ER Stress Response in Human Cellular Models of Senescence

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Abstract

The aging process is characterized by progressive accumulation of damaged biomolecules in the endoplasmic reticulum, as result of increased oxidative stress accompanying cellular senescence. In agreement, we hypothesized that WI-38 human cellular models of replicative senescence and stress-induced premature senescence (SIPS) induced by hydrogen peroxide (H2O2-SIPS) or copper sulfate (CuSO4-SIPS) would present endoplasmic reticulum chaperoning mechanisms impairment and unfolded protein response activation. Results show that in replicative senescence and CuSO4-SIPS, immunoglobulin binding protein, calnexin, protein disulfide isomerase, and ER oxireductin-1 levels adjust to restore proteostasis and inositol-requiring enzyme-1 (IRE1)-, activating transcription factor 6 (ATF6)-, and pancreatic ER kinase (PERK)-mediated unfolded protein response are activated. However, H2O2-SIPS does not exhibit IRE1 and ATF6 pathways activation but a PERK-mediated upregulation of CCAAT/enhancer-binding protein homologous protein, showing that CuSO4-SIPS mimics better the endoplasmic reticulum molecular events of replicative senescence than H2O2-SIPS. Moreover, unfolded protein response activation is required for both SIPS models induction, because PERK and IRE1 inhibitors decreased senescence-associated beta-galactosidase appearance. In CuSO4-SIPS, the decrease in senescence levels is associated with PERK-driven, but IRE1 independent, cell cycle arrest while in H2O2-SIPS cell proliferation is PERK independent. These results add a step further on the molecular mechanisms that regulate senescence induction; moreover, they validate CuSO4-SIPS model as a useful tool to study cellular stress responses during aging, hoping to postpone age-related health decline.

Key Words: ER stress—Replicative senescence—Copper—Human fibroblasts—SIPS

The increase of elderly population raised interest in the mechanisms underlying the progressive functional decline and homeostasis imbalance that associate with aging. At the molecular level, it is characterized by the continued accumulation of damaged biomolecules, as proteins, which impinge on cell and tissue function. Actually, a role was ascribed to unfolded/misfolded proteins in the normal aging process and the establishment of age-related entities as Alzheimer and Parkinson diseases (1,2). Oxidative stress seems to be a major contributor to such age-related accumulation of abnormal proteins (3,4).

The endoplasmic reticulum (ER) is a target for endogenously generated reactive oxygen species along aging, as evidenced by increased
specific ER resident proteins oxidation (5) and consequent organelle activity impairment (6). Nevertheless, to prevent protein misfolding, accumulation and aggregation, ER possesses a protein quality control chaperoning system that includes key chaperones and enzymes, such as immunoglobulin binding protein (BiP), calnexin, protein disulfide isomerase (PDI), and ER oxireductin-1 (Ero1). A fine regulation of their expression must exist to avoid protein aggregation and cellular damage, but when proteostasis disruption appears, an adaptive, coordinated ER stress response or unfolded protein response (UPR) is activated (7). At the cellular level, this complex response is mediated by three ER transmembrane sensors: pancreatic ER kinase -like ER kinase (PERK), activating transcription factor-6 (ATF6), and inositol-requiring enzyme-1 (IRE1). These UPR pathways limit ER abnormal protein load and alleviate ER stress by attenuating protein translation (8), upregulating ER chaperones (9), and degrading misfolded proteins in the proteasome by an ER-associated degradation process (10). If these protective cellular responses do not restore normal ER functioning, apoptosis is activated (7,11).

The efficiency of this protein quality control system seems to be compromised during aging, as several studies demonstrated an age-related decrease on the expression levels of some ER molecular chaperones and folding enzymes (12,13). However, most of them used different tissues from aged rodents (14–16), whereas ER stress response in in vitro nonmodified human cellular models of senescence was never addressed.

Cellular senescence was firstly described by Hayflick and Moorhead, who demonstrated that serially cultivated human diploid fibroblasts (HDFs) ceased dividing after a number of population doublings (PDs) and became unresponsive to mitogenic stimuli, thus entering in a condition termed replicative senescence (RS) (17). In addition, cells in RS exhibit dramatic changes in morphology, gene expression and organelle structure, mass, and function when compared with proliferating cells (for a review see Ref. (18)).

Besides RS, a senescent phenotype may be prematurely achieved when HDFs are submitted to subcytotoxic doses of oxidative stress inducers such as hydrogen peroxide (19), ultraviolet-B radiation (20), or copper sulfate (21). Cells in this stress-induced premature senescence (SIPS) condition display features of RS, including the typical senescent morphology and gene expression profile (22). Such induced models are frequently used to study molecular mechanisms of cellular senescence.

