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FULL LENGTH ARTICLE

Elevation of gene expression of calcineurin, calmodulin and calsyntenin in oxidative stress induced PC12 cells

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Abstract In normal physiological conditions, reactive oxygen and nitrogen species are used as important signaling molecules in the cell. However, in excess it causes the disruption of cell resulting in their death. Oxidative stress causes influx in intracellular calcium levels leading to higher concentrations of calcium in the cell. This accelerated calcium affects both the mitochondria and nuclei leading to excitotoxicity in neurons. Intracellular calcium levels are controlled by voltage dependent calcium channels located in the plasma membrane, calcium stores like endoplasmic/sarcoplasmic reticulum and majorly by calcium binding proteins. Our study was aimed at analyzing the gene expression of major calcium binding proteins namely calcineurin, calmodulin, calreticulin, synaptotagamin and calsyntenin in stress induced PC 12 cells. Rotenone (1 μM), Peroxynitrite (10 μM), H_2O_2 (100 μM) and High glucose (33 mM) were used to induce oxidative stress in PC12 cells. Results obtained from the study suggest that calcineurin, calmodulin and calsyntenin gene expression were enhanced compared to the control due to oxidative stress. However, synaptotagmin and calreticulin gene expression were down regulated. Further, Akt protein expression (stress marker) was enhanced in PC12 cells with all other stress inducers except in hyperglycemic condition.

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Introduction

Oxidative stress is a shift in the cellular redox state to a more oxidized state. Such a shift is due to exposure of cells to toxins, heavy metals, UV irradiation, heat shock and infections.[1] ROS are generated as by-products of mitochondrial respiratory chain and also by extra mitochondrial enzymes such as NADPH oxidases, lipoxygenase, xanthine oxidase, nitric oxide synthase, cytochrome P450, cyclo oxygenase and myeloperoxidase etc.[2] Aerobic metabolism involving the activity of pyruvate, α-ketoglutarate and isocitrate-dehydrogenases as well as ATP synthesis are modulated by the Ca^{2+} concentrations.[3–5] ROS at sub toxic levels acts as signaling molecules in various cellular processes like cell growth and cell death.[6] Calcium is of critical importance to neurons in the transmission of the depolarizing signal and for synaptic activity.[7] It is well known fact that calcium homeostasis is critical for cell physiology, but its deregulation can lead to neurodegeneration through complex and diverse pathways.[8] Intracellular Ca^{2+} levels are mainly controlled by voltage dependent calcium channels and Ca^{2+}pumps located in the plasma membrane. Sarcoplasmic/Endoplasmic reticulum and Calcium binding proteins (CBPs) are the main regulators of calcium homeostasis in addition to hormonal regulation.[9] However, only few calcium binding proteins namely calbindin and S100 Proteins have been studied with respect to oxidative stress.[10] Hence the present study was performed to understand the expression pattern of important calcium binding proteins during oxidative stress in PC12 cells.

Calcium binding proteins (CBP) are broad group of EF hand helix loop helix motif proteins participating in numerous cellular functions especially in Ca^{2+} homeostasis and Ca^{2+} signaling. Although there structure and functions are heterogeneous in nature, CBP selectively and reversibly bind Ca^{2+} for their activity.[11] One such important calcium binding protein is calcineurin (CaN), a calmodulin dependent serine/threonine phosphatase expressed in different parts of brain.[12] It is a heterodimeric protein with a catalytic subunit calcineurin A and Ca^{2+}-binding subunit calcineurin B.[13] CaN activates after cytoplasmic Ca^{2+} influx where it inhibits further Ca^{2+} influx into the cytosol. CaN does it in numerous ways, where it slows down the Ca^{2+} influx from plasma membrane by weakening voltage-gated Ca^{2+} channels and also by regulating Ca^{2+} release from the ER by negatively controlling IP3 and RyR through dephosphorylation.[14,15] Inhibition of calcineurin activity has led to the formation of α- Tau proteins in Alzheimer's and also has an important role in Huntington's disease.[16,17] Hence understanding the role of calcineurin in regulating calcium homeostasis with different oxidative stress environment may reveal their diversified roles in PC12 cells.

