Elevation of gene expression of Btg2, Gadd 153, and antioxidant markers in RONS-induced PC12 cells

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Abstract

Background: Free radicals generated in the biological system bring about modifications in biological molecules causing damage to their structure and function. Identifying the damage caused by ROS and RNS is important to predict the pathway of apoptosis due to stress in PC12 cells. The first defense mechanisms against them are antioxidants which act in various pathways through important cellular organelles like the mitochondria and endoplasmic reticulum. Specific biomarkers like Gadd153 which is a marker for endoplasmic reticulum stress, Nrf2 which responds to the redox changes and translocates the antioxidant response elements, and Btg2 which is an antioxidant regulator have not been addressed in different stress conditions previously in PC12 cells. Therefore, the study was conducted to analyze the gene expression pattern (SOD, Catalase, Btg2, Gadd153, and Nrf2) and the protein expression pattern (iNOS and MnSOD) of the antioxidant stress markers in differential stress-induced PC12 cells. Peroxynitrite (1 μM), rotenone (1 μM), H_2O_2 (100 mM), and high glucose (33 mM) were used to induce oxidative and nitrosative stress in PC12 cells.

Results: The results obtained suggested that rotenone-induced PC12 cells showed a significant increase in the expression of catalase, Btg2, and Gadd153 compared to the control. Peroxynitrite-induced PC12 cells showed higher expression of Btg2 compared to the control. H_2O_2 and high glucose showed lesser expression compared to the control in all stress marker genes. In contrast, the Nrf2 gene expression is downregulated in all the stress-induced PC12 cells compared to the control. Further, MnSOD and iNOS protein expression studies suggest that PC12 cells exhibit a selective downregulation. Lower protein expression of MnSOD and iNOS may be resulted due to the mitochondrial dysfunction in peroxynitrite-, high glucose-, and H_2O_2-treated cells, whereas rotenone-induced cells showed lower expression, which could be the result of a dysfunction of the endoplasmic reticulum.

Conclusion: Different stress inducers like rotenone, peroxynitrite, H_2O_2, and high glucose increase the NO and ROS. Btg2 and Gadd153 genes were upregulated in the stress-induced cells, whereas the Nrf2 was significantly downregulated in differential stress-induced PC12 cells. Further, antioxidant marker genes were differentially expressed with different stress inducers.

Keywords: Nitrosative stress, Peroxynitrite, PC12 cells, Rotenone, iNOS, Gadd153, MnSOD

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1 Background

Oxidative stress is a disparity in the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) to the antioxidants produced in the cellular system [1]. ROS and RNS are highly reactive secondary species formed during the aerobic metabolism or in response to stress [2]. Superoxide radicals, hydrogen peroxide, and peroxynitrite are the intermediate products of the metabolic pathway in cellular signaling cascades [3]. For a few decades, ROS and RNS were considered harmful by-products of the aerobic metabolism process of the cell. But, in the last few years, there have been evidences that controlled ROS and RNS generation are responsible for regulating cell signaling and cellular redox homeostasis [4]. ROS are generated in numerous cells, and 90% of ROS are produced by the mitochondrion in the cell. Aerobic respiration generates ROS and RNS as a by-product in the electron transport chain [5]. Mitochondrial ROS were considered as harmful species implied in a range of diseases and pathologies [6], but their importance in the cellular signaling pathway is being addressed and has given new scope to it. RNS induce reactions including nitrosylation of sulfhydryls (S-nitrosylation) or metals and nitration of tyrosine residues. In addition, ROS and RNS chemically or functionally exacerbate harmful effects in the living system [7]. It is known that when RNS production becomes excessive, it results in a deleterious effect on target cells. Thus, excessive production of RONS is the primary reason leading to several degenerative pathologies/diseases [8].

Several oxidative stress markers have been reported previously, Btg2 was primarily said to be an antiproliferative gene. However, it also plays a vital role in the regulation of oxidative stress. In recent years, functions of Btg2 in the nervous system and in other systems are studied extensively. Btg2 gene expression is induced during the neurogenesis process and transiently expressed in rat PC12 cells after the treatment with NGF and also helps in the survival of the PC12 cells during the stress time. Btg2 acts as a transcriptional modulator in various model systems, where their molecular mechanisms are undefined and unclear in most of the systems. Previous studies show that expression of Btg2 was also enhanced in response to stress through p53-dependent mechanisms. Enhanced expressions of epidermal growth factor (EGF) and tumor promoter agent (TPA) or the addition of serum to starved cells have also shown enhanced expression of Btg2 [9]. In stroke-affected neurons, Btg2 is present in the cytoplasm and nucleus; however, the expression of Btg2 in the cytoplasm was upregulated [10]. Further, Btg2 inhibits the apoptotic cell death by blocking the caspases in stress-induced hippocampal neurons. Btg2 is also a nuclear protein, which regulates genetic transcription and inhibition of cell cycle at the G1

checkpoint [11]. ROS/RNS regulates Btg2 expression through the PKC-NFκB pathway [12]. Btg2 is majorly regulated by NFκB under oxidative stress conditions through the ROS NFκB pathways and enhances MnSOD expression during stress conditions, since the prominent role is played by Btg2 in cell proliferation, apoptosis, differentiation, and defense mechanisms [12]. Thus, understanding the role of Btg2 in differentially stress-induced PC12 cells would give insights into their expression pattern in different conditions of oxidative stress.

