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Research Article

Dual Role of GSK-3β in Vegetative Cells of *Chlamydomonas reinhardtii* Exposed to Osmotic Stress Conditions

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Abstract

The enzyme Glycogen Synthase Kinase-3β (GSK-3β) has been reported to exhibit an intriguing capacity to either increase or decrease the apoptotic threshold in animals. However, the pro-apoptotic and anti-necrotic role of GSK-3β has not been studied. In the present study, we show the paradoxical involvement of GSK-3β in KCl-induced apoptosis and NaCl-induced necrotic-like cell death pathways of *Chlamydomonas reinhardtii*. We show here that apoptotic events were abolished by lithium chloride, a GSK-3β inhibitor, resulting in reduced cell death and a reversal of the classical apoptotic hallmark, viz. laddering, upon exposure to apoptotic stress (200 mM KCl). On the other hand, lithium chloride potentiated cell death and DNA shearing in case of necrotic stress that was induced by 200 mM NaCl. Studies of this eccentric ability of GSK-3β to oppositely influence two types of cell death signaling have shed light on the important regulatory role of this enzyme both in apoptosis and necrosis. To the best of our knowledge, this is the first such report on the dual role of GSK-3β in a photosynthetic organism. Further, this will provide the foundation for the rational use of GSK-3β inhibitors on cell death occurring in photosynthetic organisms.

Keywords: GSK-3β; Apoptosis; Necrosis; Oxidative Stress; Osmotic Stress

Introduction

Isolated more than 25 years ago, the multifunctional enzyme, glycogen synthase kinase-3 (GSK-3) is a serine/threonine kinase present in all eukaryotes. It was isolated as a protein kinase that inactivated glycogen synthase via phosphorylation [1] and is the final and rate-limiting enzyme in glycogen biosynthesis. Accumulating data showed its involvement in a variety of signaling pathways that control cellular motility, protein translation, cell proliferation, growth, differentiation and apoptosis [2]. Mechanisms that regulate the functions of GSK-3 include phosphorylation, protein complex formation and subcellular distribution. Therefore, any aberration in the expression and/or regulation of GSK-3 is obviously linked to several pathological conditions, such as diabetes, neuronal abnormalities, Alzheimer's disease, schizophrenia, dopamine-associated behaviors, bipolar disorders, Parkinson's disease and cancer [3].

Plant genomes harbour GSKs and are encoded by a multigene family [4]. Arabidopsis has ten different GSKs. These different plant GSKs are known to be also involved in diverse processes including hormonal signal transduction, development and biotic/abiotic stress responses. In Caenorhabditis elegans, GSK-3 is involved in the endoderm-mesoderm cell-fate decision [6]. While in Dictyostelium, GSK-3 is required for proper cell-fate specification between stalk and spore cells [7]. The yeast Saccharomyces cerevisiae has four genes encoding the GSKs, viz. MDS1 (RIM11, ScGSK-3), MCK1, MRK1 and YOL128c. The functions of MRK1 and YOL128c are unknown. Mammals generally express two homologues of GSK-3, GSK-3α and GSK-3β, which are encoded by separate genes.

Of these two, the β isoform (GSK-3β) regulates cellular growth, differentiation and proliferation, cell cycle progression, embryonic development, apoptosis, insulin response as well as motility [8]. Aberrant GSK-3β expression leads to many pathological condi-

tions. Its dual role as a tumor suppressor and tumor promoter in cancer is very intriguing and was believed to be dependent on both the cell type and it is signaling environment. For example, GSK-3β manifests its role as a tumor suppressor by inhibiting androgen receptor-stimulated cell growth in prostate cancer [9]. On the other hand, its abundant expression in colorectal cancer [10] and participation in nuclear factor-κB (NF-κB)-mediated cell survival in pancreatic cancer [11] makes it a tumor promoter candidate. GSK-3β induces apoptosis under various conditions like hypoxia, endoplasmic reticulum stress, polyglutamine toxicity in patients with Huntington's disease and DNA damage [12-15]. It induces apoptosis by forming a complex with pro-apoptotic transcription factors like p53 and also by inhibiting pro-survival transcription factors like heat shock protein-1 and CREB [16,17]. In *In Vitro* cell cultures like primary neurons, HT-22 cells, PC12 cells, human SH-SY5Y neuroblastoma cells etc. inhibiting GSK-3β resulted in reduced or prevented apoptosis [13,18]. Though there is substantial evidence for the pro-apoptotic role of GSK-3β, it was also seen in neuroblastoma cells that inhibition of GSK-3β promotes apoptosis and decreases cell viability [19]. GSK-3β has been typically identified as an activator of p53-mediated apoptosis, though contradictory reports suggest that inhibition of GSK-3β prevents activation of MDM2 by reducing Ser-254 phosphorylation, which prevents p53 degradation and promotes apoptosis [13]. Damalas and co-workers [20] showed in neuroblastoma cells that GSK-3β shows anti-apoptotic effect under basal conditions through MDM2-dependent degradation of p53 and pro-apoptotic effect by over-expressing a negatively regulated signalling factor, i.e. β-catenin. Similarly, in one study, inhibition of GSK-3β prevented the mitochondrial release of cytochrome c, and initiates apoptosis showing its pro-apoptotic role [13], while in another study inhibition of GSK-3β was shown to promote apoptosis by Phosphatidylinositol 3-kinase (PI3-kinase)/Akt signaling pathway depicting its anti-apoptotic role [21]. These data suggest that more in depth research is required to know the dual role of GSK-3β under different conditions.

