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Der Pharmacia Lettre, 2016, 8 (9):64-69
(http://scholarsresearchlibrary.com/archive.html)

ISSN 0975-5071
USA CODEN: DPLEB4

Effects of oxidative stress on modulating unfolded protein response signaling pathway in K562 chronic myeloid cell line

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ABSTRACT

Unfolded protein response (UPR) is a signaling pathway originating from endoplasmic reticulum (ER). UPR activates upon aggregation of unfolded proteins within ER lumen (known as ER stress), and is mediated through three ER membrane anchored proteins. UPR causes either survival or apoptosis of underlying cells. Wide range of UPR triggering conditions has been studied including oxidative stress. However, the role of oxidative stress on UPR activity is somehow controversial. We evaluated if oxidative stress could potentially suppress UPR activation in specific conditions. Multiple cellular stress categories were designed using different combinations of hydrogen peroxide (H_2O_2), Tunicamycin (Tm) and Thapsigargin (Tg).Then, expression of UPR target genes, Grp94 and Gadd153, assessed by real time PCR. We observed that the expression of UPR target genes was modified by oxidative stress depending on oxidative stress timing of induction. Simultaneous and especially previous association of oxidative stress with ER stress inhibited UPR target genes expression in a variable manner. However, exposure to oxidative stress after induction of ER stress showed a different partial-suppressive gene expression pattern. We also observed that preferential expression of apoptotic (Gadd153) gene could be resulted from ER/oxidative stress interaction. Suppressing effect of oxidative stress on expression of UPR target genes in combinational states with ER stress may partly explain the pathology of diseases which are associated with both oxidative and ER stress but unable to respond appropriately by activating UPR.

Keywords: Endoplasmic reticulum (ER) stress; Oxidative stress; unfolded protein response (UPR)

INTRODUCTION

ER stress is defined as a condition disturbing normal endoplasmic reticulum (ER) function as a result of formation of unfolded proteins in ER lumen [1, 2]. Aberrations in cellular or ER Ca^{2+} hemostasis, alternations in protein glycosylation, mutations affecting folding characteristics of peptides and production of excessive reactive oxygen species (ROS) have been designated as common situations causing ER stress[3]. ER stress signaling pathway, UPR (unfolded protein response), consists of cellular transcriptional/translational responses augmenting the cellular ability to cope with stress stimuli. In irresolvable states, however, UPR leads to apoptosis in order to protect organism from injured cells [4, 5].

UPR originates from three ER membrane adaptor proteins. IRE1 (Inositol-requiring protein 1), PERK (protein kinase R-like ER kinase) and ATF6 (activating transcription factor 6) which trigger three UPR branches each performs specific roles in signaling process [6]. These signaling pathways ultimately will cause either survival or death of underlying cells via inducing expression of survival or apoptotic target genes respectively[7]. IRE1 branch (survival pathway) activates Xbp1(X-box binding protein1) transcription factor that induces expression of Grps (glucose regulating proteins) molecular chaperons. On the other hand, Gadd153, induced by PERK, participates as one of the main controller of UPR associated cell death [8].

Role of oxidative stress in UPR activity is not well understood. Scavenging Reactive Oxygen Species (ROS) interfere with UPR activation in human skin fibroblasts (Hs68) and keratinocytes (HaCaT)[9]. Oxidative stress may cause Ca^{2+} release from ER lumen and subsequently activate PERK-Gadd153 apoptotic branch of UPR [9]. Oxidative stress may also disturb function of specific proteins within cytoplasm or ER lumen, and results in UPR activation [10, 11]. In contrast to these studies which show solely an inducing role for oxidative stress, some studies have proposed that oxidative stress may have more complex regulatory effects on UPR activity. Acrolein, a major component of cigarette smoke and an oxidative stress inducer, was shown to induce Gadd153 expression preferentially, and leads to apoptosis while sparing activation of protective genes including Grp78 and Grp94 [12]. In contrast, preferential expression of protective (Grp78) UPR gene along with bypassing Gadd153 expression has been observed in Hepatitis C virus (HCV) infected cells [13]. Interestingly, oxidative stress also contributes in pathogenesis of HCV infection [13]. In another study, it is been indicated that ROS may be a preferential activator of apoptotic rather than protective UPR [14]. Although it has been suggested that apoptotic UPR may be executed through increasing production of ROS within cells, however, subjectivity of objectivity of ROS regarding UPR activity is uncertain [15]. These results suggest a role for oxidative stress in modulating or diverging UPR activity. Nevertheless, this is not well understood and needs to be more investigated.