Calmodulin (CaM) is a sensor CBP which regulates the activity of calcineurin. CaM binds to a regulatory domain within CaN causing conformational change that removes an autoinhibitory domain (AID) from the active site resulting in activation of the calcineurin.[18] The prolong efflux of calcium from endoplasmic reticulum activates the calmodulin dependent kinases to bring about the cell death.[19] During stress conditions, oxidation of Met144 and 145 inhibits the interaction of CaM with CaMKII, thus activates CaMKII and increases the conductance of glutamate receptors. Thus, oxidation of specific residues in CaM is tightly linked to calcium/CaMKII related signaling.[20,21] The above findings prompted to know the effect of calmodulin and calcineurin in different stress conditions in PC12 cells.

Earlier studies have showed that calreticulin gene (Molecular chaperone in ER) is increased during stress. Misfolding of proteins during stress conditions evokes pro apoptotic proteins as a response and prolonged ER injury leads to apoptosis of cells.[22] It is believed that calreticulin acts both as buffer and chaperone, where it binds to misfolded proteins and prevent their export to golgi.[23] However, less is known about the calreticulin expression during the stress condition in PC12 cells. Therefore, calreticulin gene was studied to know the pattern in different stress conditions.

Calsyntenins (Clstn) are the group of transmembrane proteins belonging to cadherin family which are predominantly expressed in excitatory neurons and GABAergic neurons.[24] Clstn1 is down-regulated in AD and PD. Decreased expression of Clstn1 is mediated through si-RNA which protects from the toxic Aβ generation in AD.[25] Clstn 2 & 3 are correlated with the cognitive effects where they reduce the inhibitory synapses resulting in diminishing the parvalbumin interneuron formation.[26] However, a precise role of calsyntenins in oxidative stress is yet to be explored in PC12 cells. Synaptotagmins (Synt) are the set of membrane trafficking proteins which plays an important role in vesicle docking, exocytosis and endocytosis etc.[27] It mediates the calcium triggered neurotransmitter release at hippocampal synapses.[28] Synt 1 has been the calcium sensor protein regulating the SNARE complex vesicle fusion in response to the increased calcium concentration.[29] However precise role of these calcium binding proteins and their gene expressions in different stress environments are not explored in PC12 cells.

In the present study, we have investigated the gene expression profile of calcium binding proteins using different stress inducers in PC12 cells and also the role of Akt protein using RT-PCR and Western blotting methods respectively.

Materials and methods

Culturing of PC12 cells

PC12 cells obtained from National Centre for Cell Sciences (NCCS, India) were washed with PBS to remove cell debris and suspended in growth medium. They were re-suspended on a collagen coated dish with RPMI growth medium consisting of 10% heat inactivated Horse Serum, 5% Fetal Bovine serum and Penicillin streptomycin (25 μg/ml). Cells were grown as monolayer at 37 °C in a humidified atmosphere of 5% CO_2. To detach the cell, 3 ml of trypsin EDTA solution was added to the monolayer and incubated at 37 °C for 5 min when they attained 80% confluency. Cells were re suspended in 5 ml of fresh growth medium and further used for MTT assay.

MTT assay

The cells were treated with different concentrations of Rotenone (1 μM), Hydrogen peroxide (100 μM),

Peroxynitrite (10 μM) and High glucose (33 mM) for 24 Hr in 96 well plates. After treatment, cells were incubated with MTT dye for 2 Hr at 37 °C (5 mg/ml). After incubation, the blue-colored formazan was dissolved in DMSO. The intensity of formazan was measured by spectrophotometer (Schimadzu UV-1800) at 570 nm.

Real Time-PCR

Total RNA was isolated using Trizol method (Himedia Laboratories, LLC, PA). In brief, cells were scraped and homogenized in sterile water with TRI reagent. After 5 min, homogenate was extracted from chloroform. RNA's in the aqueous phase was precipitated by isopropanol. RNA pellet was washed with 75% ethanol and stored in 96% ethanol at −70 °C. The purity and quantity of isolated RNA's were assessed using Nanodrop (Thermo Scientific 2000c). Reverse transcription was performed with 1.5 μg of total RNA. The lists of primers used are described in Table 1 and were designed by primer blast software. The primer concentrations of 300 nM were taken and reverse transcribed. Same set of primers were used for amplification with Hi-SYBR Green Real-Time PCR Master Mix (Himedia Laboratories, LLC, PA). Amplification was performed for over 40 cycles. A melt analysis was run for the reaction to confirm specific amplification. Results were evaluated by Graph Pad Prism software 5.0 as a peak area for every well and quantified relative fold expression compared with control i.e GAPDH.

