Investigating the Age-Related Loss of Endoplasmic Reticulum Stress Response

Background Information

The Endoplasmic Reticulum and its Function:

The ER is an organelle comprising 10-20% of the cell's total volume where one third of all processing and folding of proteins occurs. Due to its importance, conditions that negatively affect the ER are often detrimental to the cell. The ER is the main organelle in the processing and trafficking of nearly 40% of cellular proteins (Voet, 2004). The fate of these proteins includes integration in the cell membrane, incorporation within lysosomes, and secretion outside the cell. There are several mechanisms designed to direct proteins both to and away from the ER.

Proteins destined for the ER contain stretches of hydrophobic N-terminal signal sequences which attract the binding of a Signal Recognition Particle (SRP). The SRP aids in the transport of the protein/ribosome complex to the ER membrane, where the protein will enter the ER lumen co-translationally through a transmembrane channel known as a translocon. Once inside the ER lumen, signal sequences are cleaved by signal peptidases. If proteins are to postranslationally enter the ER, they are kept in the primary structure by Hsp 70 chaperones until the signal sequence is recognized by the Sec 62/63 ER receptor protein. Upon recognition, the unfolded protein becomes pulled through the translocon by the Hsp 70 chaperone known as BiP (Cooper, 2003). Close to 25% of proteins synthesized are integral proteins destined for association within another membrane (ex: golgi, plasma membrane) (Voet, 2004). These proteins contain hydrophobic stop-transfer

sequences in the middle of the protein which cause the translocon to dissociate. This section of the protein becomes embedded within the hydrophobic interior of the ER membrane as an alpha helix. After the ribosome finishes translating the final portion of the protein in the cytosol, the membrane embedded protein will form a vesicle that leaves the ER for incorporation within another cell membrane (Cooper, 2003).

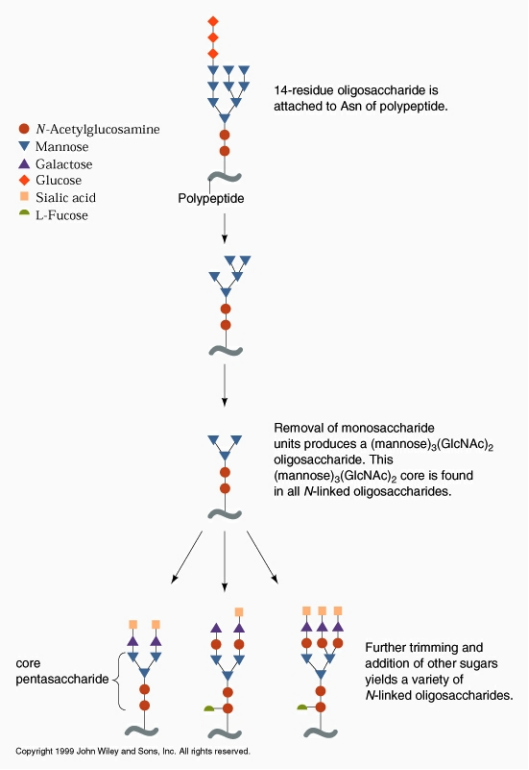

Fig. 1 N-linked glycosylation and further modification

During N-linked glycosylation, N-acetylglucosamine, mannose, and glucose residues become transferred to an Asn of a growing polypeptide. Further modifications include subsequent removal of sugar residues.

For proteins that ultimately enter the ER lumen, many posttranslational processes take place. Aside from aiding in the transport of the protein into the ER, BiP will also aid in the folding process. Disulfide bond formation among cysteine residues in the protein is made possible by the oxidative environment within the ER along with the enzyme protein disulfide isomerase (PDI). Dolicol carriers embedded within the ER membrane aid in protein glycosylation by transferring an oligosaccharide chain composed of N-acetylglucosamine, mannose, and glucose to an asparagine on the growing polypeptide (Fig.1). These glycoproteins play important roles in protein targeting and often play a role in cell-cell recognition. Other ER components, such as glycosylphosphatidylinositol (GPI) anchors, help mediate the incorporation of proteins into cell membranes (Cooper, 2003).

Once processing in the ER is complete, proteins use ER membrane components to form coated vesicles which bud off the ER and enter the cis-Golgi to undergo additional processing and targeting (Voet, 2004).

Mechanisms To Ensure Proper Protein Folding:

There are many mechanisms designed to target proteins to the ER and to aid in post translational modifications. Similarly, the ER has developed many mechanisms to ensure that proteins are properly folded before leaving the organelle.

Close to half of all proteins entering the ER become degraded, due to improper folding (Cooper, 2003). ER chaperones help to correctly fold proteins and if unsuccessful, the protein often becomes marked by ubiquitin and directed to the proteosome for degradation. One example of an ER folding mechanism is the Calnexin pathway. The chaperone, Calnexin, aids in protein folding of a glycoprotein following removal of 2 glucose residues. If properly folded, the protein exits the ER and enters the secretory pathway. If still misfolded, UDP-glucosyltransferase attaches additional glucose residues to the protein so it can be recognized by Calnexin once again. If unsuccessful folding occurs after several cycles, the protein, relocates to the cytosol, gets marked by ubiquitin and becomes degraded in the proteosome (Cooper, 2003).