We examined if oxidative stress could influence induction of UPR target genes by ER stress inducers in various combinational patterns of oxidative/ER stress. We exploited two common ER stress inducers; Tunicamycin (Tm) (which cause aberration in protein glycosylation) and Thapasigargin (Tg) (which interferes with ER calcium hemostasis) along with Hydrogen Peroxide (H_2O_2) as oxidative stress inducer.

MATERIALS AND METHODS

This study was conducted in Avicenna Research Institute and Cancer molecular pathology research center, Imam Reza Hospital of Mashhad University of Medical Sciences, in 2014 and financed by MUMS.

Cell culture: K562 cell line (Pastor Institute, Iran) was cultured in RPMI1640 medium supplemented with 5 % FBS (Gibco) and 1% pen-strep. Cells were incubated in 5% CO_2 condition. After confirmation of 95% cell viability through Trypan Blue staining, cells were subjected to treatments.

Treatments: Treatments of K562 cell line were induced by H_2O_2, Tg and Tm with 3µM, 5 µg/l and 0.1 µM concentrations respectively. One million cells were seeded in 6 well plates. Treatments were conductedin10stress groups. Individual stress conditions were: 1- H_2O_2 , 2-Tg and 3- Tm. Simultaneous association of H_2O_2 with Tg and Tm was evaluated in groups 4 and 5 respectively. We also incorporated a stress group of Tg and Tm simultaneous combination(group 6). Groups 7 and 8 were entitled to prior (4 hours) treatment with H_2O_2 respective to either Tm or Tg. Finally, subsequent treatment with oxidative stress after 4 hours' time period of exposure to either Tm or Tg was examined in groups 9 and 10. For control (unstressed) group we used 0.01% DMSO. Table 1 summarizes all treatment groups. Total time in each group was 8 hours. In groups7, 8, 9 and 10, RNA was extracted after 4 hours of second treatment.

Table 1.Stress groups assessed in current study to evaluate effects of oxidative stress on UPR activity in synergy with ER stress. Total time period was 8 hours. in priority combinational states (groups 7, 8, 9, 10), RNA was extracted after 4 hours of induction of second treatment (Tm: Tunicamycin; Tg: Thapasigargin)

Stress Groups	Agents
Group 1	H_2O_2
Group 2	Tm
Group 3	Tg
Group 4	H_2O_2 + Tm*
Group 5	H_2O_2 + Tg*
Group 6	Tm + Tg*
Group 7	H_2O_2 + Tmϕ
Group 8	H_2O_2 + Tgϕ
Group 9	Tm + H_2O_2ϕ
Group 10	Tg + H_2O_2ϕ
Group 11	Control (0.01% DMSO)
** Both agents were added simultaneously* *ϕ Second treatment was applied after 4 hours of initial treatment.*	

RNA extraction: Total RNA was extracted using total RNA extraction kit (Parstous, Iran) in order to manufacture instructions. At least 1 million cells were used to obtain good quality of RNA which was confirmed by observation of ribosomal RNA on 2% Agaros gel electrophoresis.

cDNA synthesis : cDNA was synthesized using cDNA synthesis kit (Parstous) in order to manufacture instructions. cDNA synthesis was confirmed by RT-PCR on housekeeping GAPDH gene.

Real-time polymerase chain reaction

Comparative real-time by Syber green dye (parstous) was used to measure Grp94 and Gadd153 genes expression. The Primer sequence used in this study were as forward:5'- TCGCCTCAGTTTGAACATTGAC-3' and reverse: 5'-CTTCTGCTGTCTCTTCAGGTTCTTC-3' for Grp94 and forward: 5'- TGGAAATGAAGAGGAAGAATCAAAA-3' and reverse: 5'- CAGCCAAGCCAGAGAAGCA-3' for Gadd153. Primers were designed using Primer3 and Pubmed databases. Thermal profile was set at 10 minutes at 95°C, 40 cycles at 95°C for 15 seconds, followed by 1 minute at 60°C and 30 seconds in 72°C. Reaction mixture contained 10 µl Syber Green dye, 1 µl primer mix with 10 picomol concentration, 1 µl cDNA, and 0.4 µl ROX dye. Total reaction volume was reached to 20 µl by diluted water. Reaction was done on Strategene Mx3000 instrument.