Taking into account the increased oxidative stress accompanying RS and SIPS human cellular models of aging, it was hypothesized that such conditions would favor ER stress establishment, disturbance of ER chaperoning mechanisms, and UPR pathways activation. In fact, in this study, the activation of protective IRE1-, ATF6-, and PERK-mediated ER stress responses is shown in RS and SIPS human cellular models. Moreover, our data show that PERK and IRE1 activation are necessary for the induction of senescence in CuSO₄- and H₂O₂-SIPS models.

Methods

Cell Culture

WI-38 HDFs, purchased from the European Collection of Cell Cultures, were cultivated in complete medium composed of basal medium Eagle (BME) supplemented with 10% fetal bovine serum, at 37°C in a 5% CO₂ atmosphere. The WI-38 HDFs are considered to be young below 30 PDs and enter senescence at 45 PDs or above. Cells unable to make a PD within 3 weeks were in RS. For the induction of SIPS with hydrogen peroxide (H₂O₂-SIPS) or copper sulfate (CuSO₄-SIPS), young WI-38 HDFs were exposed to subcytotoxic concentrations of H₂O₂ (50, 75, or 100 µM) or CuSO₄ (250 or 350 µM) for 2 or 24 hours, respectively. Then, cells were washed with phosphate buffered saline (PBS) and replaced with fresh complete medium. After a 72-hour resting period, cells were processed for protein or gene expression assessment techniques. Control conditions for each cellular model were: young HDFs for RS, BME for H₂O₂-SIPS, and sodium sulfate for CuSO₄-SIPS. For the inhibition of PERK- or IRE1-mediated ER stress response, 120 nM of PERK-inhibitor GSK2606414 or 10 µM of IRE1-inhibitor 4µ8c (Cat No. 516535 and 412512, respectively, Calbiochem) were added to the medium 1 hour prior to SIPS induction and were maintained throughout the experiments. Control cells were incubated with the inhibitors respective vehicle.

Cell Viability Assay

Cell survival was evaluated using neutral red assay immediately after exposure to CuSO₄ or H₂O₂ either in the presence or absence of 4µ8c or GSK2606414 inhibitors, and compared to controls. In brief, after treatments, the medium was removed and cells were incubated with neutral red in BME (40 µg/mL) for 3 hours at 37°C. They were subsequently washed with PBS, and the dye was extracted from viable cells with 1% acetic acid in 50% ethanol. Optical density was measured at 540 nm using a microplate reader (Infinite 200—TECAN). Control cells represented 100% viability.

Senescence-Associated β-Galactosidase Detection

Cells were seeded in six-well culture plates, 20,000 cells/well; after 48 hours, the senescence biomarker senescence-associated β-galactosidase (SA beta-gal) was detected as described (23). The proportion, in percentage, of SA beta-gal positive cells in each condition was determined by microscopically counting 400 total cells/well from at least three independent experiments.

Cell Proliferation

To assess the different conditions effect on cell proliferation, sulforhodamine B (SRB) assay was used for cell density determination, based on cellular protein content (24). Briefly, 10,000 cells/well were plated onto 96-well plates, submitted to the treatments, and fixed at different time-points after stress (0, 1, 2, 3, and 4 days) with 10% trichloroacetic acid (TCA), 1 hour at 4°C. The TCA-fixed cells were stained for 30 minutes with 0.057% (w/v) SRB in 1% acetic acid solution and then washed four times with 1% acetic acid. Bound dye was solubilized with 10 mM Tris base solution and the absorbance at 510 nm of each well was recorded using a microplate reader (Infinite 200—TECAN).

Western Blot

WI-38 cells were washed with PBS and scrapped on ice in a lysis buffer (10 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 0.1% Triton X-100) supplemented with protease inhibitors cocktail (Sigma-Aldrich). Upon Bradford assay, 20 µg of protein from each cell extract were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Proteins were blotted into a nitrocellulose membrane; after blocking with 5% nonfat dry milk diluted in Tris-buffered saline supplemented with 0.1% tween 20 (TBST), were probed with specific primary antibodies (anti-BiP #3177, anti-calnexin #2679, anti-Ero1 #3264, anti-PDI #3501, anti-IRE1 #3294, anti-PERK #5683, anti-phospho-eIF2α #3398—Cell Signaling Technology; anti-P-JNK sc-6254, anti-ATF6.
sc-14250—Santa Cruz Biotechnology, Inc., and anti-tubulin T5168—SigmaAldrich) overnight at predetermined optimal dilutions. After washing with TBST, immunoblots were incubated with appropriate peroxidase-conjugated secondary antibodies for 1 hour, detected using ECL western blotting substrate (Pierce—Thermo Scientific), and visualized with ChemiDocTM XRS (BioRad Laboratories). Results were quantified by densitometry using the Image Lab software.