Western blotting

Western blotting was carried out according to the manufacturer's protocol (Abcam Biotech, Cambridge, USA). After the induction of stress, cells were washed with PBS and lysed using lysis buffer. Cell lysates were centrifuged at 12,000 rpm for 30 min in 4 °C and supernatent was taken further to determine the protein content by Bradford method. Equal amounts of protein were loaded on to SDS PAGE and electrophoresis was carried out. Following electrophoresis, protein bands from the gel were transferred on PVDF membrane by electro blotting. Membrane was blocked overnight at 4 °C in TBST containing 5% non fatty milk. Membranes were then probed with specific primary Ab's overnight at 4 °C for Akt (1: 1000). Three washes were done with TBST and membrane was re-incubated with horse radish peroxidase conjugated secondary antibody for 1 Hr at room temperature. Images of the bands were taken by gel documentation system and densitometric analysis was carried out by Image studio lite.

Statistical analysis

Values are reported as mean of standard error of the mean. To compare the results, relative fold differences were determined by calculating the difference between the Ct values and reference. Statistical analysis was performed using the Graph pad prism 5.0 software. One-way ANOVA was used for multiple comparisons, followed by a Turkey multiple comparison with significance of 0.05.

Results

Inhibition of PC12 cell proliferation with different stress inducers

As described in the methods, the effect of different stress inducers on proliferation of PC12 cells was carried out using the MTT assay. PC12 cells treated with Rotenone (1 μM), H_2O_2 (100 μM), Peroxynitrite (10 μM) and High glucose (33 mM) showed significant decrease in cell proliferation compared to control, with $P < 0.05$ [Fig. 1(A—D)].

Effect of stress inducers on the gene expression of calcium binding proteins

Expression analysis of calcium binding protein (Calcineurin, Calmodulin, Calsyntenin, Calreticulin and Synaptotagmin) genes involved in stress induced PC12 cells was done. Gene expressions of the calcium binding proteins varied differently with varying stress conditions. Calcineurin, calmodulin and calsyntenin gene expression were upregulated whereas calreticulin and synaptotagmin genes were down regulated in peroxynitrite and H_2O_2 induced PC12 cells. High glucose and Rotenone induced PC12 cells showed downregulation with respect to all the calcium binding proteins except calreticulin (See Fig. 2).

Table 1 Sequence of Primers used in RT-PCR Studies.

Gene	Primer Sequence (5—3)	Annealing Temperature
Calcineurin	F: AGTAACTTTCGAGCCAGCCC R: GGGGGTCTGACCACAAGATG	62
Calmodulin	F: TGCGGAAGTTAGGAGTGCTG R: GCACAGCATAATGGAAGGCG	60
Calreticulin	F: GAGGCTTGGCGGAAGTATGA R: CTCTGTTGGACTCGGAAGCTAA	61
Synaptotagmin	F: AAAAAGTGCACTCCCAACGC R: ACCACTTCCTTCTGCTGTGG	59
Calsyntenin	F: ACAAAGCAACCGTGCACATC R: GAGACGCCGTCCACATAGAG	60

F- Forward; R — Reverse.