Necrosis which is another form of cell death that results from sudden physical damage or toxic insults [22]. It has been shown by multiple researchers that inhibiting apoptosis leads to necrosis [23,24]. The process was not clearly understood though - researchers did not have a view into either the signaling molecules or the exact molecular mechanism that determines a type of cell death. The earliest research on the role of GSK-3β in necrosis was carried out by Yang and co-workers [25]. They looked into the mechanisms that are involved in GSK-3β inhibition-induced cell death. Their findings included the fact that inhibiting GSK-3β results in a strong autophagic response, which then leads to necrotic cell death.

GSK3β enzyme being a protein kinase requires magnesium ions for its catalytic function. Lithium acts as a non-competitive inhibitor under both *in vitro* and *in vivo* conditions. Also known as tau protein kinase 1, GSK-3β phosphorylates Microtubule Associated Proteins (MAP) thereby contributing to the stability of microtubules, such as MAP1B and tau. It was shown in *C. reinhardtii* that MAP1B phosphorylation by GSK-3β increases its affinity for binding to microtubules, resulting in increased microtubule stability. The inhibition of GSK-3β by lithium and its ability to regulate microtubule stability suggest that the lithium-sensitive target involved in flagellar length regulation may be regulated by GSK-3 [26]. Previous studies have also shown that, in mammals, lithium plays an important role, by preventing deleterious effects caused by oxidative stress in neuronal cells [27]. As there is little information about the interplay between GSK-3β regulation and cell death stimuli in promoting apoptosis and necrosis, more studies are required to clearly understand the effects of regulating GSK-3β on the multiple signaling pathways involved in growth, development and metabolism. Therefore, the present study aims at preliminary understanding the role of GSK-3β under osmotic-stress induced cell death in the vegetative cells of *C. reinhardtii*.

Materials and Methods

Culturing, maintenance and exposure of *Chlamydomonas reinhardtii* cells to cells

Free-living, vegetative cells of *Chlamydomonas reinhardtii* were grown and maintained on TAP-agarified medium plates that were sub-cultured once a week. For experimental purposes, a single colony from the plate was inoculated into 50 ml of TAP liquid medium and grown in an incubator (25^0C, continuous light) with continuous shaking. After the liquid culture attained a cell count of ~1 - 1.2 x 10^6 cell/ml, about 50 ml of it was inoculated in 400 ml TAP medium. This culture was harvested and spun at 1,100 X g for 5 minutes. The cell pellets were then re-suspended in fresh TAP medium and a stress agent of required concentration (KCl and NaCl) were added to it. Cells were then harvested at the time-points as mentioned in the results section.

Death assay using Evans blue

The assay was performed as mentioned in [27]. Briefly, two ml of a 50 ml culture was harvested and to the pellet was added 1.8 ml of fresh TAP liquid medium and 200 µl of 1% Evans Blue dye. Post an incubation period of 30 minutes at RT, cells were spun at 1100 x g for 5 minutes followed by two washes, spinning each time at 1100 x g for 5 minutes. The final pellet was then re-suspended in 200 µl of 1% SDS+ 50% methanol solution and incubated at 50^oC, for 30 minutes. The solution was spun at 2800 x g, for 15 minutes.

Ten µl of this supernatant was diluted with 190 µl of DDW and the OD600 measured. A positive control was obtained by exposing 2 ml of control cells to a temperature of 100°C, for 30 minutes prior to adding Evans Blue dye. After obtaining the O.D, percentage death was calculated by considering this positive control value as 100% death and a graph was plotted.