Real-Time PCR
Total RNA extracted (PureLink RNA Mini Kid, Ambion) from cells derived from at least three independent cultures was converted into cDNA by reverse transcription reaction. Amplification reaction assays contained SYBR Green Mastermix (Maxima SYBR Green/ROX qPCR Master Mix, Thermo Scientific) and primers (STAB VIDA, Lda.) at optimal concentration. The primer sequences were: BiP, 5-AGTT CTTCGCC TCAAGGTG-3 and 5-TGGTACAGTAACACTGATG-3; CCAAT/enhancer-binding protein homologous protein (CHOP), 5-CAGAGACTGCA GGCTT CCT-3; and TATA box binding protein (TBP), 5-TCAACC AGAATTTGCTCTTAT-3, and 5-CCT GAATCCCTT TAGATA GGTTAGA-3. The protocol used for the quantitative real-time polymerase chain reaction (qPCR) was: 95°C (10 minutes), 40 cycles of 95°C (15 seconds), and 60°C (1 minute). The specific amplification of the spliced variant of human X box-binding protein 1 (XBP1s), the primer sequences used were: 5-GTGCTG TGCTTCGCCAGCAGG-3 and 5-GCCCTTGATATATGCGG-3; and the qPCR protocol was: 95°C (10 minutes), 40 cycles of 95°C (15 seconds), 55°C (30 seconds), and 72°C (30 seconds). The qPCR was performed in the StepOnePlus thermal cycler (Applied Biosystems). The TBP was the selected housekeeping gene when calculating relative transcript levels of the target genes.

Statistical Analysis
Student’s t test was used to compare the means between two different conditions. A p value lower than .05 was considered statistically significant.

Results
Cell Viability, Proliferation and SA β-gal Detection in CuSO4- and H2O2-SIPS Cells
The CuSO4-SIPS cellular model was recently established by exposing WI-38 fibroblasts to 250 μM CuSO4 for 24 hours (21). As 500 μM CuSO4 was found to greatly affect cell viability, here, we evaluated the cytotoxicity of an intermediate concentration (350 μM) to include a higher sublethal dose of copper in all experiments. As presented in Figure 1A, fibroblasts treated with 250 or 350 μM CuSO4 exhibited 75% and 61% viability, respectively. Moreover, cells treated with 50, 75, or 100 μM H2O2, the most commonly used SIPS inducer, had 97%, 94%, or 87% of cell survival when compared with controls.

Both SIPS and RS conditions had a significant increase in the percentage of senescent cells as assessed by the positivity for SA beta-gal activity (Figure 1B). Control cells presented 4% of SA beta-gal positive cells, whereas cells submitted to 250 and 350 μM CuSO4 presented 36% and 39%, respectively. In addition, fibroblasts treated with 50, 75, and 100 μM H2O2 exhibited 20%, 27%, and 34% of stained cells, respectively. Finally, in RS, 80% of the total number of cells were positive for SA beta-gal.

Cell proliferation inhibition is another typical and frequently evaluated marker of the senescence phenotype. Similarly to previous observation (21), 3 days after removing the stressor, cells exposed to 250 or 350 μM CuSO4 presented 49% and 74% of cell proliferation inhibition, respectively, when compared with controls (Figure 1C). In addition, cell proliferation was 40% and 56% inhibited in fibroblasts treated with 75 or 100 μM H2O2, respectively, whereas 50 μM H2O2 treated cells did not present significant inhibition (3%) compared with controls (Figure 1D). In turn, RS cells inhibition was 90% of young actively proliferating cells, as expected (Figure 1C and 1D).

