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Title; What Signals Are Responsible for the Exercise-Induced Increase in the Endoplasmic Reticulum Unfolded Protein Response?

Garth Hunt, Undergraduate Student, Center for Advanced Nutrition, Major: Dietetic
Advisor: MieJung Park-York, PhD

Abstract

The Unfolding Protein Response (UPR) located within the Endoplasmic Reticulum protects against the pathological effects of abnormal proteins made in neurodegenerative diseases by activating three main stress sensors that are responsible for the refolding and/or degradation of proteins and apoptosis. Neurotrophins are protective hormones released during habitual exercise that protect the body from many neurodegenerative disorders. Using the HT22 hippocampal cell line and a combination of common ER stressors, we investigated the role of neurotrophins in regulating UPR activation and the induction of apoptosis. This report will examine the ER stress response of HT-22 cells to Tunicamycin (TM), Dithiothritol (DTT), and Thapsigargin (TG) and the effect of the neurotrophin NGF on this UPR mechanism. By understanding the relationship between exercise, neurotrophins and the UPR mechanism we will be able to reduce or eventually inhibit the incidence of AD and other neurodegenerative disorders.

Introduction

ER stress, caused by the accumulation of unfolded and misfolded proteins, has been suggested to play a major role in the onset and development of Alzheimer's disease and other neurodegenerative disorders. This stress has a major inhibiting effect on the synthesis, development and transport of proteins throughout the body (3). It is through the UPR that the encoded chaperone genes refold or degrade the problem causing misfolded proteins in order for the ER to return to homeostasis. If ER stress is prolonged, an apoptotic signaling pathway (as indicated by activation of C/EBP homologous protein [CHOP] or Janus Kinase [JNK]) will be activated, leading ultimately to cell death. By folding all of the proteins correctly through the activation of the UPR, the cell is able to survive reducing chances of the development of diseases and other disorders.

The presence of unfolded proteins activates genes related with encoding ER resident protein chaperones by initiating the dissociation of the glucose-related protein78 (GRP78, BiP, or Hspa5) from luminal sensors which facilitate protein folding [4-5]. The UPR system is composed of three sensory arms: inositol requiring enzyme 1(IRE1), activating transcription factor 6 (ATF6), and PKR-like ER-resistant kinase (PERK). The first sensor molecule, IRE-1, acts as an endoribonuclease which takes part in splicing the pre mRNA of xbp1. Through this splicing, unspliced xbp1 (xbp1u) is converted into spliced xbp1 (xbp1s) which is then translocated into the nucleus where it triggers transcription of downstream target genes encoding proteins that promote folding of unfolded proteins [11-14]. The second arm, ATF6, is a basic/leucine zipper transcription factor. Upon ER stress, the cytoplasmic domain of ATF6 is cleaved and moved into the nucleus where this active form stimulates expression of the chaperon gene,

BiP/GRP78. The third ER stress sensor pathway, PERK, is a transmembrane kinase that affects the posttranscriptional regulation of protein synthesis. When ER stress activates PERK by phosphorylation, it phosphorylates cytoplasmic eukaryotic initiation factor 2α (eIF2α). During prolonged ER stress, if the three signaling pathways can't deal with the misfolded proteins they begin to induce Caspase 3, and a Caspase 12 gene known as CHOP. All three signals help to signal CHOP but the PERK pathway has a greater influence and allows CHOP to promote apoptosis (2). The Endoplasmic Reticulum(ER) is the cellular location in which many secretory and membrane proteins fold into the completed tertiary structures before transport to their final destinations [1-2]. Various stress conditions lead to an increase in the level of unfolded proteins in the cell. This initiates the unfolded protein response (UPR) the ultimate purpose of which is to lower the burden of the cell for unfolded protein, leading to decrease ER stress [3].

Dr. York's lab has shown that voluntary exercise induces stress on the ER and activates the UPR mechanism (1), but unlike other stressors such as TM, DTT, or TG, exercise does not activate the apoptosis pathway. It has also been known that exercise is protective against a variety of neurodegenerative disorders that consistently plague the human population. Exercise increases the synthesis and secretion of neurotropins, e.g. brain-derived neurotrophic factor (BDNF), Nerve growth factor (NGF), vascular endothelial growth factor (VEGF), galanin, and these responses may underlie the neuroprotective effects. Our aim is to examine if any of these neurotrophins are also directly responsible for both UPR activation or inhibition of the apoptotic pathway. NGF is a common neurotrophin that aids in transport of proteins in the cell and helps supply nutritive support and cell survival. The protectivity of the neurotrophin NGF will be examined in this report along with experiments to identify the optimal concentration of ER stressors for HT-22 cells.