Genomic DNA extraction

45 ml of cell culture was spun at 1100 x g, for 5 minutes. The spent medium was discarded, and the pellet was suspended in 500 µl of CTAB buffer, 9 µl of β-mercaptoethanol was also added to it. The solution was incubated at 65°C, for 1 hour. To the tube, 500 µl of PCI was added, mixed gently and spun at 16,000 x g, for 5 minutes, at 25°C. The clear supernatant was extracted to a new vial. This extraction step was performed twice. Isopropanol in the ratio 1: 0.7 was added to the pooled supernatants, mixed thoroughly and incubated at -20°C for 1 hour. It was then spun at 16,000 x g, for 20 minutes, at 4°C. White pellet of nucleic acid was observed at this step. Isopropanol supernatant was discarded, and the pellet was washed with 500 µl of 70% ethanol thrice at 16,000 x g, for 10 minutes. Remaining ethanol was completely dried, and the pellet was finally dissolved in 24 µl of TE Buffer. In order to degrade the RNA, 2 µl of RNase A was added to this solution and incubated at 37°C, for 1h. The resulting purified DNA was electrophoresed on 1% agarose gel and observed under Trans-UV light in the gel documentation unit.

Results and Discussion

Earlier studies have shown that when the vegetative cells of *C. reinhardtii* are exposed to abiotic stress, an apoptosis-like process ensues, and cells die a programmed cell death [27-30]. This PCD is mitochondrion-mediated and either caspase-dependent [27-29] or caspase-independent [30]. However, when independently exposed to NaCl at concentrations above 200 mM, necrotic cell death occurred [29]. This led to the understanding that *C. reinhardtii* genome harbored molecules that could conduct cell death mediated by at least three different signaling pathways. In trying to dissect the molecular mechanism of PCD and necrosis in the organism, insights from other organisms were sought. GSK-3 is one such molecule who's originally discovered role was that of regulating glycogen metabolism. Its demonstrable role in regulating protein translation, cell proliferation and differentiation, microtubule dynamics, apoptosis (including neuronal death) and motility clearly expanded its cellular function. Use of lithium as an established GSK-3 inhibitor to reverse stressful situations such as DNA damage, free-radical formation and lipid peroxidation in diverse model organisms has been reported [26]. In fact, inhibiting GSK-3β effectively reduces apoptosis following a number of pro-apoptotic stimuli [31].

In order to test the role of GSK-3β in these stress-induced cell death processes, lithium was used as an inhibitor. The effect of inhibiting GSK-3β with lithium when *C. reinhardtii* cells are exposed to abiotic stress that induces cell death was monitored using Evans Blue dye-retention cell death assay (Figure 1). This assay is based on the principle that only dying cells take up the dye and score positive in the dye-binding assay. *Chlamydomonas reinhardtii* cells were pre-treated with different concentrations of LiCl (20, 30 and 40 mM) for one hour and then exposed to respective stress agents followed by quantifying cell death. Cells are treated with KCl alone, ~50% cell death was scored (Figure 1A); but, when GSK-3β was inhibited with different concentrations of LiCl (20, 30 and 40 mM), there was a reduction in the cell death (~36%, 33% and 29% respectively; Figure 1A). Contrary this fact, when the cells were exposed to 200 mM NaCl alone, ~50% of the cells showed death (Figure 1B); and, upon pretreatment with different concentrations of LiCl and then exposed to NaCl, an increased (~58%, 66% and 81% respectively; Figure 1B) cell death was observed. These results clearly indicate that inactivation of GSK-3β enzyme prior to its exposure with KCl resulted in an alleviation of cell death as opposed to the increase seen when exposed with NaCl. This indicated the involvement of this enzyme in the cell death process with a likely dual role.

In order to ascertain whether the cell death process is apoptotic or necrotic the DNA laddering assay was performed. A distinct feature of PCD is the extensive cleavage of nuclear DNA into oligonucleosome-sized fragments. The laddering assay was performed on cells treated with different concentrations of LiCl (20, 30 and 40 mM) and then exposed to different osmotic stress agents (200 mM KCl and 200 mM NaCl). In sync with our earlier observation, we found DNA laddering in cells that were exposed for 200 mM KCl for 24h depicted the characteristic ladder [30] (Figure 2A, lane 3). But, when pretreated with different concentrations of LiCl and then exposed to KCl, no DNA laddering was evident (Figure 2A, Lanes 5, 7, 9). The DNA was the same as was seen for the control, unexposed lane (Figure 2A, Lane 2). This clearly indicated that inhibiting GSK-3β by LiCl rescued the cells from undergoing PCD. However, in contrary, cells exposed to 200 mM NaCl showed DNA shearing (a characteristic feature of necrosis); but, when GSK-3β is inhibited by LiCl and then exposed to NaCl, there was enhanced DNA shearing (Figure 2B), indicating that inhibiting GSK-3β under necrotic stimuli enhanced necrosis.