Gadd153 is a transcriptional factor which is also called CHOP (C/EBP homologous protein). This family of transcription factor regulates different genes which are involved in metabolic processes, immunity, differentiation, proliferation, etc. [13]. It acts as an inhibitor of the C/EBP role and also as an activator of several apoptotic and modulating genes. CHOP is expressed ubiquitously at minimal levels. But it is observed to be expressed in high amounts during stress in different cells. It is localized in the cytosol under non-stressed conditions, but oxidative and genotoxic stress results in inducing CHOP and thus accumulates in the nucleus [14]. Gadd153 is also induced by nutrient depletion, for example, glucose and amino acid depletion. Glucose deprivation induces ER stress by inhibiting N-linked protein glycosylation in the ER. The expression of CHOP is majorly regulated at the transcriptional level. It works as transcriptional factors regulating the genes involved in both survival and death. Overexpression of CHOP plays an important role in the regulation of differentially induced apoptosis pathways and translocation of Bax from the cytosol to the mitochondria [15, 16]. CHOP has a major transactivator ATF4; in its absence, it affects antioxidative genes like glutathione synthesis and also inhibits the amino acid transport [17]. CHOP regulates the ER stress, for instance, long term with minimal ER stress results in apoptosis in β cells through CHOP leading to diabetes [18]. NO induces the depletion of ER Ca^{2+} and induces CHOP in neuronal cultures and also in pancreatic cells [19]. ER stress-mediated apoptosis occurs in the development of Alzheimer's disease and works through CHOP activation. CHOP-mediated apoptosis could also be involved in the progression of Parkinson's disease, polyglutamine disease, and other neurodegenerative diseases [14]. Therefore, the above findings encouraged us to know the effect on gadd153 in differentially stress-induced PC12 cells, which could lead us in understanding ER stress and their effect in different conditions.

Nrf2 is one of the important transcription factors which lead to the expression of the antioxidant responsive element (ARE) during oxidative stress [20]. The Keap-Nrf2 pathway is shown to play a major role in protecting cells from oxidative stress, especially during the

endoplasmic reticulum stress. It regulates the gene expression of detoxification enzymes and antioxidant proteins by binding to an enhancer sequence of the ARE [21]. During stress, activation of the Nrf2 pathway protects the cell from cell death and various neurodegenerative diseases like Alzheimer's disease, Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis which are induced by oxidative stress. GSH is observed in higher amounts and could be the major reason for the protection of cells due to Nrf2 activation [22]. In normal conditions, Nrf2 protein is at low levels due to repeated synthesis and degradation. Keap1 senses the redox change during oxidative stress in the cell. Keap1-dependent ubiquitination is done through three cysteine residues (Cys151, 273, 288) for Nrf2. In stress conditions, electrophiles/molecules modify cysteine residues of Keap1, thus resulting in conformational changes in Keap1 which releases Nrf2. As a result, Nrf2 is released and translocates into the nucleus which binds ARE genes [23]. These reports were encouraged to understand the mechanistic role of Nrf2 in stress-induced PC12 cells.

Oxidative and nitrosative stress leads to changes in physiological, molecular, morphological, and genetic factors in the living system. Certain changes in the gene expression, structure, shape, and function of organelles or the factors/effects of change can be an identity for diseases/pathologies. During stress, some of the main organelles in the system, namely the endoplasmic reticulum and mitochondria, attempt to resist the damage caused by oxidative stress. They can produce or lead any secondary molecules to produce antioxidants against the free radicals by neutralizing/diminishing their effects caused in the cellular system. Specific biomarkers like Gadd153 which indicate the ER stress, Btg2 which is a multirole protein, and Nrf2 which responds to the redox change and activates the ARE genes during ER stress have to be analyzed in their gene expression level in PC12 cells. iNOS and MnSOD act as biomarkers where their gene and protein expression patterns are changed (upregulation/downregulation) due to specific stress when compared to normal conditions. Therefore, in the present study, the gene expression pattern of stress markers with nitrosative stress inducer (peroxynitrite) and oxidative stress inducers (rotenone, high glucose, and H_2O_2) is investigated in PC12 cells. Further, the protein expression of iNOS and MnSOD protein were also analyzed by Western blotting.

2 Methods
2.1 Materials
The materials are as follows:

A). Rotenone preparation (MW-394.4, Sigma Aldrich, St. Louis, USA): 1.972 mg of rotenone dissolved in 0.5 ml of DMSO to get stock of 10 μM and it was diluted 10 times with DMSO to obtain 1 μM.