Expression of Key ER Chaperones and Enzymes Is Altered in RS and SIPS Fibroblasts
BiP, an hsp70 family member (25), is one of the first molecular chaperones encountered by newly ER synthesized polypeptides. Its expression in all three cellular senescence models was evaluated by qPCR (Figure 2A). Fibroblasts exposed to 250 or 350 μM CuSO4 presented a 2.9- and 5.7-fold increase in BiP transcript level, respectively, compared with controls, and RS cells exhibited a 24-fold BiP mRNA increase comparatively to young cells. In contrast to copper-treated cells, H2O2-SIPS cells showed BiP mRNA decrease after exposure to 50, 75, or 100 μM H2O2 (0.7-, 0.6- and 0.6-fold variations, respectively) when compared with controls. To confirm these gene expression variations, BiP protein levels were assessed by western blot in the different senescence models (Figure 2B and 2C). Fibroblasts exposed to 250 or 350 μM CuSO4, or in RS exhibited 2.5-, 2.2- and 5.9-fold increase in BiP content; in turn, H2O2-treated cells did not present significant changes in BiP protein levels when compared with controls.

Another analyzed chaperone was calnexin, an ER transmembrane lectin, responsible for the quality control of newly synthesized glycoproteins. Calnexin protein levels decreased in all cellular models (Figure 2B and 2C). Fibroblast exposure to 250 or 350 μM CuSO4, or to 50, 75, or 100 μM H2O2 resulted in calnexin level reduction of 28% or 23% and 38%, 29%, or 32%, respectively, when compared with controls. Similarly, for the RS cellular model, calnexin levels were also lowered by about 33%.

Disulfide bond formation is an additional critical step in the folding of most newly synthesized proteins that translocate through ER membrane. PDI, a thioredoxin superfamily of proteins member, catalyzes the process through thiol-disulfide oxidation, reduction, and isomerization (26). Moreover, it is promoted with the help of Ero1 that maintains a suitable oxidizing environment and is thus, together with PDI, a major intervenor in protein disulfide bond formation. The PDI and Ero1 protein levels were evaluated in the three senescence models (Figure 2B and 2C). Fibroblasts exposed to 250 or 350 μM CuSO4, or to 50, 75, or 100 μM H2O2 showed PDI protein decrease of 28% or 23% and 38%, 29%, or 32%, respectively, when compared with controls. Similarly, for the RS cellular model, calnexin levels were also lowered by about 33%.

BiP mRNA increase comparatively to young cells. In contrast to previous studies, Ero1 protein levels were also lowered by about 33%.
ER Stress Response Transduction in RS and SIPS Human Cellular Models

ER stress is transmitted to the cytoplasm through IRE1, PERK, and ATF6 transmembrane ER sensor proteins that remain inactive while bound to BiP through their luminal domain. However, when unfolded/misfolded proteins accumulate in ER lumen, BiP is recruited to chaperone them, which results in sensors unbinding and consequent activation of their transduction pathways (27).

IRE1 Signaling: XBP1 Splicing and Jun N-Terminal Kinase Activation

IRE1 has an ER luminal N-terminal domain, a single-pass transmembrane segment and a C-terminal cytosolic region containing both a Ser/Thr protein kinase and a endoribonuclease (RNase) domain (28). In ER stress conditions, BiP is released from IRE1 N-terminal domain allowing oligomerization and phosphorylation of its cytosolic domain, which activates IRE1 signaling pathway (Figure 3A). Once activated, C-terminal cytosolic domain of IRE1 acquires RNase activity and cleaves 26 nucleotides from XBP1 mRNA generating an mRNA spliced variant (XBP1s). In addition, IRE1 also plays a role in signaling because its activated kinase domain interacts with TNF receptor-associated factor 2 leading to the phosphorylation/activation of the c-Jun N-terminal kinase (JNK) (29). Activated JNK can then determine downstream events that may contribute to cell survival or to apoptosis (11,30).

To evaluate whether WI-38 fibroblasts senescence activates the IRE1 branch of ER stress response, its levels were assessed in the three cellular senescence models (Figure 3B and 3C). The IRE1 protein was increased by 2- and 2.5-fold in cells exposed to 250 and 350 μM CuSO4, respectively, when compared with controls. In addition, RS cells presented a 6.7-fold augmentation in IRE1 relatively to young fibroblasts. In addition, both RS and CuSO4-SIPS imparted IRE1 functional changes that activated its kinase and C-terminal RNase domains. Phosphorylation levels of JNK (P-JNK) were raised in copper-treated cells (1.5-fold) and in RS model (3-fold) when compared with their controls. Furthermore, assessment of XBP1s transcript levels by qPCR revealed that both models presented a near 2.5-fold increase in XBP1s mRNA content (Figure 3D). In contrast, H2O2-treated cells did not evidence significant variations in IRE1 and P-JNK levels (Figure 3B and 3C), but showed a dose-dependent decrease on XBP1s transcripts (Figure 3D), when compared with controls.