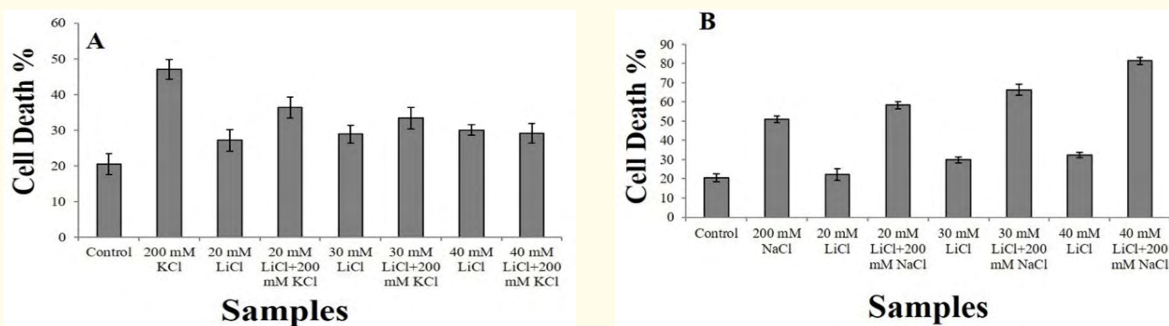

Figure 1: Evans blue cell death assay to detect the effect of LiCl pre-treatment. Effect of pre-treatment with LiCl on (A) KCl and (B) NaCl treated cells, where, inhibition of GSK-3β by LiCl reduces the amount of cell death in case of KCl (A) while it enhances in NaCl (B).

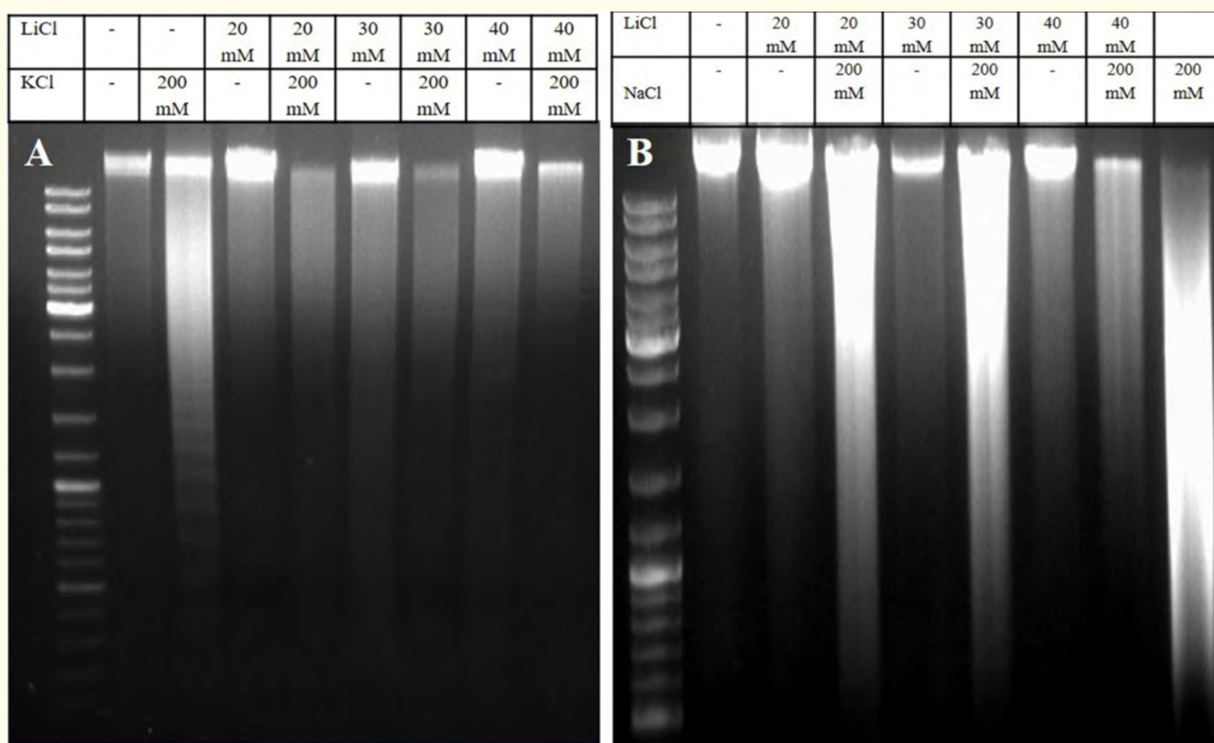

Figure 2: Agarose gel electrophoresis of the genomic DNA extracted from stressed and control cells. (A) Cells treated with 200 mM KCl and with LiCl pre-treatment showed absence of DNA ladder. (B) Cells treated with 200 mM NaCl and with LiCl pretreatment showed enhance DNA shearing.

