons. It is hoped that the results would provide the cellular and molecular basis for designing of new strategies for the prevention and treatment of aging-related brain diseases.

**Materials and Methods**

**Cell culture**

Human neuroblastoma cell line SH-SY5Y was obtained from American Type Culture Collection (Rockville, USA) and cultured in DMEM/F12 (Giboc, Gaithersburg, USA) containing 10% FBS (Giboc) in an incubator with 5% CO$_2$ at 37°C.

**Gnaq-SY5Y and Vector-SY5Y cell line construction**

The Gnaq overexpression SY5Y cell line (Gnaq-SY5Y) and control cell line (Vector-SY5Y) were constructed by Hanbio Co. Ltd. (Shanghai, China). In brief, the whole codon sequence of Gnaq (NM_002072.4) was amplified by PCR with the following primers: forward 5'-AATCTCGAGATGACTGAGTTGCTCCATCAT-3', reverse 5'-ATAGCGGCGCGTCTAGACGATTGTACT-3'. The PCR product was cloned into lentivirus vector pHBLV-RES-ZsGreen-PK-puro (pHBLV) between Xbol and Norl sites. The sequence integrity was verified by sequencing. The pseudotyped lentiviruses were produced by cotransfecting 293 T cells with pHBLV-Gnaq or pHBLV together with two other packaging vectors: pspx2 and pMD2G. SY5Y cells in 6-well plates were transduced with lentivirus (MOI = 30) and 8 μg/ml polybrene (Sigma, St Louis, USA) and medium was renewed after 24 h. Full medium with puro was used to select the target cells. PCR and western blot analysis were used to detect the overexpressing efficiency of Gnaq gene.

**Cell viability**

Cell viability was detected by MTT assay. SY5Y, Gnaq-SY5Y and Vector-SY5Y cells were respectively seeded in a 96-well plate at 2 × 10$^4$ cells/well. Twelve hours after seeding, cells were incubated with different concentrations (50, 100, 200, and 400 μM) of H$_2$O$_2$ (Sigma) for 0.5, 1, 2, or 4 h. Subsequently, 10 μl of MTT stock solution (5 mg/ml) was added to each well. After 4 h of incubation, the supernatants were carefully aspirated and 100 μl dimethyl sulfoxide (DMSO) was added into each well. Plates were covered by tinfoil and gently shaken for 10 min. The absorbance (540 nm) was determined using a Microplate Reader (Thermo, Waltham, USA).

**Intracellular ROS measurement**

The dihydroethidium (DHE) method was used to determine the level of intracellular ROS. Gnaq-SY5Y and Vector-SY5Y cells were seeded in a 6-well plate at 3 × 10$^5$ cells/well. Twelve hours after seeding, cells were incubated with H$_2$O$_2$ (200 μM) for 4 h at 37°C. After the addition of 10 μM DHE solution, the reaction was allowed to proceed for 60 min in the dark, then the fluorescence was detected using DM LS2 microscope (Leica, Solms, Germany) and flow cytometry (BD, Franklin Lakes, USA).

**Apoptosis assay**

Hoechst33342/PI apoptosis assay kit was purchased from Beijing Leagene Biotechnology Co., Ltd. (Beijing, China). Experiments were performed according to the manufacturer’s protocols. In brief, Vector-SY5Y cells and Gnaq-SY5Y cells were seeded in 6-well plates in a concentration of 0.5 × 10$^4$ cells/ml. Twelve hours after seeding, cells were treated with 200 μM H$_2$O$_2$ for 4 h, and control cells were treated with the same volume of PBS. Then, medium was aspirated and replaced by reaction buffers (cell stain buffer 900 μl, Hoechst 33342 stain 5 μl, and PI stain 5 μl for each well). After 30 min of staining in the dark, the reaction buffers were discarded and cells were washed with PBS, then the fluorescence was detected using DM LS2 microscope (Leica) at the wavelength of 450 nm.

**RNA extraction and PCR analysis**

Cells cultured to 70%–80% confluence were collected and total RNA was prepared by using Trizol reagent (Invitrogen, Carlsbad, USA). A total of 2 μg RNA were used to generate cDNA by using the SuperScript™ III reverse transcriptase (Invitrogen). The cDNAs...
were amplified by using GoTaq® DNA polymerase (Promega, Madison, USA). Primers were designed and synthesized by Hanbio Co. Ltd.: Gnaq 5′-AGAAGTTGATGGAGAAGG-3′ (forward), 5′-GGACTGAATCTAAAGG-3′ (reverse); GAPDH 5′-TGCC TCTGACACCAACT-3′ (forward), 5′-CCGGTTCAGCTCA GGGATGA-3′ (reverse).