B). Peroxynitrite preparation hydrogen peroxide (30%, MW-34.01, Nice Laboratories): isoamyl nitrite (MW = 117.15, Himedia), DTPA (MW = 393.35, Himedia), NaOH (MW-40, Sisco Research Lab), dichloromethane (MW-84.93, Himedia), and manganese dioxide (MW-86.94, Himedia). Peroxynitrite was prepared as per the procedure of Uppu and Pryor [24]. Briefly, H_2O_2 buffer was prepared by adding 25 ml of cold H_2O_2 to 40 ml of NaOH. Further, 5 ml of 0.04 M DTPA was dissolved in 0.05 N NaOH and made up to 100 ml with distilled water along with 27 ml of isoamyl nitrite. This solution was stirred vigorously overnight at room temperature. The obtained aqueous phase was siphoned off, and the remaining solution was washed five times with acetone. The obtained yellow solution was purified with MnO_2, and the elution obtained was measured at 302 by diluting it 1000 times with distilled water and stored at − 20 °C.

C). Hydrogen peroxide preparation (30%, MW-34.01, Nice Laboratories): 0.5 ml of H_2O_2 diluted in 49.5 ml of water (100 times) to obtain 100 μM concentration.

D). High glucose preparation (MW-180.16, Sigma-Aldrich, St. Louis, USA): 59.45 mg of glucose was added in 10 ml of distilled water to obtain a 33-mM glucose solution.

E). Lysis buffer preparation (10×): lysis buffer was prepared using 5 ml of Tris (10×, pH 7.5, MW-121.14, Himedia), 100 μl of EDTA (0.5 M, MW-372.24, Himedia), 0.01 ml of SDS (0.1%), and 1.5 ml of NaCl (1 M, MW-58.44, Himedia) dissolved in 10 ml of double-distilled water. Ten microliters of PMSF (1×, MW-174.19, Himedia) was added before use to inhibit the protease activity.

2.2 Culturing of PC12 cells
PC12 cells were obtained from the National Centre for Cell Sciences (NCCS), Pune. PC12 cells were washed with PBS to remove cell debris and were suspended in a growth medium. The next day, they were again resuspended on a collagen-coated dish which consisted of 85% RPMI, 10% heat-inactivated horse serum, 5% fetal bovine serum, and penicillin and streptomycin (25 μg/ml). Cells were grown as a monolayer culture in a humidified atmosphere of 5% CO_2 at 37 °C. At ~ 80% confluency, 3 ml trypsin EDTA solution was added to the monolayer and incubated at 37 °C for 5 min for cells to detach. Further, cells were resuspended in 5 ml of fresh growth medium and counted using a hemocytometer.

All the chemicals were purchased from Himedia Pvt Ltd., Mumbai.

2.3 Estimation of nitric oxide

The NO production was measured using the Griess assay [25]. Briefly, PC12 cells (1×10^4) were seeded into a 6-well plate with DMEM containing 5.5 mM glucose/well and 20% FBS and incubated at 37 °C with 5% CO_2. After 48 h, the cells were washed with fresh medium and treated with rotenone (1 μM), peroxynitrite (1 μM), and H_2O_2 (100 μM) in DMEM containing 1% serum. Cells were incubated for 16 h at 37 °C with 5% CO_2. Fifty microliters of the supernatant obtained and 50 μl of the Griess reagent were added, incubated at 37 °C for 10 min. Absorbance was read at 540 nm. Further, cells were washed with PBS and lysed using lysis buffer for the estimation of protein. The normalization of the nitric oxide was done for the estimation of protein content.

2.4 Cellular protein estimation by the Bradford method

Protein estimation was carried out according to the procedure of Bradford [26]. Briefly, BSA stock solution was prepared with distilled water (10 mg/ml), and the working BSA standards were prepared ranging from 50 to 250 μg/ml of the stock. Five microliters each of the cell lysate sample, standard, and lysis buffer were prepared and used as blank; 250 μl diluted Bradford reagent was added to the samples and standard, incubated for 5 min at room temperature. Absorbance was read at 595 nm against the blank. A standard curve was plotted, and calculation of the protein was done by correlating the absorbance of the samples with the standard curve.

2.5 Estimation of reactive oxygen species

Cells were treated with stress inducers and incubated at 37 °C with 5% CO_2 for 24 h. After incubation, the medium was removed and DCFH-DA dye was added (20 μM concentration in phenol red-free DMEM). Further, Hoechst dye was also added (10 μM final concentrations in phenol red-free DMEM). Cells were incubated for 1 h at 37 °C. Furthermore, cells were washed with PBS and lysed with lysis buffer (150 μl). One hundred microliters of clear cell lysate was transferred into a separate well, and 100 μl of lysis buffer was taken as blank in separate wells of the same plate. Absorbance was read at 485 nm excitation and 528 nm emission for ROS. Data are expressed in a bar graph where the y-axis indicates the relative fluorescence intensity of ROS (RFUROS) against the control.