ER Stress and the Unfolded Protein Response

The manner in which the ER copes with unfolded/misfolded proteins is extremely important in maintaining homeostasis within the cell and within an organism. ER stress is

characterized by the accumulation of unfolded/misfolded proteins in the ER and can be caused by multiple factors, including oxidative stress and lack of glycosylation. Other causes of ER stress include changes in calcium homeostasis or oxidative environment, elevated secretory proteins, expression of misfolded proteins, glucose deprivation, altered glycosylation, and overloaded cholesterol. All of these factors lead to the accumulation of unfolded proteins in the ER lumen and threaten the cell (Zhang, 2004).

The ER has evolved several signaling pathways that cope with the buildup of unfolded/misfolded proteins in the ER and collectively, these homeostatic mechanisms make up the Unfolded Protein Response (UPR). In 1993, Cox, *et al,* investigated components of the UPR in the budding yeast *Saccharomycess cerevisiae*. They identified a gene, IRE1, that encodes a serine/threonine kinase and aids in communication between the ER lumen and the nucleus. IRE1 mutants lacked the ability to produce BiP and PDI. In addition, IRE1 was also shown to play a vital role in cellular survival (Cox, 1993).

The mammalian analog to Ire1p is IRE1α, which is expressed in most cell types and IRE1β, which is found in the intestinal epithelium. The domains of IRE1 include a luminal N-terminal ER stress-sensing domain, a transmembrane domain, a serine/threonine kinase domain, and a cytosolic C-terminal endoribonuclease domain (Zhang, 2004).

Additional UPR initiators, including the protein kinase-like ER kinase (PERK) and the activating transcription factor 6 (ATF6), both of which are found on the ER membrane. PERK, contains both an ER luminal domain that senses stress and a cytosolic domain which phosphorylates the eukaryotic translation initiation factor (eIF2 α). ATF 6

contains a cytosolic N-terminal basic leucine zipper domain and works to sense ER stress with its C-terminal ER luminal domain (Zhang, 2004).

The UPR transducers IRE1, PERK, and ATF6 work to lower the protein load on the stressed ER by minimizing overall translation initiation, and to upregulate specific genes whose products may alleviate the stress experienced by the ER (Zhang, 2004).

BiP, the Hsp 70 chaperone which aids in protein translocation and protein folding, also plays a significant role in proper ER function during ER stress by activating the UPR transducers. Bertollotti, *et al*, investigated the manner in which BiP activates IRE1 α and PERK (Bertollotti, 2000). They used enriched sources of the UPR transducers from pancreatic cells and showed that both PERK and IRE1α complexes with BiP in unstressed cells. PERK and IRE1α had similar functions in the luminal domain, and cells treated with the ER stress inducing agent thapsigargin (which induces an ER stress by altering Ca^{2+} levels) exhibited a dissociation of BiP from both PERK and IRE1α. This was followed by oligomerization, as indicated by an increase in molecular weight and phosphorylation, as indicated by a retarded mobility on a SDS page gel. This suggests that when no BiP is attached, oligomerization induces the kinase activity. Following ER stress, BiP reassociated with PERK and IRE1α (Bertollotti, 2000).

The group led by Chen, *et al*, investigated the mechanism behind ATF6 action. The transmembrane transcription factor ATF 6, upon ER stress is allowed to proceed in the secretory pathway towards the Golgi. The luminal domain has been shown to be a requirement for this. Once at the Golgi, ATF6 is cut by site 1 protease. Subsequent cleavage by site 2 protease then allows for the translocation into the nucleus and the up regulation of certain UPR target genes (Chen, 2002).

The activation of these UPR transducers results in several ER stress coping mechanisms.

Upon activation, IRE1 cleaves the mRNA of a transcription factor known as the X-box DNA-binding Protein (XBP1) and subsequently activates the transcription factor allowing it to promote the upregulation of many UPR target genes (Fig.2). In addition, IRE1 helps to regulate ER-associated degradation (ERAD). When proteins become trapped in low energy-unfolded states, the proteins will retrotranslocate to the cytoplasm through the translocon and become marked for degradation. A deletion in IRE1 has been shown to inhibit this process (Zhang, 2004).

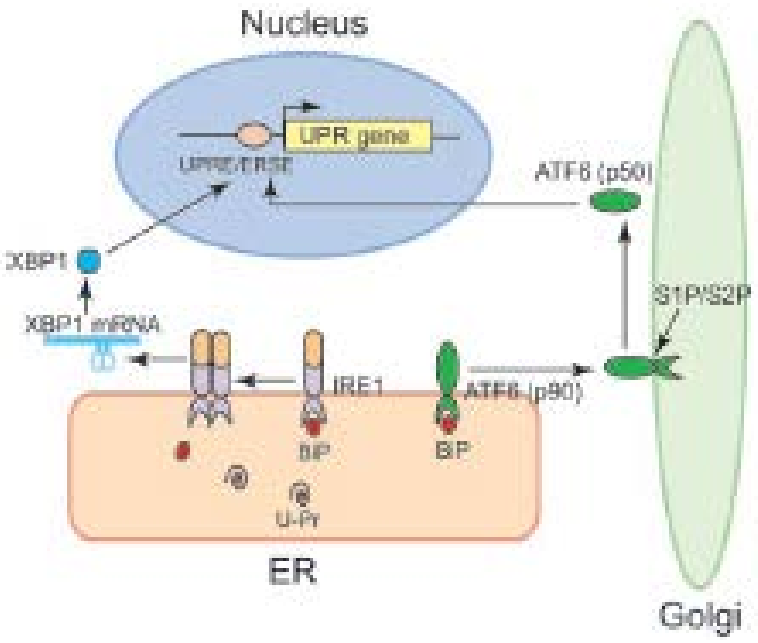

Fig. 2 Signaling by IRE1, ATF6, and XBP

Upon activation, ATF6 goes to the Golgi, becomes cleaved by S1P and S2P and translocates to the nucleus to up regulate UPR genes. Upon activation, IRE1 cleaves XBP1 mRNA, after which, XBP1 will enter the nucleus and up regulate UPR genes (Zhang, 2004).