Protein extraction and western blot assay
Cells cultured to 70%–80% confluence were harvested and lysed in RIPA buffer (Solarbio, Beijing, China). The lysates were centrifuged at 14,000 g at 4°C for 15 min and the supernatant was collected. Protein concentrations were determined by Bradford assay (Bio-Rad, Hercules, USA). Expressions of Gnaq, Bcl-2, Bax, NF-κB/p65, Erk1/2, and phosphorylated Erk1/2 were determined by western blot analysis. Briefly, total protein (20 μg) of each sample was electrophoresed by Tris-glycine SDS-PAGE. Then target proteins were transferred to PVDF membranes (Millipore, Boston, USA) which were blocked with 5% skim milk for 3 h. Then, membranes were incubated for 8 h or overnight with the appropriate dilutions of the primary antibodies (CST, Boston, USA) obtained from Cell Signaling Technology (Beverly, USA). After being properly rinsed, PVDF membranes were reacted with the HRP-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, USA), followed by detection with the ECL kit (Kodak, New York, USA). Images were captured by AI600 system and gray levels were measured by ImageJ software (NIH, Bethesda, USA).

Statistical analysis
Statistical differences between groups were analyzed by using one-way ANOVA (SPSS 17.0, Chicago, USA). Data were presented as the mean ± SD. The P value < 0.05 was regarded as statistically significant.

Results
Gnaq was stably overexpressed in SY5Y cells by gene recombinant engineering and lentivirus transfection
By lentiviral-transfection technique and puro-selection process, qualified Gnaq-SY5Y model cell line and Vector-SY5Y control cell line were obtained, in which all the cells stably expressed green fluorescent protein (GFP), the gene of which was recombined into the lentivectors in advance (Fig. 1A). PCR and western blot analysis showed that Gnaq gene was successfully overexpressed in SY5Y cells (Fig. 1B,C).

Overexpression of Gnaq significantly improved antioxidative as well as anti-apoptotic abilities of SY5Y cells
MTT assay showed that the amount of survived cells of SY5Y (control) and Vector-SY5Y cells, instead of Gnaq-SY5Y cells, decreased significantly with the increase of H2O2 concentration (Fig. 2A). When treated with 200 μM of H2O2 for 0.5, 1, 2, and 4 h, the amount of survived cells of SY5Y (control) and Vector-SY5Y cells, instead of Gnaq-SY5Y cells, decreased significantly (Fig. 2B). Hoechst33342/PI apoptotic assay also showed that more apoptosis was induced in Vector-SY5Y cells than that in Gnaq-SY5Y cells after being treated with 200 μM H2O2 for 4 h (Fig. 2C).

Overexpression of Gnaq significantly decreased ROS level in SY5Y cells with or without H2O2 treatment
Dihydroethidium (DHE) labeled intracellular ROS determined by fluorescence microscopy showed that the basic amount of ROS in Gnaq-SY5Y cells was lower than that in Vector-SY5Y cells (Fig. 3A; the red of panel H was dimmer than panel B, meanwhile, the blue of panel I was brighter than panel C). After stimulation with H2O2, the level of ROS in Gnaq-SY5Y cells was also lower than that in Vector-SY5Y cells (Fig. 3A; the red of panel K was dimmer than panel E, and the blue of panel L was brighter than panel F). Flow cytometry analysis also demonstrated that the level of ROS in Gnaq-SY5Y cells were significantly lower than that in Vector-SY5Y cells after H2O2 stimulation (Fig. 4).

Expression levels of signal proteins NF-κB/p65, p-Erk1/2, and apoptosis-related proteins Bcl-2, Bax were significantly changed after overexpression of Gnaq in SY5Y cells and additional stimulation with H2O2
Western blot results showed that the Gnaq expression level was very low in Vector-SY5Y cells, but much stronger in Gnaq-SY5Y cells,
cells, instead of Gnaq-SY5Y cells, decreased significantly with the increasing concentrations of H2O2. (B) After treatment with 200 μM of H2O2 for 0.5, 1, 2, and 4 h, the viability of SY5Y (control) and Vector-SY5Y cells, instead of Gnaq-SY5Y cells, decreased significantly (*P < 0.01 compared with control and vector group, n = 3). (C) Apoptosis assay with Hoechst and PI staining showed that more apoptotic cells (cells with bright blue nuclear) were induced in Vector-SY5Y cells than in Gnaq-SY5Y cells after H2O2 treatment. Most importantly, the levels of NF-κB/p65 and p-Erk1/2 were significantly down-regulated in Gnaq-SY5Y cells but significantly up-regulated in Gnaq-SY5Y cells (Fig. 3B). There was no significant change in the Bax expression between Vector-SY5Y cells and Gnaq-SY5Y cells; however, Bax was significantly up-regulated in Gnaq-SY5Y cells and control cell model Vector-SY5Y was successively constructed by gene recombination and lentivirus transfection techniques. Here we provided strong experimental evidence demonstrating that Gnaq can protect SY5Y cells from oxidative damage.