Effect of TM on xbp1

The first stressor used was Tunicamycin (TM), a toxin that can activate the UPR by inhibiting the placing of an oligosaccharide on proteins. This is known as glycosylation. The oligosaccharide is important in the quality of the protein and the length of time it can spend in the ER. Without it proteins degrade quite rapidly (4).

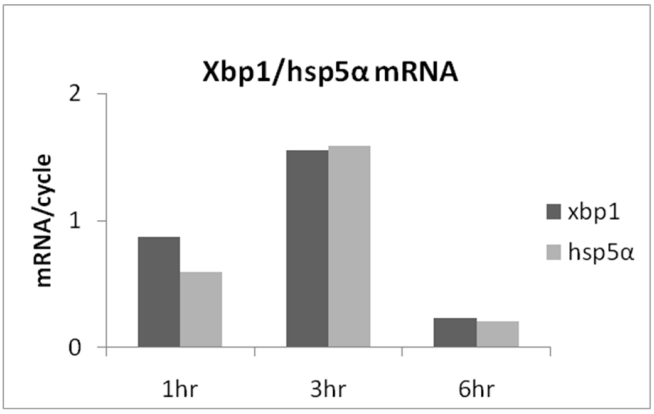

Figure 1. Tunicamycin induces ER stress which activates the UPR mechanism. 3 Hrs incubation proved to show a maximum increase of xbp1 and hsp5α.

We began incubations with 0.2µg/mL concentration of TM and increased consecutively in duplicates up to 1µg/mL. Incubation times were varied from one, three, and six hours after which RNA was extracted using Tri-Reagent and then cleaned up with the Turbo DNase kit. After RNA concentrations were measured, reverse transcriptase reaction was performed to make cDNA. Primers for xbp1 were used to measure their expression level by PCR. Cyclophilin primers were used as a control.

One hour and three hour incubation was shown to increase the effect of xbp1 in HT22 cells proving that the UPR mechanism had been activated but three hours of incubation with TM proved to give maximum increase of the UPR genes xbp1 and hasp5α (Fig. 1). Six hours increased xbp1 at a low dose but decreased at a high dose. This may be due to the cell death by high dose of TM incubation for a longer period of incubation (Fig. 2). Reimertz C et al showed in their reports as well, that prolonged incubation with TM proved to end in apoptosis (3). As TM did not induce the UPR in a consistent manner in HT22 cells, we also tested DTT as another ER stressor.

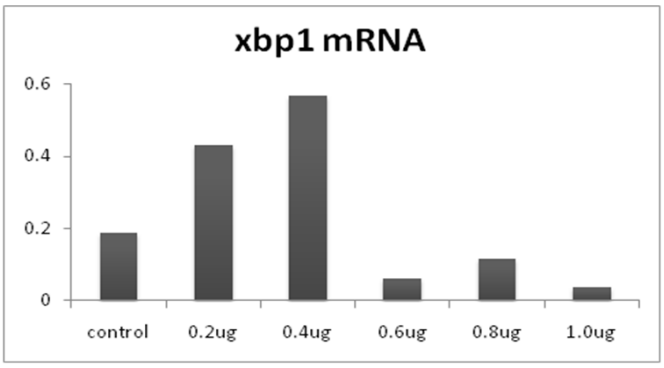

Figure 2. Incubation with TM (either 0.2 or 0.4 µg/ml) for 6 hour increases Xbp1 gene expression while higher dosages decreased xbp1 gene.

Effect of DTT on xbp1

Initial incubations with DTT proved to induce xbp1 in HT22 cells. Our initial concentrations of 1mM and 2 mM showed to highly induce the effect of UPR. Three hours incubation induced xbp1 about 7 fold but these results could not be replicated nor were they consistent. We lowered the dosage to range from 100 to 400µM/mL and found UPR induction was increased at 100µM but decreased at the higher doses.

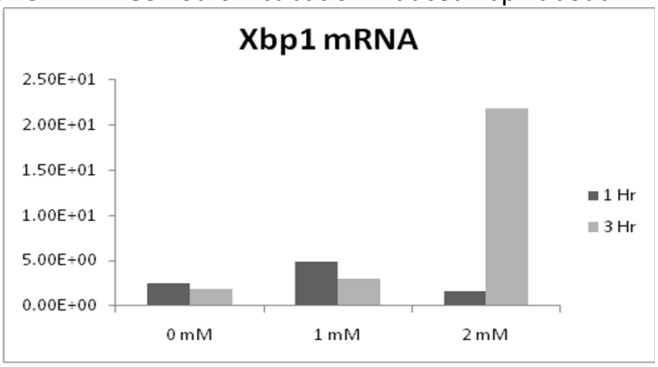

Figure 3. The gene expression of xbp1 was highly increased with 1mM and 2 mM DTT. Incubation with 2mM DTT for three hours showed a major increase.