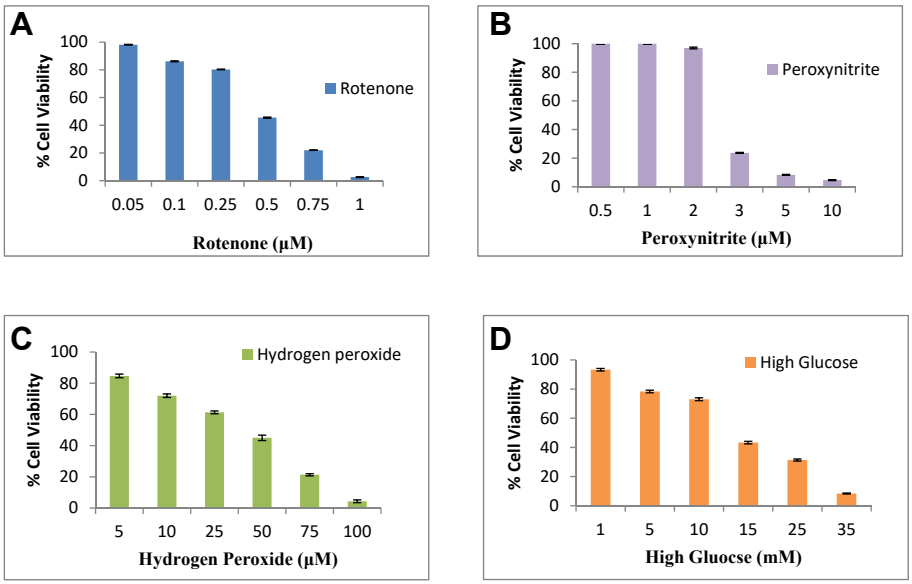

Figure 1 Effect of oxidative stress on PC12 cells proliferation. **(A)** PC12 cells induced with rotenone with different concentrations from 0.05 µM—1 µM, where 1 µM of rotenone induced PC12 cell show maximum cell death after 24 Hr incubation. **(B)** PC12 cells induced with Peroxynitrite with different concentrations ranging from 0.5 µ M—10 µM, where 10 µM of Peroxynitrite induced PC12 cells show maximum cell death after 24 Hr incubation. **(C)** PC12 cells induced with Hydrogen Peroxide with different concentrations from 5 µM—100 µM, where 100 µM of rotenone induced PC12 cell show maximum cell death after 24 Hr incubation. **(D)** PC12 cells induced with High Glucose with different concentrations ranging from 1 mM—35 mM, where 35 mM of High Glucose induced PC12 cells show maximum cell death after 24 Hr incubation.