Aerobic cells are known to produce a variety of ROS during their metabolic processes. A proper concentration of ROS acts as signal molecules and plays important roles in the activation of transcription factors, induction of cellular proliferation and differentiation [20]. However, abnormal high concentration of ROS can lead to OS, which would result in cell apoptosis, autophagy and necrosis [21]. The aerobic metabolism level of cells in vitro mostly depends on their proliferation speed. The faster the proliferation, the higher the aerobic metabolism level of cells

Discussion

The SY5Y cells, which were from human neuroblastoma, have the morphological, physiological, and biochemical characteristics of normal neurons. The cells which emit obvious extending processes and can produce and secrete the neurotransmitter dopamine, have been extensively used as cell models for the investigation of neurodegenerative diseases such as Parkinson’s disease [18,19]. In the present study, the stable Gnaq-overexpression cell model Gnaq-SY5Y and control cell model Vector-SY5Y were successively constructed by gene recombination and lentivirus transfection techniques. Here we provided strong experimental evidence demonstrating that Gnaq can protect SY5Y cells from oxidative damage.

OS occurs when cells generate and accumulate excess ROS or cells suffer from extracellular stimulation (such as treated with H2O2). With the imbalance between oxidation and antioxidation, many undesirable oxidative products are produced, which conceivably would weaken the cells. There are two antioxidative systems in the organism: enzymatic and non-enzymatic [23]. The enzymatic antioxidative system includes superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px). The non-enzymatic

Figure 2. Gnaq-overexpressing SY5Y cells had stronger ability against antioxidative damage than the control cells  (A) The viability of SY5Y (control) and Vector-SY5Y cells, instead of Gnaq-SY5Y cells, decreased significantly with the increasing concentrations of H2O2. (B) After treatment with 200 μM of H2O2 for 0.5, 1, 2, and 4 h, the viability of SY5Y (control) and Vector-SY5Y cells, instead of Gnaq-SY5Y cells, decreased significantly (*P < 0.01 compared with control and vector group, n = 3). (C) Apoptosis assay with Hoechst and PI staining showed that more apoptotic cells (cells with bright blue nuclear) were induced in Vector-SY5Y cells than in Gnaq-SY5Y cells after 4 h of treatment with 200 μM H2O2. Images were the representatives from three independent experiments.

Figure 3. ROS levels in cells detected by fluorescent microscopy  (A, D, G, J) Images of cells without initiation of fluorescent. (B, E, H, K) Red fluorescence was produced by oxidized DHE. The stronger red fluorescence represents the higher level of ROS in cells, and the results were H < B, and K < E. (C, F, I, L) Blue fluorescence was produced by non-oxidized DHE. The stronger blue fluorescence represents lower level of ROS in cells, and the results were I > C and L > F. Images were representatives from three independent experiments (magnification ×200, scale bar: 75 μm).
system includes glutathione (GSH), melatonin and vitamin E. Once OS occurs, these antioxidative systems are initiated to protect the cells \[24\]. In the present study, we found that overexpression of Gnaq protected SY5Y cells from H2O2 treatment, and the ROS level in Gnaq-SY5Y was significantly lower than that in Vector-SY5Y. It was therefore reasoned that there must be a correlation between overexpressed Gnaq and activity of antioxidative systems of the transfected cells.

OS can induce cell apoptosis by several signal pathways \[25\]. In the present study, we found that Vector-SY5Y cells were significantly induced to apoptosis after H2O2 treatment, yet the same phenomenon did not happen in Gnaq-SY5Y cells. Further investigation of apoptosis-related proteins revealed that overexpression of Gnaq in SY5Y cells had no significant influence on the expression levels of Bcl-2 and Bax when cells were in natural states; however, after H2O2 treatment, Bcl-2 was up-regulated and Bax was down-regulated significantly in Gnaq-SY5Y cells and the opposite effects were observed in Vector-SY5Y cells. The above results implicated that overexpression of Gnaq could protect neural cells from apoptosis induced by OS through increasing Bcl-2 and decreasing Bax. Bcl-2 is a verified anti-apoptotic protein and Bax can promote apoptotic process. Both factors are regulated by NF-kB and MAPK/Erk signal pathways \[26,27\].