Figure 1. Subcytotoxic concentrations of CuSO4 and H2O2 induce senescence in WI-38 human diploid fibroblasts. (A) Cell viability was measured using neutral red assay immediately after exposure to the different concentrations of CuSO4 or H2O2, assuming that the control condition exhibited 100% of viable cells. (B) The percentage of cells positive for senescence associated beta-galactosidase activity (SA beta-gal) is plotted for cells in H2O2-stress-induced premature senescence (SIPS), CuSO4-SIPS, or replicative senescence (RS). (C and D) Cell proliferation in CuSO4-SIPS, H2O2-SIPS, and RS fibroblasts was assessed using sulforhodamine B assay. Data are expressed as mean ± SEM from at least three independent experiments.
Figure 2. Expression of key endoplasmic reticulum chaperones and enzymes is altered in both replicative senescence (RS) and stress-induced premature senescence (SIPS) fibroblasts. (A) Relative transcript levels of immunoglobulin binding protein (BiP) were quantified by qPCR after normalization to TATA box binding protein expression for the three cellular models of senescence. (B) BiP, calnexin, ER oxireductin-1, and protein disulfide isomerase proteins were detected by Western blot and (C) their relative protein levels were quantified based on densitometry relative to tubulin in the cellular extracts from RS, CuSO₄-, and H₂O₂-SIPS cellular models. Data are expressed as mean ± SEM from at least three independent experiments. *p < .05; **p < .01; ***p < .001; and n.s. = nonsignificant when compared with control.
PERK Pathway: Phosphorylation of Eukaryotic Translation Initiation Factor 2α and CHOP Induction

PERK is an ER transmembrane protein with a cytoplasmic C-terminal domain with Ser/Thr kinase activity and a luminal N-terminal BiP binding domain, similar to that of IRE1 (31). As misfolded/unfolded proteins increase in ER lumen, BiP dissociates from PERK resulting in its oligomerization, autophosphorylation, and kinase domain activation (Figure 4A). Activated PERK phosphorylates eukaryotic translation initiation factor 2α (eIF2α), which leads to inhibition of protein translation and ER protein-folding load reduction (9). In addition, eIF2α phosphorylation results in the selective translation of activating transcription factor-4 (ATF4) (32), which promotes the expression of CHOP, a transcription factor that can either mediate ER stress-induced apoptosis (33) or an autophagy mediated cytoprotective response (34).

In this study, PERK and phosphorylated-eIF2α (p-eIF2α) protein levels were assessed by western blot (Figure 4B and 4C), and CHOP transcription levels were evaluated by qPCR (Figure 4D) in H2O2-SIPS, CuSO4-SIPS, and RS WI-38 human fibroblasts. When compared with controls, PERK protein consistently increased in all three models. Cells exposed to 250 or 350 μM CuSO4 exhibited 2.1- or 2.5-fold increase, respectively; similarly, cells treated with 50, 75, or 100 μM H2O2 presented 1.8-, 2.8-, or 2.7-fold augmentation, respectively; and RS cells showed 4.9-fold rise in PERK abundance when compared with controls. Accordingly, p-eIF2α levels were also increased in the three cellular models studied herein, corroborating PERK activation during cellular senescence: CuSO4-SIPS cells showed a 3.9- or 5.2-fold increase when exposed to 250 or 350 μM CuSO4, respectively; 75 and 100 μM H2O2-treated cells presented 2.3- and 3.0-fold higher levels of p-eIF2α, respectively; and RS fibroblasts exhibited a 3.5-fold activation of p-eIF2α when compared with controls. In contrast, CHOP mRNA levels were slightly diminished in CuSO4-SIPS, RS, and 50 μM H2O2-treated fibroblasts, corresponding to a decrease of about 25%, whereas cells exposed to 75 or 100 μM H2O2 exhibited a statistically significant 1.3-fold increased CHOP expression when compared with controls.

ATF6 Activation by Proteolytic Cleavage

ATF6 is a 50kDa ER transmembrane protein with a DNA binding motif in its cytosolic portion. In ER stress conditions, BiP dissociates from ATF6 and allows its transport to the Golgi compartment, where it is activated by proteolytic cleavage (Figure 5A). The 50kDa cleaved form – ATF6(p50) – now a transcription factor, binds to a ER stress response element and regulates the expression of genes encoding ER chaperones and folding enzymes (3) and also XBP1 and CHOP (9).