Upon activation, PERK phosphorylates eIF2α and prevents the formation of the eIF2-GTP-$tRNA^{Met}$ initiation complex that is required for translation. This leads to a decrease in total mRNA translation and helps to reduce the workload of the stressed ER (Zhang, 2004). In addition, phosphorylated eIF2α can also work to promote translation of certain mRNA whose products lead to attenuation of ER Stress (Zhang, 2004). One of the transcription factors that phosphorylated eIF2α is known to activate NF_kB. This activation is correlated with an ER overloaded with membrane proteins and reactive oxygen species, and an increased permeability to Ca^{2+} efflux. Once NF_kB is activated, it helps to alleviate ER stress by regulating immune and anti-apoptotic responses (Oyadomari, 2004).

Another target of the PERK kinase is the transcription factor nuclear factor erythroid2-related factor 2 (Nrf2). In 2003 Cullinan, *et al*, identified this novel PERK substrate. During normal conditions, Nrf2 is bound to the cytoskeleton anchor Keap1. The group found that upon induction of ER stress, PERK phosphorylates Nrf2 and allows subsequent activation and dissociation from Keap1. Upon activation by PERK, Nrf2 translocates to the nucleus and binds to the Antioxidant Response Element (ARE) promoter sequences (Cullinan, 2003). Activated AREs promote the expression of phase II detoxification enzymes which work by adding polar substances to toxins in an organism's liver cells to solubilize them for excretion (Klaasen, 1996).

Nrf2 is shown to upregulate the transcription of γ-glutamylcysteine ligase (GCL), a rate controlling enzyme necessary for glutathione synthesis. Glutathione helps to ensure cellular survival by regulating the redox balance within a cell (Suh, 2004).

Another phase II detoxification enzyme is the NADPH quinone oxido reductase (NQO1). NQO1 acts as an electron donor and aids in the direct conversion of quinones to hydroquinones, thus inhibiting the production of semiquinone radicals that can cause oxidative damage (Jaiswal, 2000).

The up regulation of these and other phase II detoxification enzymes can protect the cell from oxidative damage and can thus promote cell survival during times of ER stress (Fig.3).

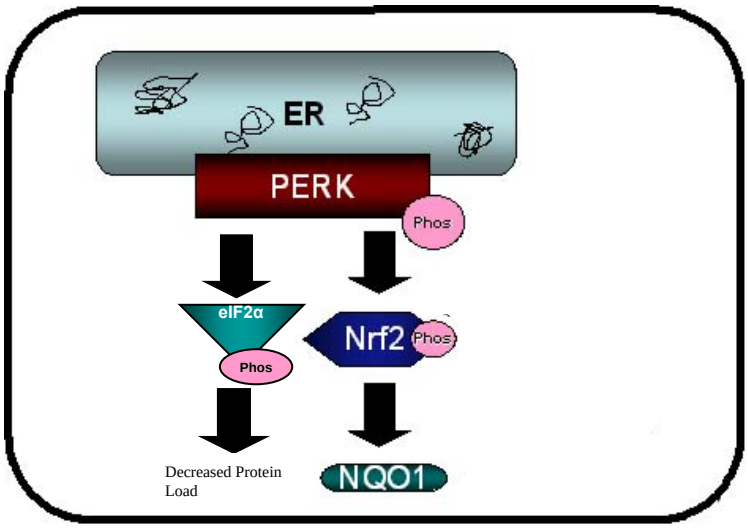

Fig. 3 UPR pro-survival pathways

Upon phosphorylation and activation, PERK phosphorylates and activates eIF2α. This prevents eIF2 α from forming the initiation complex required for protein translation and thus total protein levels decrease. PERK also works to phosphorylate and activate the transcription factor Nrf2. Upon phosphorylation, Nrf2 translocates to the nucleus and up regulates phase II detoxification enzymes that aid in cellular survival.

The UPR regulates mechanisms that help ensure cellular survival, however if excessive stress accumulates for a prolonged period, the cell undergoes self-mediated

death or apoptosis (Fig. 4). One major player in this death route is the C/EBP homologous protein (CHOP) also commonly referred to as growth arrest and DNA damage-inducible gene 153 (GADD 153) (Oyadomari, 2004). This bzip transcription factor becomes activated upon various ER stress inducing factors including tunicamycin and thapsigargin (Zhang, 2004).

In 1993, Larsson, *et al*, investigated the effects of added tunicamycin on A31 cells and their SV 40 transformants (SV A31). They found that the agent tunicamycin, which is an inhibitor of N-linked glycosylation, induced cell death in the post mitotic SV A31 cells (Larsson, 1993).