2.6 Real-time PCR

The total RNA was isolated by TRIzol Reagent. Briefly, cells were scraped out and homogenized using

sterile water and TRI reagent. After 5 min, the homogenate was extracted using chloroform. RNAs in the aqueous phase were precipitated with isopropanol. RNA pellet was washed and stored in ethanol at −80 °C. The purity and quantity of isolated RNAs were assessed using a spectrophotometer (Schimadzu UV-1800 spectrophotometer, Japan). Reverse transcription was done using 1.5 μg of total RNA. The lists of primers used for the gene expression analysis are described in Table 1 and were designed by the primer blast software. The concentration of the primer used for reverse transcription was 300 nM. The same set of primers was used for the Hi-SYBR Green Master Mix for real-time PCR. Amplification was performed over 40 cycles. A melt analysis was run for all the products to confirm specific amplification. The results were evaluated by the GraphPad Prism software 5.0 as a peak area for every well, and the resultants were quantified by relative fold expressions compared with control, i.e., GAPDH.

2.7 Western blotting

Western blotting was performed according to the manufacturer's protocol (Abcam Biotech, Cambridge, USA). Briefly, after inducing stress to PC12 cells, they were washed with PBS and lysed using lysis buffer. Further, the cell lysates were centrifuged at 12,000 rpm for 30 min at 4 °C, and the supernatant was taken to determine the protein content. Equal amounts of protein were loaded onto SDS-PAGE, and electrophoresis was carried out (100 V). Protein bands from the gel were transferred to the PVDF membrane by electroblotting. Further, the membrane was blocked overnight at 4 °C in TBST containing 5% non-fat milk. The membranes were then probed with specific primary Abs overnight at 4 °C for iNOS (1:1000) and MnSOD (1:1000). The membrane was washed three times with TBST and was re-

Table 1 Forward and reverse primers used in RT-PCR studies

Gene	Primer sequence (5-3)	Annealing temperature
Catalase	**F: CCT ATT GCC GTC CGA TTC**	**60**
	R: AGG GTC CTT CAG GTG AGT TT	
SOD	**F: AAT ACA CAA GGC TGT ACC**	**57**
	R: GAG ATC ACA CGA TCT TCA A	
BTG2	**F: CCCGGCTACACTGTATATTGACTTGG**	**67**
	R: GGGTTTCATCACGTTGGTCAGGAT	
Nrf2	**F: CCC AGC ACA TCC AGA CAG AC**	**64**
	R: TAT CCA GGG CAA GCG ACT C	
GADD153	**F: AAC CAG CAG AGG TCAC AAG C**	**60**
	R: AGC CGT TCA TTC TCT TCA GC	

F forward, *R* reverse

incubated with a horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Images of the bands were taken by gel documentation system, and densitometry analysis was carried out using Image Studio Lite, where the relative protein expression (AU) of MNSOD and iNOS is analyzed against control, i.e., B-actin.

2.8 Statistical analysis

The experimental data are reported as mean ± standard deviation. To compare the results, relative fold differences were determined by calculating the difference between the Ct values and the reference. Statistical analysis was performed using the GraphPad Prism 5.0 software. One-way ANOVA was used for multiple comparisons, followed by a Turkey multiple comparison with a significance of < 0.05.

3 Results

3.1 Effect of stress inducers on NO and ROS in PC12 cells

Protein concentrations present in the culture samples collectively were found to be 100 μg/ml, where BSA was used as the standard. The total protein content of the samples is necessary to determine the nitric oxide concentration. As described in the "Methods" section, NO generation was observed in PC12 cells after the exposure of rotenone (1 μM), peroxynitrite (1 μM), H_2O_2 (100 μM), and high glucose (33 mM) for 24 h. An increase in the NO was seen with different stress inducers, i.e., 0.0213 μmol/mg, 0.0505 μmol/mg, 0.1079 μmol/mg, and 0.1043 μmol/mg, respectively, compared to the vehicle control (Fig. 1). ROS production is expressed in relative fluorescent intensity (RFUROS) produced in PC12 cells after the exposure of different stress inducers against the control. Results were found to be 73%, 78%,

70%, and 73% for rotenone (1 μM), peroxynitrite (1 μM), H_2O_2 (100 μM), and high glucose (33 mM), respectively, compared to the control which was 58% (Fig. 2).

3.2 Effect of stress inducers on gene expression of antioxidant enzymes

Gene expression studies of SOD and catalase were done to evaluate the amount of oxidative damage undertaken by the stress inducers in PC12 cells. Alterations in the level of mRNA expression of antioxidant marker genes exposed to rotenone (1 μM), peroxynitrite (1 μM), high glucose (33 mM), and H_2O_2 (100 μM) are presented in Fig. 3a, b. A significant ($p < 0.05$) upregulation of catalase gene expression is seen in rotenone-induced stress (3-fold) in PC12 cells. Further, catalase was upregulated in peroxynitrite-induced stress (1.15-fold) but downregulated in high glucose and H_2O_2. Another important antioxidant SOD was downregulated in all the stress-induced PC12 cells significantly ($p < 0.05$) as shown in Fig. 3b.