Conclusion

Taken together, the results of the cell death assay corroborated with that of the DNA laddering, indicate the clear involvement of cellular GSK-3β with an activity that effectively reduces death when exposed to a pro-apoptotic stimulus (Figure 2A) and, another activity that increases death when exposed to a pro-necrotic stimulus (Figure 2B). In other words, with an apoptotic stimulus, GSK-3β gets activated and induces PCD, thereby acting as a pro-apoptotic marker; while, it gets inhibited under necrotic stimuli and induces necrosis behaving as a pro-necrotic marker. Therefore, this interesting finding of one enzyme, glycogen synthase kinase-3 (GSK-3), having the intriguing capacity to either increase or

decrease the necrotic and apoptotic threshold is probably due to its paradoxical effects of oppositely regulating the two major cell death pathways (Figure 3). Studies of this unique ability of GSK-3β to oppositely influence two types of cell death signaling pathways would shed light on important regulatory mechanisms in apoptosis and necrosis, especially in a unicellular, free-living, photosynthetic unicellular organism such as *Chlamydomonas reinhardtii*. It might be plausible that the upstream molecule is obviously different in both the cell death pathways that probably converge on the same molecule that is affected under both conditions.

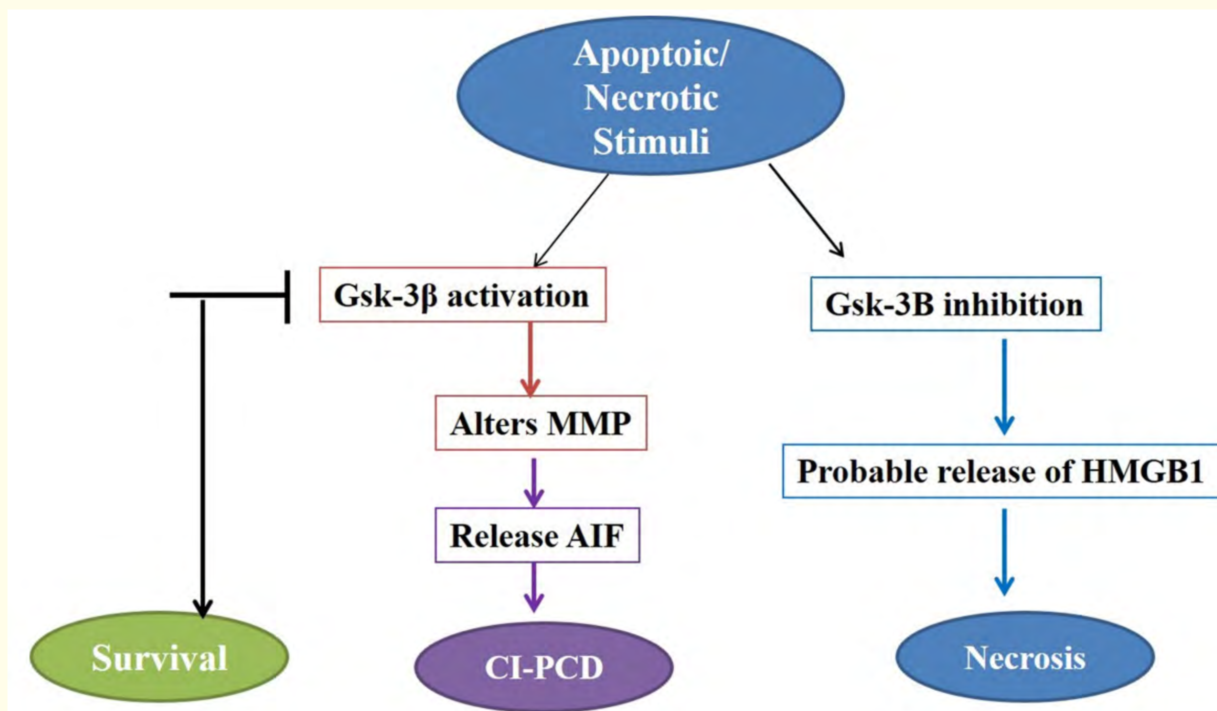

Figure 3: Schematic representation of the probable mechanism of GSk-3β in *C. reinhardtii*.

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Conflict of Interest

There is no conflict of interest exists among the authors.

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