To evaluate ATF6 activation, ATF6(p50) was semi-quantified in extracts from the three cellular models (Figure 5B). Cells exposed to 250 or 350 μM CuSO4 presented a significant 1.7- or 2.2-fold increase in ATF6(p50) levels; RS cells, although in a statistically nonsignificant fashion (p = .09), exhibited higher levels (4.5-fold) of activated ATF6 when compared with controls.
H₂O₂ showed a trend to higher (∼1.5-fold) levels of ATF6(p50) when compared with controls, but only the lowest dose tested (50 µM) yielded a statistically significant variation (p = .04).

ER Stress Response Inhibition Attenuates the Appearance of SIPS

The importance of ER stress response activation during cellular senescence was evaluated by inhibiting IRE1 or PERK activities in WI-38 fibroblasts prior to the induction of SIPS. The IRE1 inhibitor 4µ8c and PERK inhibitor GSK2606414 were already tested in several cell lines and were shown to selectively inhibit IRE1-dependent XBP1 splicing and eIF2α phosphorylation, respectively (35, 36). Herein, the efficacy of each one of these inhibitors was verified in cells treated with dithiothreitol (DTT), a potent ER stress inducer. It was demonstrated that the marked increased in XBP1s mRNA and p-eIF2α protein levels induced by DTT was greatly prevented by the presence of the IRE1 and PERK inhibitors, respectively (see Supplementary Figure 1). Because CuSO₄-SIPS cells exhibit both IRE1- and PERK-mediated ER stress response activation (Figures 3 and 4), whereas H₂O₂-SIPS cells just present PERK pathway induction (Figure 4), IRE1 inhibition with 4µ8c was performed prior to SIPS induction with copper sulfate, whereas PERK was inhibited with GSK2606414 before SIPS induction with copper sulfate or hydrogen peroxide. As presented in Figure 6A, GSK2606414 or 4µ8c did not affect cell viability. Regarding cell proliferation, PERK inhibition with GSK2606414 prior to H₂O₂-SIPS induction had no effect when compared with H₂O₂-treated cells without the inhibitor (Figure 6B). However, pretreatment of CuSO₄-treated cells with GSK2606414 resulted in a statistically significant increase in cell proliferation as compared to CuSO₄-treated cells without the inhibitor (Figure 6C). A divergent result was obtained using 4µ8c: the inhibition of IRE1-mediated ER stress response prior to CuSO₄-SIPS induction did not affect copper ability to inhibit cell proliferation (Figure 6D). It is noteworthy to mention that inhibition of IRE1-pathway per se results in a marked inhibition of cell proliferation in WI-38 fibroblasts (control vs. control 4µ8c). This anti-proliferative effect of 4µ8c was already described in KP4 tumor cell line (36), and IRE1 has actually been identified as a cell cycle regulator, as it controls cyclin A1 expression promoting cell proliferation through XBP1 (37).

To evaluate the effect of ER stress response inhibition in the appearance of typical senescence biomarkers, cells were pretreated...
with GSK2606414 or 4µ8c before being exposed to hydrogen peroxide or copper sulfate and the percentage of SA beta-gal positive cells and p21 mRNA relative levels were quantified. The PERK inhibition prior to H$_2$O$_2$-SIPS induction resulted in a decreased number of cells positive for SA beta-gal from 33.7% to 20.1% and in an attenuation of p21 mRNA levels, from 16.4- to 2.1-fold (Figure 7A and 7C). In addition, PERK- and IRE1-inhibition prior to cell exposure to 350 µM CuSO$_4$ resulted in a diminished percentage of positive cells for SA beta-gal from 38.6% in CuSO$_4$-treated cells to 17.1% and 17.2% in the presence of GSK2606414 and 4µ8c, respectively (Figure 7B). Moreover, fibroblasts treated with 350 µM CuSO$_4$ exhibited a 4.1-fold increase in p21 mRNA relative levels when compared with control cells (Figure 7D) and this increase was also significantly prevented by the presence of GSK2606414 (2.0-fold). Regarding IRE1-pathway inhibition, cells treated with 4µ8c prior to CuSO$_4$-SIPS induction did not show any significant variation on p21 transcript levels (3.5-fold) when compared with CuSO$_4$-treated cells.