3.3 Effect of stress inducers on gene expression of Btg2, Nrf2, and Gadd 153

Alterations in the level of mRNA expression of Btg2, Nrf2, and Gadd 153 in PC12 cells exposed to rotenone (1 μM), peroxynitrite (1 μM), high glucose (33 mM), and H_2O_2 (100 μM) are presented in Fig. 4a, b, c. Upregulation in the expression of Btg2 genes was seen in all the treated cells (Fig. 4a), wherein the maximum upregulation was exhibited by peroxynitrite-treated cells (3-fold) followed by high glucose (2-fold), rotenone (1.8-fold), and H_2O_2 (1.6-fold). Surprisingly, Nrf2 was downregulated significantly ($p < 0.05$) (Fig. 4b) in all the stress-induced cells. Further, Gadd 153 was significantly upregulated in the rotenone-induced
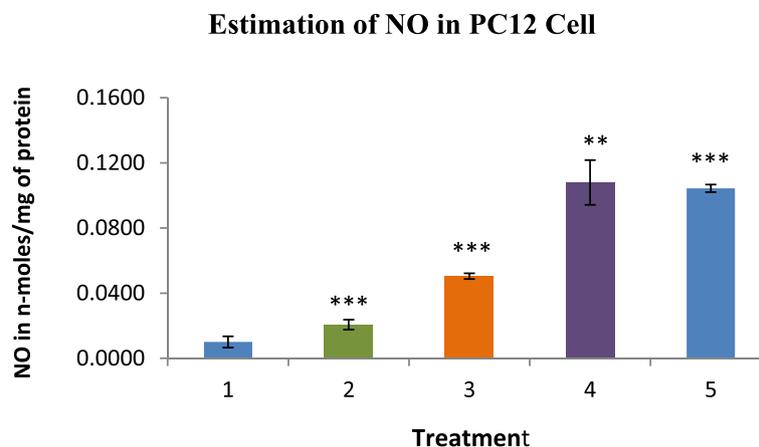

Fig. 1 Effect of stress inducers on NO production in PC12 cells during oxidative stress. (2) Rotenone (1 μM). (3) Peroxynitrite (1 μM). (4) Hydrogen peroxide (100 μM). (5) High glucose (33 mM). Data are expressed as moles/milligram of protein compared to the (1) vehicle control. The data provided are the mean ± SD from three independent experiments. *$p < 0.05$, **$p < 0.001$, ***$p < 0.001$ compared to the control
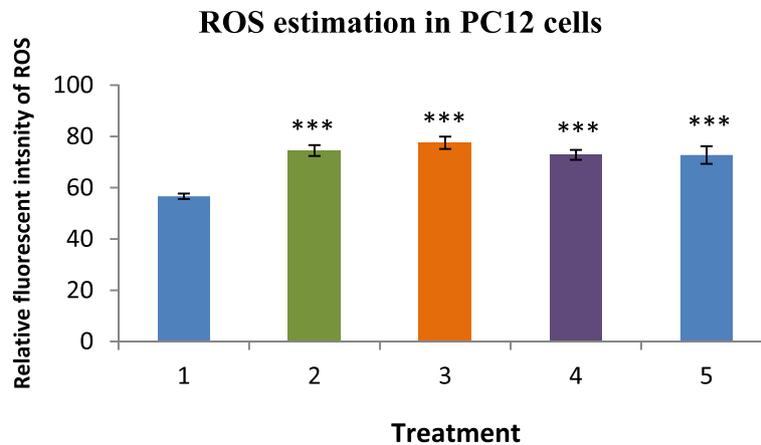
Fig. 2 Effect of stress inducers on ROS production in PC12 cells during oxidative stress. PC12 cells were treated with (2) rotenone (1 μM), (3) peroxynitrite (1 μM), (4) hydrogen peroxide (100 μM), and (5) high glucose (33 mM) to estimate the NO concentration. Data are expressed as a percentage compared to the (1) vehicle control. The data provided are the mean ± SD from three separate experiments. Data are expressed in the bar graph where the y-axis indicates the relative fluorescence intensity of ROS (RFUROS) against the control. *$p < 0.05$, **$p < 0.001$, ***$p < 0.001$ versus control

(5-fold) PC12 cells ($p < 0.05$) (Fig. 4c) compared to peroxynitrite (1.2-fold) and high glucose (1.1-fold), whereas H_2O_2-treated Gadd 153 expression was downregulated.