The same year, Wong, *et al*, treated GH_3 cells with thapsigargin, a tumor promoting sesquiterpene lactone. The substance perturbs Ca^{2+} homeostasis and leads to an inhibition of protein synthesis (Wong, 1993). Tunicamycin and thapsigargin are now commonly accepted as ER stress inducing agents. There are several UPR key players that aid in the activation of CHOP. PERK's activation of eIF2α, eIF2α's up regulation of ATF4 and ATF4's subsequent activation of CHOP is one pathway that activates CHOP. XBP (activated by IRE1 and also upregulated by ATF6) and ATF6 are transcription factors that act to up regulate CHOP expression (Oyadomari, 2004).

In 1998, Wang, *et al*, also showed that the mammalian form of IRE activated CHOP induction. COS1 cells were transfected with mIRE expression plasmids and stained for CHOP. Following tunicamycin treatment, a dominant negative form of IRE1 showed diminished CHOP expression, further supporting the notion that IRE1 is a mode in which CHOP activity is up regulated. Furthermore, CHO cell lines containing mIRE showed noticeable induction of cell death following treatment with thapsigargin. These

results showed that in response to ER stress, the mammalian version of IRE1 activates CHOP and leads the cell towards apoptosis (Wang, 1998).

CHOP then acts to regulate the expression of genes, such as caspases that promote apoptosis (Zhang, 2004). The mitochondria has been shown to be a receiving and amplifying site for the CHOP death signal. CHOP may also work to induce apoptosis by disrupting glutathione levels in the cell and thus altering a homeostatic redox status (Oyadomari, 2004).

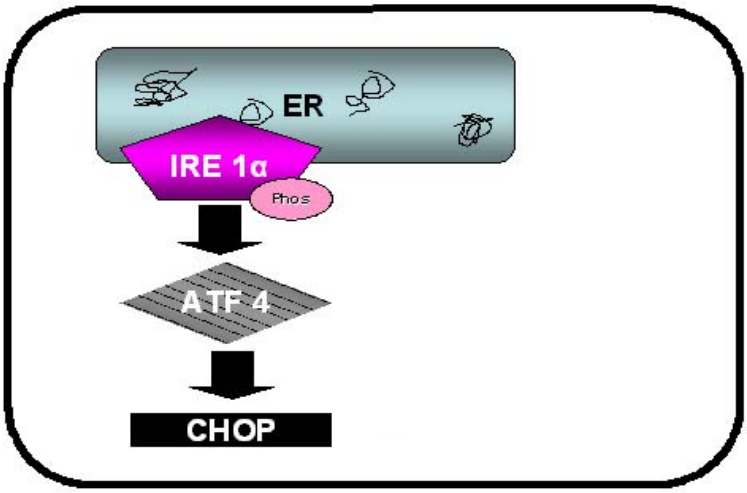

Fig. 4 A UPR pro-apoptosis pathway

Upon activation of IRE1α, a pro-death pathway becomes activated. One of the means in which this pathway acts is through the activation of ATF4 which subsequently up regulates CHOP activity. Downstream signaling of CHOP results in cell programmed death or apoptosis.

The Gap in Knowledge

The aging of the human body is a fate feared by most and inevitable to all. The decline in health that accompanies old age reminds us of our mortality and drives scientists to study the causes of age-related illnesses. On a cellular level, a hallmark of

aging includes vulnerability to a variety of oxidative (Cullinan, 2004), environmental, toxicological, (Cribb, 2005) and pathological (Tsai, 2002) stresses. While diverse in their chemical nature and mode of action, it is interesting to note that many of these stresses commonly target the endoplasmic reticulum (ER).

The maintenance of proper apoptotic levels in an organism plays an extremely important role in maintaining homeostasis. A high rate of apoptosis could lead to neurogenerative diseases through a loss of post-mitotic neurons. An excessive loss of leukocytes could also lead to diminished immune functions (Oyadomari, 2004). Possible clinical implications that could be involved in ER stress and, in particular, the CHOP pathway include: diabetes, heart disease, Alzheimers, Parkinson's and Huntington's.

It is well established that the ER is a crucial organelle in the cell and conditions that affect ER-dependent protein processing are highly detrimental. This is seen in the diseases that are thought to be linked to the accumulation of misfolded proteins and ER stress. Many of the diseases that have been linked to ER stress, such as Parkinson's, Huntington's, and Alzheimers, are also diseases that typically have an age-related onset. This observation raises the question of whether there is a link between ER stress and aging. This phenomenon constitutes a gap in knowledge, because it hasn't been thoroughly investigated. We studied how the survival and death pathways of the UPR alter with age in order to try and bridge this gap.

Materials and Methods

Animals

Young and old Fisher 344 rats were used as an aging model. The young rats were 3 months old. The old rats were 24 months old. Referring to the methods established in Suh, *et al*, these rats were sacrificed and the hepatocyte cells were isolated from their liver. The animals were obtained from the National Institute of Aging animal colonies and were adapted in the Oregon State University animal facilities on a standard chow diet before sacrifice. To control for diurnal variation, the animals were sacrificed between 9 a.m and 10 a.m. each morning. The rats were given diethyl ether as an anesthetic after which an abdominal midline incision was made. The anticoagulant heparin was administered via the iliac vein. A Hanks A salt buffer (pH 7.4) was administered for 5 min through the portal vein to remove blood. After quick removal of the liver, the livers were placed in a calcium chloride and collagenase containing Hanks solution. This solution helps break down the tissue to its cellular components. Following this step, gentle massaging of the liver resulted in hepatocytes separating from the tissue. A Krebs solution (pH 7.3) was used to solubilize and concentrate the cells.