**Discussion**

This study evaluates the levels of ER chaperoning system interveners and the activation of ER stress response during cellular senescence. The occurrence of ER stress was reported in human senescence induced by tumor-associated mutations (38) or chemotherapeutic agents in neoplastic cells (39), but never in nonmodified senescent cells in vitro. Using RS, H$_2$O$_2$- and CuSO$_4$-SIPS human cellular models, it is shown that BiP, calnexin, PDI, and Ero1 levels are adjusted to deal with senescence-associated imbalance in proteostasis. Moreover, RS triggers a cellular protective ER stress response mediated by IRE1, ATF6, and PERK. This behavior is mimicked by CuSO$_4$-SIPS but not by H$_2$O$_2$-SIPS, which does not exhibit the activation of IRE1 and ATF6 axis of ER stress response and, instead, reveals PERK-mediated upregulation of CHOP.

The BiP chaperone expression is usually induced when misfolded polypeptides accumulate in the ER (40). Aging is likely to result in the impairment of this mechanism as BiP expression was lower in the liver (6), brain cortex (41), and other tissues (16) of aged rodents. In contrast, in this study, the human cellular models of RS and CuSO$_4$-SIPS, but not H$_2$O$_2$-SIPS, had enhanced BiP mRNA and protein levels when compared with controls. One likely explanation for this discrepancy is the effective percentage of senescent cells present in each case. In fact, aged baboons (42,43), monkeys (44), and humans (23) skin biopsies had senescent cells ranging from 0.1% to 15% of the total number. This implies that most BiP in old tissues results from nonsenescence cells which precludes a direct comparison of tissue homogenates with the senescent cell enriched in vitro cellular models. We are convinced that during cellular senescence, either RS or CuSO$_4$-SIPS, moderate oxidative damage on proteins promotes their misfolding/unfolding and aggregation. In such a proteostasis disruption condition, ER stress response is activated, leading to enhanced BiP transcription in an attempt to assist protein folding and restore proteostasis.

Calnexin and PDI were reduced in aged rodent hippocampus (15) and liver (6,14). In addition, calnexin levels in RS and H$_2$O$_2$-SIPS fibroblasts exhibited significant decrease when compared with young cells (45). The current investigation confirmed calnexin downregulation in the three models and further evidenced a similar PDI protein change. The Ero1 protein levels variation along aging, largely unknown so far, evidenced here a senescent-associated increase, probably as a result of an oxidative stress induced effect on transcriptional regulation, as reported in yeast (46). Besides these chaperones and enzymes, aging modulates ER stress response components such as the PERK, IRE1, and ATF6 transmembrane sensors. The efficiency of these cell protective pathways declines during aging, disturbs protein quality control, and can lead to cell death (13–16). Age-related variations in PERK signaling
Figure 6. Pancreatic ER kinase (PERK) and inositol-requiring enzyme-1 (IRE1) inhibition prior to stress-induced premature senescence (SIPS) induction does not affect cell viability but present differential effects on cell proliferation. (A) Cell viability was assessed using neutral red assay immediately after stress in H$_2$O$_2$- and CuSO$_4$-SIPS cells in the presence or absence of PERK (GSK2606414) or IRE1 (4µ8c) inhibitors, assuming that the control condition exhibited 100% of viable cells. Cell proliferation was evaluated using in sulforhodamine B assay at 0, 1, 2, 3, and 4 days after stress in (B) H$_2$O$_2$-SIPS or (C and D) CuSO$_4$-SIPS cells pre-treated or not with 120 nM GSK2606414 or 10 µM 4µ8c, as indicated in the respective plots. The percentage of cell mass was calculated at the fourth day after stress for H$_2$O$_2$-SIPS and CuSO$_4$-SIPS cells in the presence or absence of the inhibitors, considering that the respective control cells represented 100% of cell mass. Data are expressed as mean ± SEM from at least three independent experiments. *p < .05; **p < .01; ***p < .001; and n.s. = nonsignificant when compared with control.
were previously reported, but the results are sparse and conflicting. For instance, in aged rats hippocampus, PERK mRNA was downregulated (15), whereas PERK protein levels were increased in other tissues (16). In addition, it remained to be determined IRE1 or ATF6 pathways involvement in aging as IRE1 inactivation resulted in *Caenorhabditis elegans* decreased life span (2,47) and no change in *Saccharomyces cerevisiae* (48). This study provides compelling evidence that RS and CuSO₄-SIPS conditions activated IRE1 pathway, as shown by enhanced expression of IRE1 protein itself and its downstream effectors XBP1s mRNA and phosphorylated JNK; similarly, a clear rise in ATF6(p50) levels is consequent to ATF6 axis activation upon proteolytic cleavage; and PERK pathway activation was also verified by increased total PERK and p-eIF2α levels, although without increased CHOP transcription. In contrast, H₂O₂-SIPS fibroblasts do not reveal IRE1 or ATF6 signaling activation, but instead they exhibit PERK activation with increased p-eIF2α and CHOP overexpression. These results in RS and CuSO₄-SIPS models favor the view that senescence is accompanied by moderate ER stress which activates protective responses, mediated by IRE1, ATF6, and PERK. In this case, however, PERK-mediated UPR response activation is not upregulating CHOP as it occurs in its most classical view. Instead, although uncertain, a differentially regulated PERK-dependent response leading to CHOP downregulation may be activated in these conditions. In fact, it was recently identified a PERK-responsive microRNA (miR-211) that inhibits CHOP expression, allowing cell homeostasis re-establishment (49). However, further investigation is needed to clarify this point.