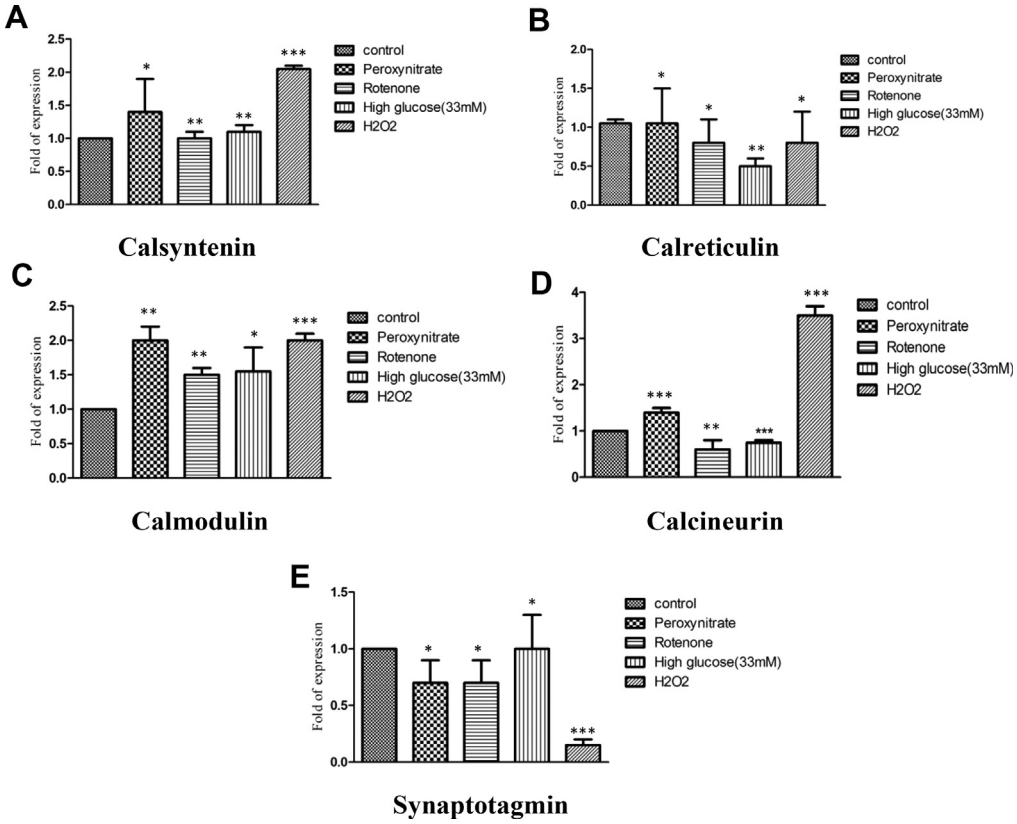

Figure 2 Real Time PCR analysis of alteration in the mRNA expression of **(A)** Calsyntenin; **(B)** Calreticulin; **(C)** Calmodulin; **(D)** Calcineurin; **(E)** Synaptotagmin in PC 12 cells exposed to stress inducers. The data provided are mean ± SD from three separate experiments. *$p < 0.05$, **$p < 0.001$, ***$p < 0.001$ versus control.

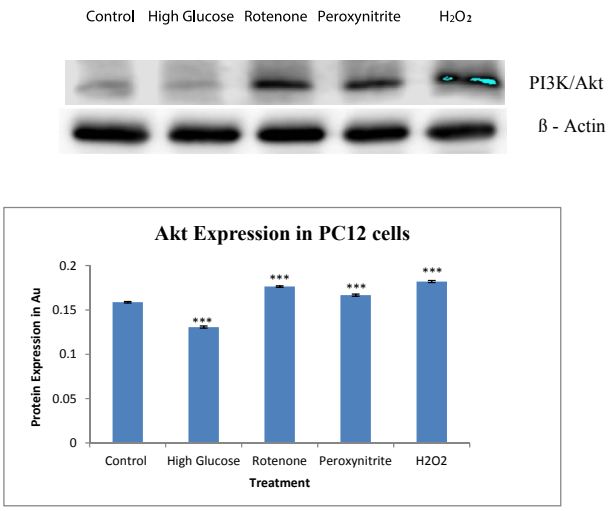

Figure 3 The protein expression of Akt in PC 12 cells with different stress induction. Lane: 1) Control; 2) Stress Induced by High Glucose; 3) Stress induced by Rotenone; 4) Stress induced by peroxynitrite; 5) Stress induced by H_2O_2. Data expressed are mean ± SD from triplicate experiment where $^*p < 0.05$, $^{**}p < 0.001$, $^{***}p < 0.001$ versus control. The bar graph represents the densitometric analysis of Akt protein expression levels.

Rotenone, peroxynitrite and H_2O_2 increases the protein expression of Akt protein

To analyse the role of Akt protein expression in PC12 cells treated with Rotenone (1 μM), Hydrogen peroxide (100 μM), Peroxynitrite (10 μM) and High glucose (33 mM). Differential expressions were seen with respect to the stress inducers. H_2O_2 induced PC12 cells showed increase Akt expression than peroxynitrite and rotenone, whereas high glucose induced PC12 cells showed decreased expression in PC12 cells (Fig. 3).

Discussion

In the present study, PC12 cells were induced with different chemical inducers like Rotenone (1 μM), hydrogen peroxide (100 μM), Peroxynitrite (10 μM) and High glucose (33 mM) to investigate the cell proliferation, calcium binding proteins gene expression and the Akt protein expression. The cells treated with different concentrations of rotenone, hydrogen peroxide, peroxynitrite and high glucose showed a dose dependent cell death in PC12 cells (Fig 1(a-d)). The proliferation of PC12 cells decreased as the concentration of stress inducers was increased. Peroxynitrite induced PC12 cells showed stability till 2 μM, however at 10 μM the viability was decreased drastically. Results obtained shows the pattern of oxidative injury in PC12 cells affecting in dose dependent manner where peroxynitrite works via neurotrophic factors[30] and rotenone affects through endoplasmic reticulum.[31,32] High glucose and Hydrogen peroxide affects the PC12 cell at higher concentrations[33,34] to induce the oxidative injury. However, the study was intended to see the changes in calcium binding protein gene expression at minimal concentrations.