Following liver homogenization, cell viability was measured using trypan blue dye exclusion and hepatocytes were plated at 200,000 cells/cm^2 on collagen coated 6 well plates.

ER Stress Induced

In a manner similar to Li, *et al*, hepatocytes were treated with 5.0 ug/ml of tunicamycin (which induces an ER stress by preventing the maturation of proteins through altered glycosylation) for four hours. After treatment, the cells were harvested at various time points (0, 4,12, 24 hours) and the samples were set aside for analysis. For every batch of cell treatments, control cells (marked 0 hours) in which no tunicamycin was added were set aside to measure normal physiological activity within cells that were not experiencing an ER stress.

qPCR of CHOP and NQO1

From each time point, a set of cells were stored in RNALater (Ambion, Austin TX) at 20°C. Homogenization was accomplished using a sonication homogenizer. Using an RNeasy Midi Kit (Qiagen, Valencia, CA), total RNA was isolated. cDNA was prepared using SuperScript II (Life Technologies, Gaithersburg, MD) and oligo (dT) primers (Qiagen). Samples were run using quantitative real-time PCR concurrently with β actin standard curves. Using gene-specific primers for CHOP and NQO1, levels of these cellular components were measured from all time points obtained from the cell treatments.

Nuclear Nrf2 levels

Nuclear extracts from frozen cell samples were made using NXTRACT CelLytic NuCLEAR Extraction Kit from Sigma-Aldrich. The cells were homogenized with a sonication homogenizer and Western Blot analysis was conducted. 40 ug of protein were loaded in precast 12% TrisHCl polyacrylamide gels (Bio-Rad). The polypeptides were then transferred to nitrocellulose membranes (Amersham Pharmacia) and probed with anti-Nrf2 antibodies. Detection was conduced by an ECL western Blotting Detection kit from Amersham Pharmacia (Suh, 2004). These samples included extracts from cells treated with tunicamycin at various time points, as well as controls.

Viability Assay

In order to ensure that the cells were indeed surviving the ER stress, two cell viability assays were conducted. The first was using trypan blue to check for membrane integrity in viable cells. An additional assay, a Cell Titer Blue Viability Assay, was conducted as a fluorometric method for estimating the number of viable cells in a multiwell plate. The assay uses the indicator dye resazurin to measure the metabolic capacity of cells as an indicator of cell viability. Viable cells retain the ability to reduce resazurin into resorufin which creates a highly fluorescent pink color. The nonviable cells rapidly lose metabolic capacity and cannot reduce the indicator dye and thus fail to generate a fluorescent signal.

Results:

All analysis were compiled from an n=3 young and n=3 old rats.

CHOP mRNA Levels Are the Same In Young and Old

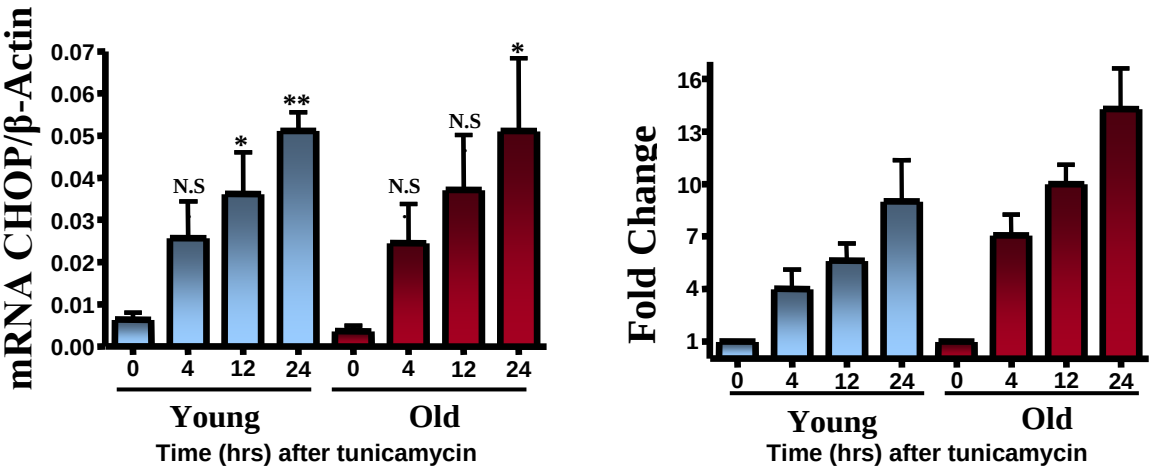

Fig. 5 CHOP mRNA induction with time following ER stress

Results of this analysis showed that hepatocytes from young, and old rats displayed a robust induction of CHOP mRNA with time compared to controls with no tunicamycin treatment. In young cells, a 9-12 fold increase in CHOP mRNA was exhibited 24 hours following tunicamycin treatment ($p<.01$). A similar trend was seen in the old cells, exhibiting a 14-16 fold increase in CHOP mRNA ($p<.05$) 24 hours following tunicamycin treatment.