3.4 Effect of stress inducers on iNOS and MnSOD protein expression in PC12 cells

Protein expression levels of inducible nitric oxide synthase (iNOS) and MnSOD exposed to rotenone (1 μM), peroxynitrite (1 μM), high glucose (33 mM), and H_2O_2 (100 μM) are presented in Fig. 5a, b and c. MnSOD protein expression levels were decreased in the order of control > peroxynitrite > rotenone > hydrogen peroxide > high glucose (Fig. 5b). iNOS was decreased in the order control > H_2O_2 > peroxynitrite > high glucose > rotenone (Fig. 5c).

4 Discussion

In the current study, PC12 cell stress biology was studied by treating them with different chemical inducers like rotenone (1 μM), H_2O_2 (100 μM), peroxynitrite (1 μM), and high glucose (33 mM) to estimate the NO concentration, ROS production, stress marker gene expressions, and protein expressions of iNOS and MnSOD. Specific biomarkers like Gadd153, Btg2, and Nrf2 gene expressions would give a brief idea in recognizing the apoptotic pathway undergone by the cells. Therefore, analyzing the gene expression pattern of these stress markers would lead us in differentiating the type of stress undergone by PC12 cells.

In the current study, the concentration of NO showed a significant increase in H_2O_2- and high glucose-induced PC12 cells compared to the control (10-folds). Peroxynitrite- and rotenone-induced PC12 cells too showed a

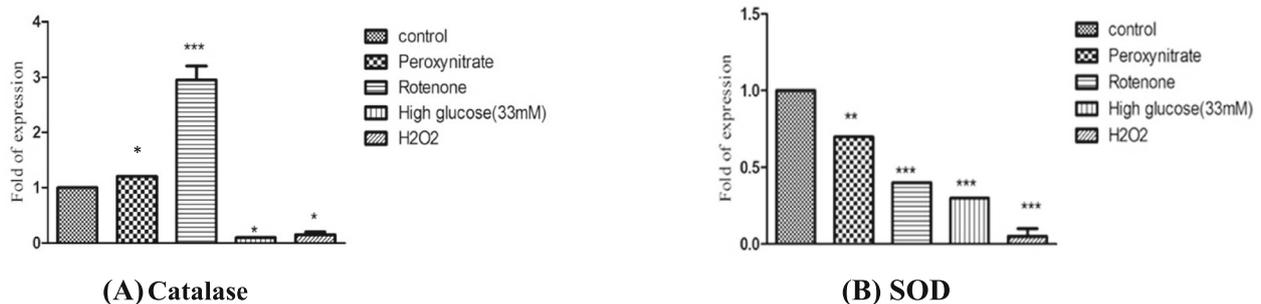
(A) Catalase

(B) SOD

Fig. 3 Real-time PCR analysis of the alteration in the mRNA expression of stress marker genes. **a** Catalase gene expression was upregulated in the rotenone stress-induced group (3-fold) compared to the peroxynitrite stress-induced group (1.2-fold) whereas downregulated in the hyperglycemic stress-induced groups (0.1-fold) and H_2O_2 stress-induced groups (0.2-fold) against the control. **b** SOD gene expression was downregulated in all stress-induced groups where the H_2O_2-induced stress groups showed the least expression (0.1-fold) compared to the hyperglycemic stress-induced group (0.3-fold), rotenone stress-induced group (0.4-fold), and peroxynitrite stress-induced group (1.25-fold) against control. The data provided are the mean ± SD from three independent experiments. *$p < 0.05$, **$p < 0.001$, ***$p < 0.001$ versus control
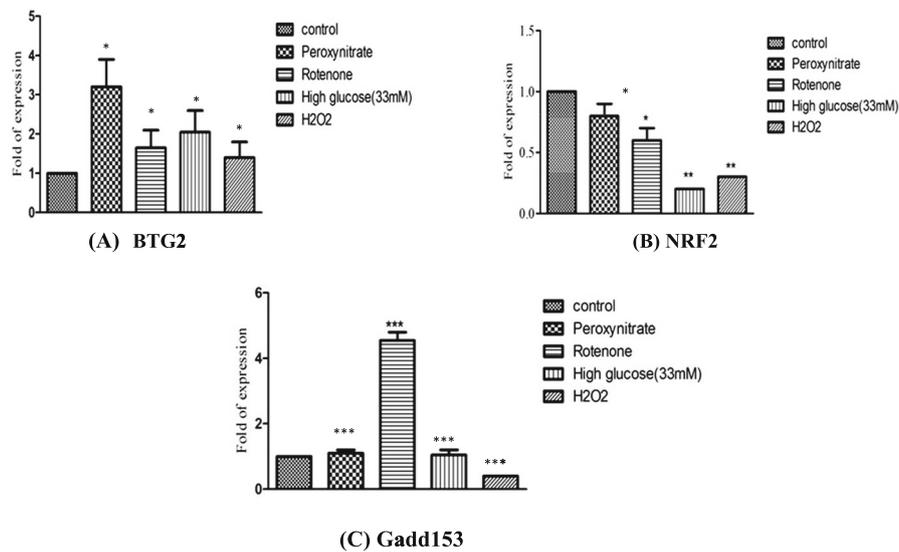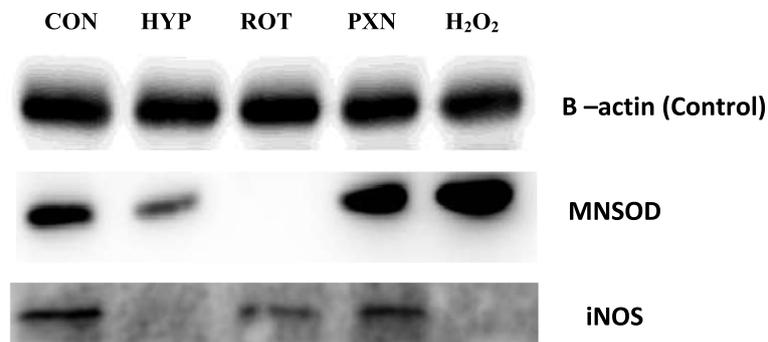
Fig. 4 Real-time PCR analysis of the alteration in the mRNA expression of stress markers. **a** BTG2 gene expression in the peroxynitrite-induced stress group showed a maximum (3-fold) increase compared to the hyperglycemia-induced stress group (2-fold), rotenone-induced stress group (1.8-fold), and H_2O_2-induced stress group (1.7-fold) against the control. **b** Nrf2 gene expression was downregulated where the hyperglycemic-induced stress group showed the least expression (0.2-fold) compared to the H_2O_2-induced stress group (0.3-fold), rotenone-induced stress group (0.65-fold), and peroxynitrite-induced stress group (0.8-fold) against the control. **c** Gadd153 expression in the rotenone-induced stress group showed a maximum (5-fold) increase compared to the hyperglycemic-induced stress group (1.2-fold) and peroxynitrite-induced stress group (1.2-fold), whereas the H_2O_2-induced stress group was downregulated (0.5-fold) against the control. The data provided are the mean ± SD from three independent experiments. *$p < 0.05$, **$p < 0.001$, ***$p < 0.001$ versus control