Statistical Analysis

Independent sample-t test was used to examine significant of difference in mean gene expression in different groups.

RESULTS AND DISCUSSION

Simultaneous combinations of Tg and Tm show a stronger synergic effects on UPR than simultaneous combinations of H_2O_2 with either Tm or Tg.

Simultaneous induction of H_2O_2 with either Tg or Tm significantly suppressed expression of protective Grp94 gene ($p=0.02$). Grp94 expression in individual H_2O_2 condition was 2.9 folds, while this expression was reduced in combinations with Tm and Tg (respective expression of 2 and 2.1, figure 1a).

Also, Gadd153fold changesin treated cells with H_2O_2+Tm and H_2O_2+Tg simultaneous induction state were1.6,showinga reduction compared to individual treatment with H_2O_2 (figure 1b). In simultaneous Tm+Tg condition, the expression of both Grp94 and Gadd153 was higher (3.8 and 2 folds respectively) than combinational states with presence of oxidative stress (figure 1).

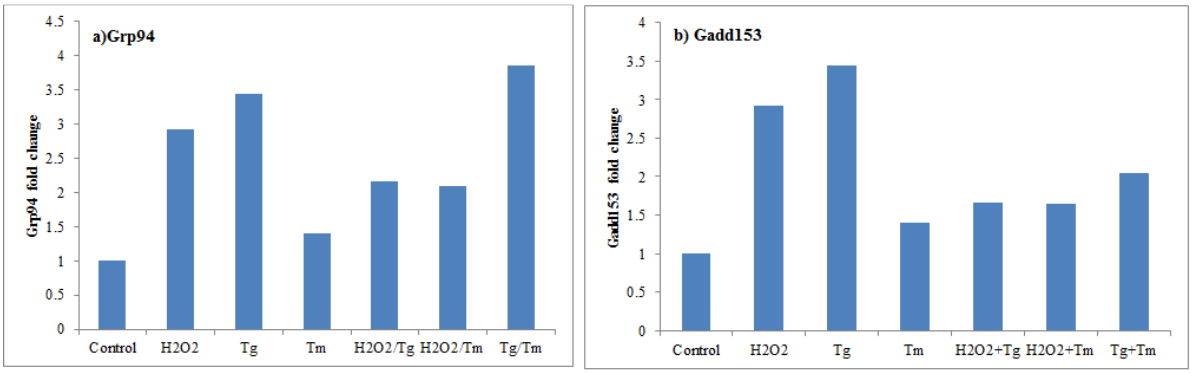

Figure 1. Unfolded protein response (UPR) pathway target genes; a) Grp94 and b) Gadd153 expression in various stress states. Stress groups included Individual exposure to H_2O_2, Tunicamycin (Tm) and Thapasigargin (Tg). In simultaneous conditions, H_2O_2 was presented with either Tm or Tg. Also, a group of simultaneous combination of Tm and Tg was considered. UPR target gene (Grp94) expression was lower in combinations of H_2O_2+Tm and H_2O_2+Tg than either individual H_2O_2 condition (*p=0.02). Grp94 and Gadd153 expression (fold changes respective to control unstressed cells) were 2.9 (H_2O_2); 1.4 (Tm); 3.4 (Tg); 2 and 1.6 (H_2O_2+Tm); 2.1 and 1.6 (H_2O_2+Tg); 3.8 and 2 (Tg+Tm) respectively. Time period of each state was 8 hours

H_2O_2 priority significantly blocked UPR target genes expression

A significant reduction in both Grp94 and Gadd153 expression was observed in conditions with prior(4 hours)exposure toH_2O_2before addition of either Tm (p=0.008)or Tg (p=0.003). See figure 2a.

When $H_2O_2$was added 4 hours after either Tm or Tg, Grp94 expression increased 3.9 and0.9 times respectively(figure 2).Gadd153 expression in Tm (4h)+H_2O_2 and Tg (4 h)+H_2O_2stateselevated6.1 and 4 folds respectively. In this expression pattern, we saw a3 fold change difference between Grp94 and Gadd153 which may indica tea domination of UPR apoptotic branch activity over pro survival.