In our goal to find the optimal concentrations of DTT that induce ER stress we lowered the dosage range further to range between 20 to 80µM/mL. This helped to show a range of where the induction increase was dose-related, but these responses could not be replicated reliably in subsequent studies. We found that we were having trouble getting quality PCR product of RNA/cDNA samples from DTT treated cells. We suspect carry over DTT may have acted as an inhibitor of reverse transcription reaction but did not pursue further to clarify.

Effect of Thapsigargin on xbp1

The last stressor that we used was Thapsigargin. It is a toxin that is used to activate the UPR by disrupting the ER Ca^{2+} levels which ultimately affects the overall charge of the ER and the calcium transport channels. This causes cells to overload with proteins that are not properly transported or exported from cells. According to Geiszt M et al (5), maximum TG concentrations should

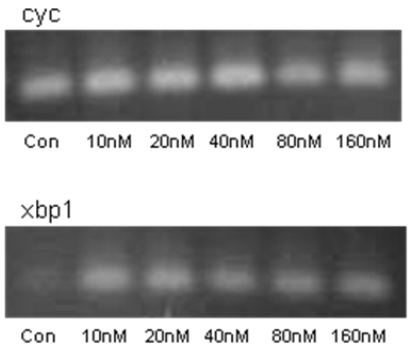

Figure 4. Xbp1 gene expression Caused by TG induced ER stress.

not exceed 50nM and should be incubated for at least 2 hours (5). To confirm this, we incubated five wells of HT-22 cells with the following TG concentrations: 10nM, 20nM, 40nM, 80nM, 160nM. After performing extraction and quantitative PCR we found TG did induce stress on HT22 cells and that 20nM TG incubated for 2 Hrs showed consistent xbp1 increase in gene expression (Fig. 4).

Effect of TG & NGF on xbp1 and CHOP

The first neurotrophin we decided to use was NGF to see if it inhibits TG induced xbp1 gene expression.The results showed in Figure 5A that NGF does not inhibit TG's effect on xbp1 gene expression. We used two different doses of NGF 200pg and 600pg. We did not see a dose effect of NGF in xbp1 gene expression. Interestingly, we observed NGF alone increased xbp1. This is interesting because exercise itself increases UPR, so it suggests neurotrophins released by exercise actually increase UPR.

The next question we asked was if this neurotrophin increases UPR, does it lead to activate apoptotic signaling pathway? We chose CHOP protein as a marker for the apoptotitic pathway to see the response to NGF in the presence and absence of TG. Figure 5B shows that TG induced CHOP protein expression as expected but NGF alone had no effect on CHOP. However, NGF inhibited TG induced CHOP activation, suggesting that NGF may be responsible for the exercise induced increase in UPR and parallel inhibition of apoptosis.

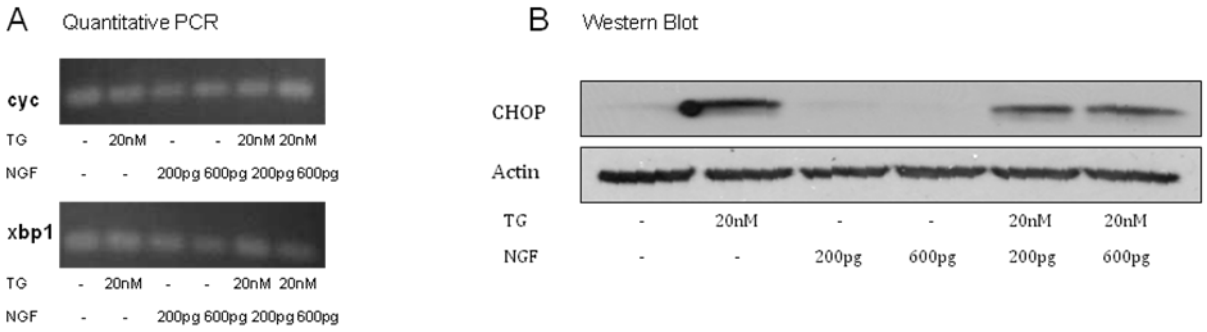

Figure 5. A) xbp1 gene expression B) CHOP protein expression. Both were incubated 2 hours with 20nM TG and 200pg & 600pg NGF.

Conclusion

Thapsigargin, a chemical that depletes the ER of Ca^{2+}, causes stress to the ER. Misfolded or mutated proteins are either refolded or degraded as a result of the UPR mechanism, by doing so it returns the ER to homeostasis. We have shown , using an in vitro cell culture system, that NGF, a neurotrophin released by physical activity, not only increases UPR but also decreases the activation of CHOP, a signaling protein in the apoptotic pathway. This proves to be a significant step in showing that NGF may mediate exercise's neuroprotective effect due to the increase of the ER capacity which is independent with the apoptotic pathway.

References

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