marked increase in the NO concentration compared to the control (Fig. 1). ROS levels were increased in peroxynitrite-, rotenone-, H_2O_2-, and high glucose-induced PC12 cells significantly compared to the control (Fig. 2). The results obtained from the study indicate that even the lower concentration of stress inducers like rotenone and peroxynitrite can elevate the levels of NO and ROS in the cells leading to their cell death, whereas earlier studies showed similar results with higher concentrations of the stress inducers in PC12 cells and other mammalian cells as well [27–30]. Thus, we report ≤ 1 μM of rotenone and peroxynitrite also exerts sufficient oxidative stress in the neuronal cells.
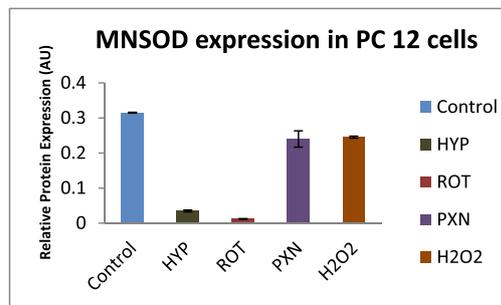
In the present study, some of the important and prominent stress markers were studied in regard to the PC12 cells in different stress conditions. In the mitochondria, Btg2 is known for sensitizing the oxidative stress wherein in this current study, elevated gene expressions of Btg2 are seen in response to minimal concentrations of peroxynitrite (3-fold), rotenone (1.8-fold), hyperglycemia (2-fold), and H_2O_2 (1.7-fold) (Fig. 4a) compared to the control indicating the role of Btg2 where it could sense the oxidative stress at minimal concentrations. These results are in agreement with the earlier studies, wherein Btg2 mediated upregulation of antioxidant enzymes in carcinoma and other mammalian cells [31, 32] via ROS-PKC-NFκB cascade [12, 33]. Further, a previous

study also reported Btg2 regulates necrosis by forming mitochondrial permeability transition pore opening during H_2O_2 stress [34]. However, the complete or in-depth study of Btg2 expression in PC12 cells during oxidative stress is poorly understood.
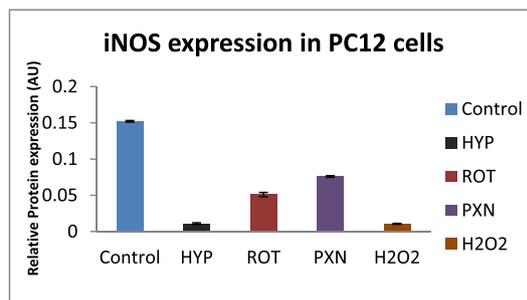
Transcriptional factor Nrf2 is a major protein, which regulates the basal and induced expression of antioxidant response element-dependent genes [35]. Thus, different stress inducers have their own effect on the expression of Nrf2 gene expression with a tissue or with a cell, whereas the gene expressions of Nrf2 showed a sharp decrease in PC12 cells when induced by different types of stress (Fig. 4b). Earlier studies have shown this type of decrease in gene expression in neuronal cells because neurons react differently compared to non-neuronal cells during oxidative stress [36]. Nrf2 gene expression was downregulated where the hyperglycemic-induced stress group showed the least expression (0.2-fold) compared to the H_2O_2-induced stress group (0.3-fold), rotenone-induced stress group (0.65-fold), and peroxynitrite-induced stress group (0.8-fold) against the control (Fig. 4b). Contrasting results of Nrf2 downregulation should be studied further to know their function in PC12 cells. Several pathways have been proposed for Nrf2/EpRE regulating the expression of antioxidant enzymes via antioxidant response elements (ARE) [37] which helps the cells in sensing oxidative stress. The