Results showed that relative to the controls which had no tunicamycin treatment, hepatocytes from young rats exhibited a robust induction of CHOP mRNA with time. (Fig 5). When observing CHOP mRNA levels (standardized against β actin), at 4, 12, and

24 hours, both young and old cells exhibited very similar trends in increased CHOP expression with time. When analyzing the results as a percentage of control (cells in which no ER-Stress inducing tunicamycin was added), the trends become more apparent. Compared with the control, the young cells at 12 hours exhibited a 5-7 fold increase in CHOP ($p<.05$) while at 24 hours, a 9-12 fold increase was exhibited in CHOP ($p<.01$). Similarly, the old cells exhibited a steady increase in the amount of CHOP induced overtime with a statistically significant 14-16 fold increase in CHOP ($p<.05$) shown at 24 hours. Results of this analysis suggest that hepatocytes from young, and old rats have a robust induction of CHOP mRNA with time, compared to controls with no tunicamycin treatment. Similar CHOP mRNA levels in young and old rat cells suggest that the death response pathway through IRE1α remains functional with age.

Tunicamycin Induces NQO1 in Young but Not Old Cells

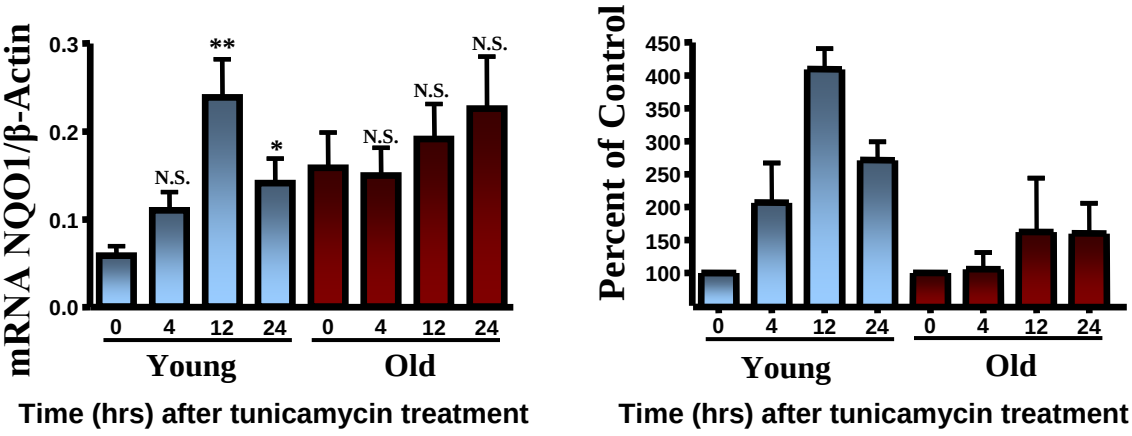

Fig. 6 NQO1 mRNA induction with time following ER stress

The young cells exhibited a 4 fold increase in NQO1 mRNA 12 hours following tunicamycin treatment ($p<.01$) and a 2-3 fold increase at 24 hours ($p<.05$). However, the hepatocytes from old animals showed no statistically significant induction of NQO1 mRNA with time following tunicamycin treatment. Results of this analysis show that hepatocytes from young animals display a robust induction of NQO1 mRNA with time compared to controls with no tunicamycin treatment. In contrast, the old cells do not exhibit a significant increase in NQO1 levels.

Results show that relative to the controls, which had no tunicamycin treatment, hepatocytes from young rats exhibited a robust induction of NQO1 mRNA with time. However, the old cells did not exhibit this trend, showing significantly less NQO1 induction compared to the young cells (Fig 6). In the young, at 12 hours, NQO1 mRNA levels were 4 fold higher than that seen in the non-treated control cells ($P<.01$). After 24 hours, the level of NQO1 dropped, however it still remained 2-3 fold higher than the control ($p<.05$). In contrast to this trend, the hepatocytes from the old rats exhibited no significant change in NQO1 induction following ER stress. At 12 and 24 hours, a 2 fold increase in NQO1 levels could be seen-less than what was exhibited in the young. Furthermore, none of the increases in NQO1 levels seen in the old animals could be verified with statistical analysis.