Figure 4. ROS level detected by flow cytometry Results showed that ROS level in Gnaq-SY5Y cells were significantly lower than that in Vector-SY5Y cells. After treatment with H2O2 for 4 h, ROS level increased in both Vector-SY5Y and Gnaq-SY5Y cells; however, the increase in Gnaq-SY5Y cells was significantly lower than that in Vector-SY5Y cells. Images were representatives from three independent experiments, and different letters of a/b/c/d above the bar indicated that there was significant difference between each two groups. \(P < 0.05, n = 3\).
NF-κB signal pathway is activated when the OS occurs [28]. The dipolymer of NF-κB/p65 in the cytoplasm is separated from its inhibitor protein IκB. When activated, NF-κB/p65 translocates into the nucleus to bind with the promoter domain or enhancer domain of target genes, which would be transcripted to mediate apoptosis and autophagy of cells [29]. When cells are stimulated by OS, the Erk1/2 is phosphorylated to form p-Erk1/2 to induce cells apoptosis. Here, our results showed that overexpression of Gnaq significantly down-regulated the expressions of both NF-κB/p65 and p-Erk1/2 in SY5Y cells. After stimulation with H2O2, the NF-κB expression level was partly re-upregulated and Erk1/2 signal pathways were activated in SY5Y cells. It was reported that ERK was overactivated in Alzheimer’s disease and decrease in the activation of ERK could reverse early memory deficit [30]. In this study, we found that the G protein β and γ subunits were not overexpressed together with Gnaq. Most of the overexpressed Gnaq would not exist as tripolymer. The results that the NF-κB p50/p65 and p-Erk1/2 were down-regulated, instead of being up-regulated by overexpressed Gnaq, tended to suggest that NF-κB and Erk1/2 signal pathways were not regulated by the above-mentioned classical G protein signal pathway. The exact mechanism by which Gnaq regulates NF-κB and Erk1/2 awaits further investigation.

In conclusion, we have demonstrated that overexpression of Gnaq protected neural cells from damage of OS, which is the main cause leading to aging and aging-associated diseases. Thus, through reducing the basic ROS level and attenuating the ROS increase after OS, as well as up-regulating Bcl-2 and down-regulating Bax, should be the protective effect of Gnaq through mediating NF-κB and Erk1/2 signal pathways.

The G protein α subunit Gnaq exists as tripolymer together with β and γ subunits, and keeps in an inactive state when bound to GDP. When G protein coupled receptors (GPCR) is stimulated by hormones such as norepinephrine and antidiuretic hormone, Gnaq is activated to initiate phospholipidase C (PLC), then the second messengers including diacylglycerol (DAG), inositol-1,4,5-triphosphate (IP3) and Ca²⁺ are produced to activate protein kinase C (PKC) which regulates cell physiological processes by phosphorylating corresponding target proteins including NF-κB and Erk1/2 [31,32]. In the present study, we found that the G protein β and γ subunits were not overexpressed together with Gnaq. Most of the overexpressed Gnaq would not exist as tripolymer. The results that the NF-κB p50/p65 and p-Erk1/2 were down-regulated, instead of being up-regulated by overexpressed Gnaq, tended to suggest that NF-κB and Erk1/2 signal pathways were not regulated by the above-mentioned classical G protein signal pathway. The exact mechanism by which Gnaq regulates NF-κB and Erk1/2 awaits further investigation.

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overexpressed Gnaq has been shown to inhibit the cellular damage aging effect mediated by NF-κB and Erk1/2 signal pathways. We have also shown that overexpressed Gnaq depresses the expressions of NF-κB p50/p65 and p-Erk1/2. Gnaq expression is tissue-specific and has been found to be rich in nervous system. Therefore, exploration and designing of a Gnaq expression enhancer may be a potential therapeutic strategy for the prevention and treatment of ageing-related diseases in which OS or ROS and the above-mentioned pathways are implicated.

**Funding**

This work was supported by the grants from the National Natural Science Foundation of China (Nos. 81260195 and 81302362) and the Special-Combined-Funds between the Department of Science-technology of Yunnan Province and Kunming Medical University (No. 2014FB004).

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