Of all the calcium binding proteins, S-100 and Calbindin D28k have been studied extensively related to PC12 cells.[35,36] Thus, the other related calcium binding proteins in PC12 cells under different stress conditions were studied. The experimental data of the gene expression of calcium binding proteins varied with different stress environment in PC12 cells. The mRNA levels of cells induced with rotenone showed down regulation of calreticulin and calcineurin gene, whereas negligible change in calsyntenin and synaptotagmin gene expression. As we expected, calmodulin gene expression was up regulated in rotenone induced injury in PC12 cells. High Glucose stress in PC12 cells had negligible amount of effect on calsyntenin and synaptotagmin gene expression which were similar to the control. Downregulation of calreticulin and calcineurin gene was observed due to high glucose whereas calmodulin gene expression was upregulated. Calmodulin gene expression upregulates with the increase of intracellular calcium concentration. Both ER and mitochondria can lead the neuronal cells to apoptosis via CaM/CaMKII pathway when there is an increase in intracellular calcium concentrations.[37] Rotenone is a known inhibitor of mitochondrial complex-I, where it increases the intracellular Ca^{+2}concentrations during stress leading to neuronal death via degrading DNA. Increase in intracellular Ca^{+2} activates the calmodulin-dependent protein kinase II (CaM-KII) pathway leading to apoptosis[38]

Although peroxynitrite and H_2O_2 induced PC12 cells show minimal expression of calreticulin. Calcineurin, calmodulin and calsyntenin gene expression were increased by several folds in PC12 cells. Calcium mediated hyperactivation of calcineurin triggers synaptic dysfunction and neuronal death.[39] Peroxynitrite and H_2O_2 stress leads to increase in the expression of CaN leading to apoptosis in PC12 cells.[40] Higher calcineurin activity can lead to overload of Ca^{2+} which makes mitochondrial membrane pore formation resulting of loss of matrix components.[41] Thus, the mitochondrial membrane potential is lost which leads to the apoptosis through the caspase 3 pathway.[40]

During peroxynitrite stress, calsyntenin acts as a neurotrophic factor[30] which up regulates rapidly for 12—24 h and decreases as the cellular stress is continuous for long duration which can be corelated with earlier studies on endothelial cells of brain.[42] Hydrogen peroxide can induce some of the gene clusters in *in vitro* gingival fibroblasts.[43] This can be applied into neuronal cells where it can induce the calsyntenin gene clusters during H_2O_2 stress where it binds to cell surface receptors and induces the production of other cytokines leading to cell death.

In high glucose induced PC12 cells, reactive oxygen species induced by hyperglycemia suppresses the Akt signaling. This may be because of PTEN phosphatase upregulation as seen previously in endothelial cells.[44] Peroxynitrite treated cells do not show increased expression of Akt compared to control. Results obtained from rotenone and H_2O_2 induced PC12 cells exhibited an increase protein expression of Akt. These two stress inducers affect the Akt phosphorylation levels in a concentration and time-dependent manner as observed in previous studies.[45—47] Higher expression of Akt represents the response of the

PC12 cells due to oxidative injury. Phosphorylation was increased at minimal concentrations of stress inducers where as high glucose was induced with higher concentration has affected the Akt phosphorylation irreversibly.

Relation between calcium binding proteins genes and oxidative stress have been established in which Peroxynitrite and Hydrogen peroxide upregulate the PC12 cell calcium binding protein gene expression which lead to the loss of mitochondrial membrane potential leading to their cell death. Rotenone and high glucose affect the gene expression differentially by affecting the mitochondria and initiates the CaMKII pathway thus leading to apoptosis. Rotenone and high glucose can further damage through endoplasmic reticulum. But the downregulation of calreticulin gene which is a stress marker of ER makes its probability lesser. Akt protein expression is suppressed in high glucose induced PC12 cell may be because of PTEN phosphatase upregulation. But the other stress inducers have responded to stress by increasing their expression in PC12 cells. Thus, the study provides a co-relation between the oxidative stress, calcium binding proteins and the Akt protein in PC12 cells stimulated with different stress inducers.

Conflict of Interests

The authors have no conflict of interests to declare.

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