Nrf2 Nuclear Localization Is Delayed With Age

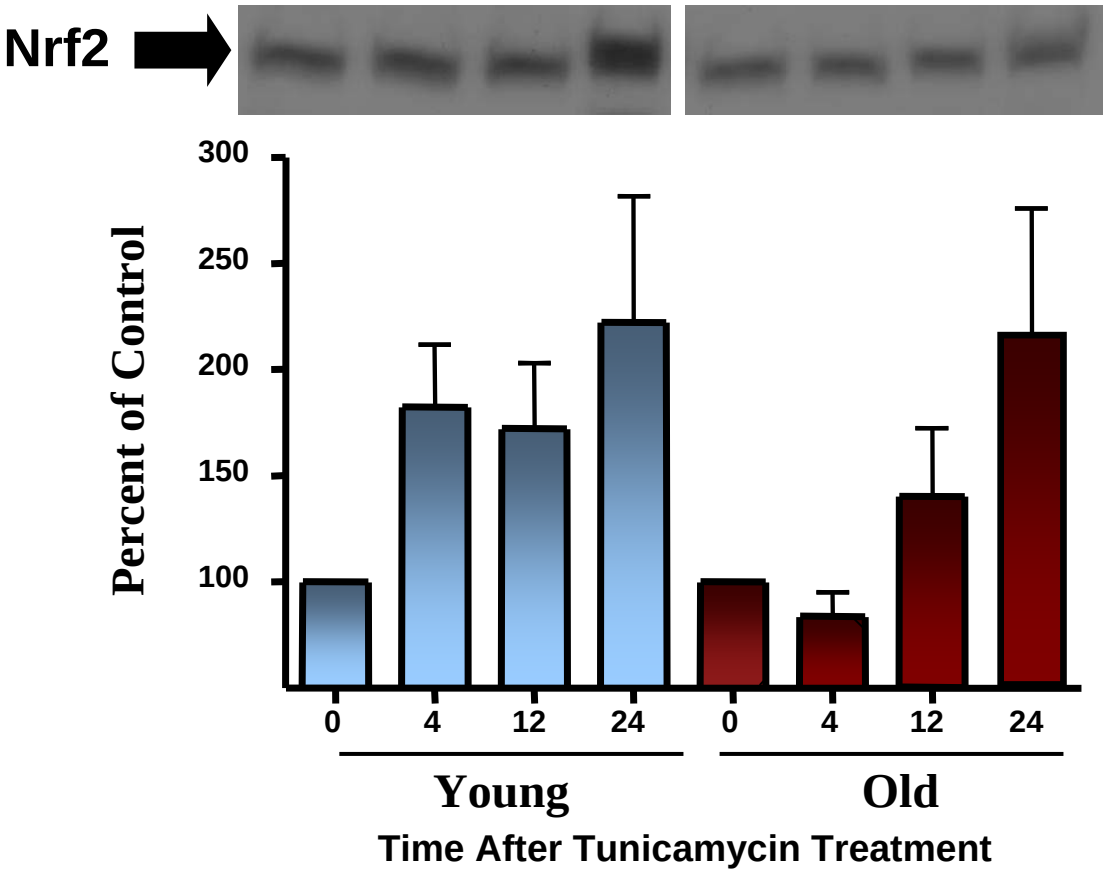

Fig. 7 Levels of nuclear Nrf2 with time following ER stress

Results of this analysis show that the young cells exhibited a 2 fold increase in nuclear Nrf2 levels as soon as 4 hours following tunicamycin treatment. At 24 hours, a 2-3 fold increase was observed. However, the hepatocytes from old animals showed no increase in nuclear Nrf2 levels at 4 hours and only at 24 hours do the old cells exhibit a similar level of nuclear Nrf2 levels (2-3 fold increase) as compared to the young cells. Results imply that hepatocytes from young animals exhibited a quick localization of Nrf2 to the nucleus compared to controls with no tunicamycin treatment. While hepatocytes from old animals also exhibited an increase in nuclear Nrf2 levels, this induction was significantly delayed as compared to the young.

Results from the western blot analyses indicated that nuclear extracts from hepatocytes of young animals showed increased levels of nuclear Nrf2 in the earlier time points, but the extracts from hepatocytes of old animals failed to exhibit the same level of nuclear Nrf2 until the later time points, suggesting that movement of Nrf2 to the nucleus upon ER stress is significantly delayed in old cells (Fig.7). In the hepatocytes isolated from young rats, at 4 hours following tunicamycin treatment, the level of nuclear Nrf2 is about 2 fold higher than that of the control with no tunicamycin treatment. At 12 hours, levels stay similar and at 24 hours, a 2-3 fold increase in nuclear Nrf2 is exhibited. However, in contrast to this, the old cells show no increase in nuclear levels of Nrf2 at 4 hours. At 12 hours, less than a 2-fold increase is exhibited. At 24 hours, the old cells, similar to the young exhibit a 2-3 fold increase in nuclear Nrf2 levels. Results suggest that while hepatocytes from old cells do eventually localize Nrf2 to the nucleus, this localization is significantly delayed when compared to young cells. Rapid movement of Nrf2 to the nucleus following ER stress is important for a cell's survival, thus the results helped to affirm the hypothesis that the aging process creates a glitch in the pro-survival pathway involving Nrf2 and PERK.

Cell Viability Assay

This assay confirmed that the cells were not significantly dying in response to treatments.

Discussion/Clinical Implications:

We conclude that, upon induction of ER stress, the pro-apoptotic pathway acting through IRE1α remains the same in cells from young and old rats. In contrast, we discovered an age-related loss of the pro-survival pathway acting through PERK and Nrf2. These data may explain the susceptibility of older individuals to various diseases. When a cell responds to an ER stress, it's essentially entering a race between survival and death. This finding suggests in young animals that survival and death pathways are nearly equally active. However, in old animals, the survival pathways may be down regulated, while death pathways remain active. These results may help explain many physical attributes of aging due to the loss of cells as they become more susceptible to the death pathway with increasing age. Understanding the dynamics of cellular death could provide important insights and treatments for age-related diseases.