Although the pattern of chaperone and ER stress sensors activation is considerably coherent in RS and CuSO₄-SIPS, it is not so in H₂O₂-SIPS model. The latter is the most frequently used in vitro model to study molecular events of cellular senescence because it largely mimics genotypic and phenotypic features of RS. Surprisingly, in this study, H₂O₂-treated cells failed to activate two of the ER stress response pathways that are triggered in RS. In fact, neither ATF6 nor IRE1 pathways were activated in H₂O₂-SIPS and, actually, XBP1s transcript level decreased in fibroblasts treated with H₂O₂ highest doses. As shown earlier in mesangial cells (50), such oxidant environment is likely to affect XBP1s mRNA, which must be stabilized to promote an efficient IRE1-mediated ER stress response (51). In contrast to ATF6 and IRE1, PERK-CHOP pathway is activated in 75 or 100 μM H₂O₂-induced SIPS fibroblasts. The CHOP, apart from its involvement in ER stress-induced apoptosis (33), was recently recognized as essential for the transcriptional activation of autophagy genes (34), preventing cell death (30). In H₂O₂-SIPS, a condition where the cells are known to acquire resistance to cell death, the slightly overexpressed CHOP might be regulating cytoprotective mechanisms as autophagy instead of inducing apoptosis.
The ER acts as a sensor of the physiological state of the cell and is able to trigger an ER stress response that will decide cell fate. It is shown here that the inhibition of ER stress response (either PERK- or IRE1-mediated) before the induction of H$_2$O$_2$- or CuSO$_4$-SIPS leads to the attenuation of the appearance of the senescence biomarker SA beta-gal. Actually, a similar effect had been already described in an oncogene-induced premature senescence model (38). The decrease of CuSO$_4$-induced senescence by GSK2606414 is reinforced by the increase in cell proliferation and decrease in p21 mRNA levels. Actually, PERK activation was already associated with cell cycle blockage, as it was shown that high levels of eIF2alpha phosphorylation are able to induce G1 cell cycle arrest in mouse fibroblasts submitted to hypoxia (52). Interestingly, PERK inhibition prior to H$_2$O$_2$-SIPS did not affect cell proliferation while p21 is still decreased. These results indicate that PERK-mediated ER stress response is implicated in copper ability to induce cell cycle arrest in CuSO$_4$-SIPS model, whereas cell proliferation inhibition by H$_2$O$_2$ is independent of PERK-pathway activation. This differential behavior observed between CuSO$_4$- and H$_2$O$_2$-SIPS cells, already discussed earlier, may arise from the different intensities of stress provoked by each SIPS inducer and the alternative pathways they might be activating in the cell. The IRE1 inhibition prior to cell exposure to CuSO$_4$ did not alter the effect of copper sulfate on cell proliferation or p21 mRNA levels. Together, the data shown here indicate that the cell cycle arrest occurring in CuSO$_4$-SIPS cells is mediated by PERK activation but does not require IRE1 activity.

This study shows that CuSO$_4$-SIPS model mimics better the ER molecular events of RS than H$_2$O$_2$-SIPS and adds further evidence on the molecular mechanisms that regulate the induction of senescence. Copper has recently been involved in the establishment of senescence, because its intracellular levels were increased in RS fibroblasts when compared with young cells (53). Such involvement can now be justified by copper ability to induce the activation of ER stress response, which we demonstrate here to be required for the induction of senescence. Actually, copper homeostasis disruption has been involved in age-related diseases, as Alzheimer’s and Parkinson’s (for a review, see Ref. (39)). Such involvement may provide a link between ER-associated protein degradation and the unfolded-protein response.

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### Supplementary Material

Supplementary material can be found at: [http://biomedgerontology.oxfordjournals.org](http://biomedgerontology.oxfordjournals.org)

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