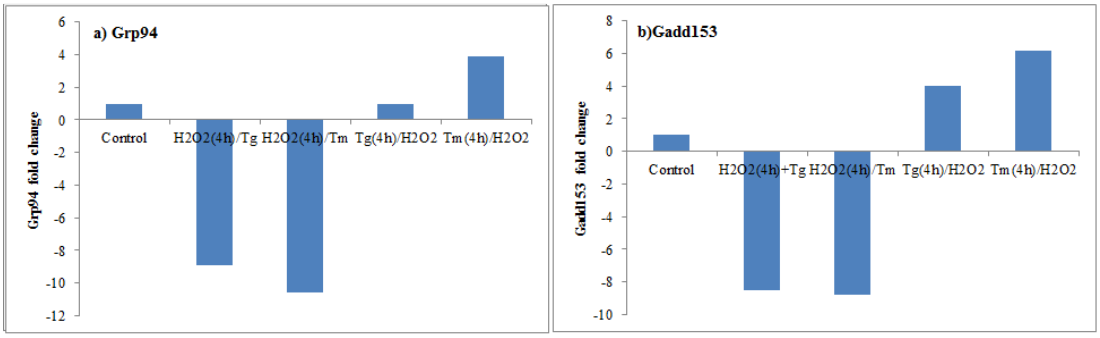

Figure 2. Grp94 (a) and Gadd153 (b) expression in prior or subsequent induction of oxidative stress respective to Tm or Tg. UPR target genes expression were significantly different regarding priority of oxidative or ER stress presentation (P values of * .008 and ** 0.003). Grp94 and Gadd153 expression showed reduction of 10.6 and 8.7 folds in H2O2(4h)+Tm and 8.9 and 8.4 in H2O2(4h)+Tg respectively. In conditions in which H2O2 was added after either Tm or Tg, Grp94 and Gadd153 folds were 3.9 and 6.1 (priority of Tm) and 0.9 and 4 (priority of Tg). A total 8 hours in each state was considered. The second treatment was introduced 4 hours subsequent to first one. In control groups, cells were not exposed to 0.01 DMSO

UPR pathway activation can result in either apoptosisor survival in stressed cells[5]. Interestingly, this is an outstanding characteristic providing a great potential for UPR to be exploited as a therapeutic strategy in either pathologic undesired hyperplasia or hypoplasia conditions. To achieving this, determinants of balance of UPR apoptotic/survival signaling should be well realized.

In present study, we observed that especially in prior and to fewer amounts in simultaneous exposure of cells to oxidative stress, ER stress inducers were unable to effectively prompt Grp94 and Gadd153 expressions. However, such suppressing effect was not observed in subsequent introduction of oxidative stress to previously ER stress induced cells. In the study carried out on neurological cell lines by Paschen et al, down regulation of UPR target genes, Grp94, Grp78 and Gadd153 was observed in Tg- induced cells that were previously exposed to H_2O_2[16].In

addition, especially in vivo, UPR activity has not always displayed a predictable outcome. Even though several potential ER stress inducers may be present in cells, UPR still could be blocked or partially activated. Interestingly, oxidative stress often participates as a mediator in situations with blocked or partially activated UPR[17].These results suggest that combinational stress situations may modify UPR gene expression pattern, however, possible mechanisms are largely unclear.

Some studies have implicated the role of oxidative stress as a partial or preferential activator of specific UPR survival or apoptotic branches[9, 12-14].We observed that in condition with primary induction of Tm and then oxidative stress, Gadd153 (apoptotic gene) expression was significantly higher than Grp94 (survival gene). Some other studies have been suggested that oxidative stress may be involved in differential expression of UPR apoptotic[9, 12] or survival[13] genes. These observations suggest that through controlling respective time of oxidative or ER stress execution within cells we may be able to manage UPR activity pattern and UPR survival/apoptosis signaling balance. Depending on hyperplastic or hypoplastic pathologic conditions, forcing of UPR survival/apoptosis balance to the desire direction offers a promising therapeutic strategy in various human disorders. However, discovering precise molecular participants in effects of oxidative/ER stress on UPR activity require more intensive research.

CONCLUSION

Our results indicate that interactions of oxidative stress with different ER stress inducers can exert aregulating effect on UPR genes expression. Considering that many serious human disease originate from death or proliferation of cells, diverging of UPR double edged sword (survival/apoptosis balance) to willing side represent a great opportunity to effectively manage pathologic hyperplasias or hypoplasias.

Acknowledgments
This study was the result of an MSc student thesis supported financially by the vice president of research at Mashhad University of Medical Sciences, Mashhad, Iran. We thank him.

Conflict of Interest
The authors declare that they have no conflict of interest.

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