Parkinson's

The group led by Ryu, *et al*, investigated cellular components that are associated with Parkinson's Disease (PD). They treated mice with the compound 6-hydroxydopamine (6-OHDA) to mimic neural degeneration that occurs in PD. Following treatment, a method known as Serial Analysis of Gene Expression (SAGE) was used to identify gene expression following 6-OHDA treatment. Many UPR transcripts were

identified, including PERK, BiP, ATF4, and CHOP. Phosphorylated levels of IRE1α and PERK were exhibited by shifts in SDS page assays, similar to what is exhibited upon cellular treatment with DTT, a known UPR causing agent. Increased cell death was exhibited in 6-OHDA treated sympathetic neurons from ganglia of mice lacking ($Perk^{-/-}$). This suggests that neurons with a defect in the ER stress coping mechanism are more sensitive to death. A correlation also existed between death promoted by 6-OHDA and CHOP induction (Ryu, 2002).

Thus, strong evidence exists that ER stress is linked to the development of PD. Considering the typical age of patients diagnosed with PD is around 60, it is clear that the disease has an age-related onset. This matches with our results that suggest that the cell loses its ability to cope with ER stress with age.

Heart Disease

Hyperhomocysteinemia, is when levels of homocysteine accumulate in the plasma. Elevated levels of homocysteine have been shown to cause damage to endothelial cells and the research team lead by Zhang, *et al*, sought to investigate if elevated levels of homocysteine caused cell death. They found that homocysteine induced cell death in human umbilical vein endothelial cells (HUVEC). This was shown to occur through the activation of the UPR and CHOP. A mutant form of IRE1 failed to activate CHOP further verifying the dependence on CHOP activation by the transmembrane kinase. Results indicated that disrupted ER function is a plausible method for

homocysteine's ability to induce cell death in a manner similar to tunicamycin (Zhang, 2001).

Levels of homocysteine are shown to increase with age and this elevation has been linked with various conditions associated with heart disease. Heart disease is the leading cause of death in the US for both men and women and the risk factors for the ailment greatly increase with age. Thus, the implication of a diminished cellular response to ER stress with age is also relevant to the study of cardiovascular health.

Bone Remodeling

The group led by Hamamura, *et al*, investigated the ways in which osteoblasts utilize ATF4 during osteogenesis. ATF4 can stimulate osteoblast specific genes and CHOP. They investigated whether ER stress stimulates ATF4 in osteoblasts, and if this up-regulation causes the activation of bone remodeling genes or CHOP mediated apoptosis. Their results suggest that the ways in which osteoblasts cope with ER stress depends on the duration of exposure to ER stress. A shorter induction of ER stress led to the expression of genes, such as Runx2 and type one collagen that aid in remodeling. However, a longer exposure to ER stress leads to apoptosis through CHOP (Hamamura, 2007).

Osteoporosis and the weakening of bones both represent the deterioration of bone that accompanies older age. Results from Hamamura, *et al*, as well as our findings imply that an excessive ER stress could possibly produce a situation in which older individuals are more susceptible to osteoblast apoptosis that lead to bone weakening.

Diabetes

In 2003, Araki, *et al*, investigated the links between ER stress and pancreatic β cell death. Pancreatic β cells secrete insulin and loss of beta cells is common in Type I and Type II diabetes. Beta cells have highly involved ER because of the quantity of insulin the cell type produces, and thus they are extremely susceptible to ER stress. Nitric oxide produced by cytokines has been shown to cause beta cell failure. This research group hypothesized that NO caused ER stress and led to a response pathway. They found that NO inducted an ER stress in mice and caused apoptosis by the ER stress pathway through CHOP. The results suggested that an increased insulin demand that would be caused by such factors as obesity or cellular insulin resistance results in an increased insulin demand from the β cells, and results in β cell overload. This 'β cell exhaustion' can lead to dysfunction and apoptosis (Araki, 2003).

While Type I diabetes is often considered juvenile diabetes, due to the onset that commonly occurs in childhood, the risks of Type II diabetes increase with age. This provides another example of where the age related loss of endoplasmic reticulum stress response can be correlated with the increased risk of developing a physiological condition with age.

Ophthalmic Disorders

One of the most common physiological declines that accompany old age is the limitations placed on vision. The death of ganglion cell is common in glaucoma, optic

neuropathies, and many other ophthalmic disorders. The research group led by Shimazawa, *et al,* investigated retinal cell death as a result of ER stress. They treated retinal ganglion cells (RGC) from rats with tunicamycin over a time course and detected CHOP. They also administered intravitreal injection of tunicamycin in the eyes of mice and noticed retinal ganglion cell layer death. Following intravitreal injections of N-methyl-D-aspartate (NMDA, also shown to induce ER stress) retinal cells also showed increases in BIP and CHOP in the retina. Cultured retinal ganglion cells also exhibited increases in BiP, phosphorylated eif2α, and CHOP following tunicamycin treatment (Shimazawa, 2007).

These findings help link deterioration in retinal cells to ER stress. Our finding which exhibited a cell's diminished ability to respond to ER stress with age, correlates with the age related onset of vision difficulties and the finding that ophthalmic problems are linked to ER stress and retinal cell death.

Future Implications*:*

A commonality that is shared by these illnesses is the up regulation of pro-apoptotic responses as a means to cope with ER stress. Future clinical therapies could be aimed at attempting to control this response and help the cell cope with ER stress in a manner that leaves the cell intact. While apoptosis is a necessary step in many physiological processes, the death of post-mitotic cells such as neurons can lead to irreversible damage. Thus, more research needs to be conducted in order to better understand the ER and the ways in which it copes with processes that induce stress and disrupt normal function.

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