Fig. 5 Western blot analysis of the protein expression of MNSOD and iNOS in PC12 cells with different stress inductions. (1) Control (B-actin). (2) Stress induced by hyperglycemia. (3) Stress induced by rotenone. (4) Stress induced by peroxynitrite. (5) Stress induced by H₂O₂. The data provided are the mean ± SD from three independent experiments. *$p < 0.05$, **$p < 0.001$, ***$p < 0.001$ versus control. The bar graph represents the densitometric analysis of MNSOD and iNOS proteins relative expression (AU) with the control

increase in NO can activate Nrf2 in PC12 cells [38] wherein peroxynitrite at narrow range concentrations may modulate apoptosis through the Nrf2-dependent pathways [39] which needs a detailed further study. Thus, a decrease in the expression of Nrf2 led to the downregulation of ARE genes during stress in turn decreasing the chances of cell survivability.

Gadd153 is one of the main oxidative stress markers expressed in the endoplasmic reticulum during the oxidative damage. The results indicated that rotenone stress-induced PC12 cells showed higher expression (5-fold) compared to the hyperglycemic-induced stress group (1.2-fold) and the peroxynitrite-induced stress group (1.2-fold), whereas the H₂O₂-induced stress group was downregulated (0.5-fold) against the control. The results obtained corroborate with the previous studies, where Gadd153 levels were downregulated during the oxidative stress, but rotenone-enhanced Gadd153 gene expression suggests that PC12 cells undergo endoplasmic reticulum stress as observed in the neurons [40]. Other results showed that oxidative damage causes a selective downregulation of the neuronal stress response activated under ER dysfunction [36]. Gadd153 is always upregulated by oxidative stress and sensitizes cells to ER stress during enhanced oxidative injury [41]. Thus, we

hypothesize that PC12 cells undergo endoplasmic stress with rotenone, whereas other different stress inducers are selectively downregulated due to the dysfunction of ER co-related with mitochondrial stress in PC12 cells.

Protein expression of MnSOD and iNOS were downregulated in all the stress-induced cells suggesting that stress inducers have some feedback inhibition mechanism. MnSOD play a critical role in inhibiting oxidative inactivation of nitric oxide, thereby preventing peroxynitrite formation and prevent mitochondrial dysfunction [42] that lead to the death of cells. However, the MnSOD protein expression is also been downregulated in all the treated PC12 cells. It can be assumed that MnSOD inhibited the iNOS pathway due to mitochondrial dysfunction. The release of MnSOD into the cytosol decreased the expression of it in the treated cells as seen previously [43].

5 Conclusion

To conclude, different stress inducers like rotenone, peroxynitrite, H_2O_2, and high glucose increase the NO and ROS. Further, antioxidant marker genes were differentially expressed with different stress inducers. Continuous and slow generation of peroxynitrite prolonged the nitrosative stress in rotenone-induced cells resulting in the elevation of the gene expression. mRNA and protein expression studies suggest that PC12 cells undergo endoplasmic reticulum stress with rotenone and other stress inducers through mitochondrial dysfunction. However, a further detailed study is required to understand the underlying mechanism controlling the different oxidative stress genes in neuronal cell models individually.

Abbreviations
RONS: Reactive oxygen and nitrogen species; ER: Endoplasmic reticulum; Gadd 153: Growth arrest and DNA damage 153; Btg2: B cell translocation gene 2; Nrf2: Nuclear factor erythroid 2-related factor 2; SOD: Superoxide dismutase; MnSOD: Manganese superoxide dismutase; iNOS: Inducible nitric oxide synthase; TPA: Tumor promoter agent; GSH: Glutathione; ARE: Antioxidant response elements; PKC: Protein kinase C; CHOP: C/EBP homologous protein; NFκB: Nuclear factor kappa B

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Not applicable

Authors' contributions
AP, HN, BRL, and KSD designed the study, analyzed the results, and wrote the manuscript. SRB and AG helped in conducting the experiments with AP. All authors have read, accepted for publication, and approved the final manuscript.

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All data generated or analyzed during this study are included in this published article

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