ER stress response in human cellular models of senescence

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Introduction

Endoplasmic reticulum (ER) possesses a "quality control" chaperoning system engaged in the prevention of protein unfolding, misfolding and aggregation. The efficiency of this system depends on the fine regulation of critical chaperones and enzymes, such as immunoglobulin binding protein (BiP), calnexin, protein disulfide isomerase (PDI) and ER oxireductin-1 (Ero1). However, when proteostasis is disrupted, an adaptive ER stress response mediated by the ER transmembrane sensors, pancreatic ER kinase (PKR)-like ER kinase (PERK), activating transcription factor 6 (ATF6) and inositol-requiring enzyme 1 (IRE1), are activated. These three pathways aim to limit protein load and alleviate ER stress, but if these pro-survival cellular response is not able to restore proteostasis, apoptosis is induced.

The aging process is associated with a progressive accumulation of damaged biomolecules, such as structurally and functionally abnormal proteins, as a result of the increased oxidative stress that accompanies cellular senescence. In agreement, we hypothesized that human cellular models of replicative senescence (RS) and stress-induced premature senescence (SIPS) would present an activation of ER stress response and an impairment of the ER chaperoning mechanisms. In the present study, the expression of BiP, calnexin, PDI and Ero1 as well as IRE1-, ATF6- and PERK-mediated ER stress response activation were assessed in RS, hydrogen peroxide (H_2O_2)-SIPS and copper sulfate ($CuSO_4$)-SIPS.

Methods

Cell culture

WI-38 human fibroblasts were cultivated in BME with 10% FBS. WI-38 cells are considered to be young below 30 population doublings (PDs) and enter senescence at 45 PDs or above. Cells unable to make a PD within 3 weeks were considered in RS. For the induction of H_2O_2-SIPS or $CuSO_4$-SIPS, young fibroblasts were exposed to a subcytotoxic concentration of H_2O_2 (50, 75 or 100 µM) or $CuSO_4$ (250 or 350 µM) for 2 or 24h, respectively. Then, cells were washed with PBS and replaced with fresh complete medium. After a resting period of 72h, cells were processed either for protein or gene expression assessment techniques. Control conditions for each cellular model were: young WI-38 for RS; BME for H_2O_2 -SIPS and sodium sulfate for $CuSO_4$-SIPS.

Western blot

WI-38 fibroblasts from the different conditions were 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 and phosphatase inhibitors cocktails (Sigma-Aldrich®). 20 µg from each cell extract was resolved on SDS-PAGE gels, blotted into a nitrocellulose membrane and probed at pre-determined optimal dilutions with specific primary antibodies (anti-BiP, anti-calnexin, anti-Ero1, anti-PDI, anti-IRE1, anti-PERK , Cell Signaling Technology®; anti-P-JNK, anti-ATF6, Santa Cruz Biotechnology, Inc.; and anti-Tubulin, Sigma-Aldrich®). Then, immunoblots were incubated with the appropriate peroxidase-conjugated secondary antibody, 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 (BioRad Laboratories).

Real time PCR

Total RNA was extracted from cells derived from at least three independent cultures and converted into cDNA by reverse transcription reaction. The protocol used for the real-time PCR was: 95°C (10 min); 40 cycles of 95°C (15 sec) and 60°C (1 min). For the specific amplification of the spliced variant of human XBP1s the protocol was: 95°C (10 min); 40 cycles of 95°C (15 sec), 55°C (30 sec) and 72°C (30 sec). The thermal cycler instrument utilized was the StepOnePlus™ from Applied Biosystems™. TBP was the selected housekeeping gene when calculating relative transcript levels of the target genes.

Acknowledgements

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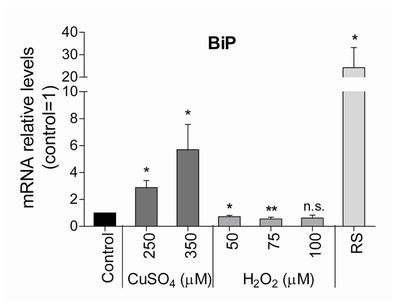
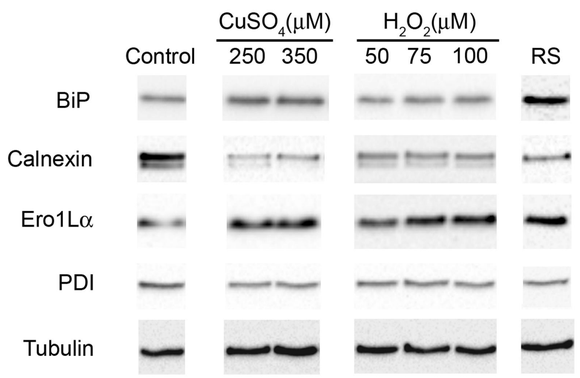
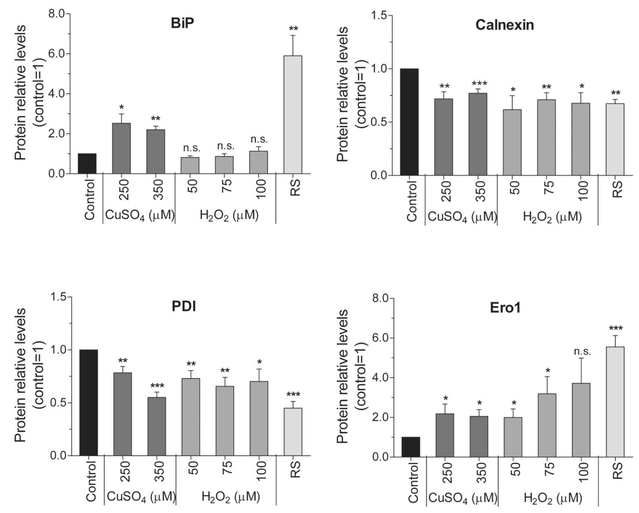
Results

Expression of key ER chaperones and enzymes is altered in RS and SIPS



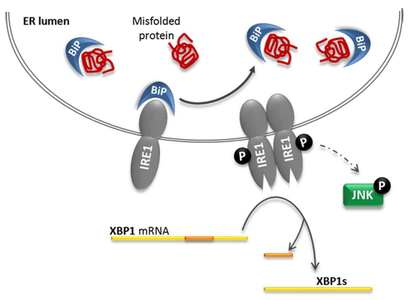
Real time PCR analysis revealed that both RS and $CuSO_4$-SIPS cells presented increased transcript levels of BiP, but, in contrast, H_2O_2-SIPS fibroblasts presented a decrease in BiP mRNA when compared to control cells.

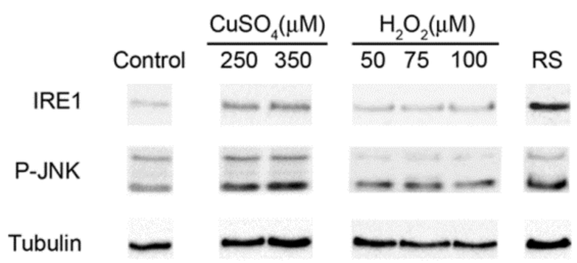


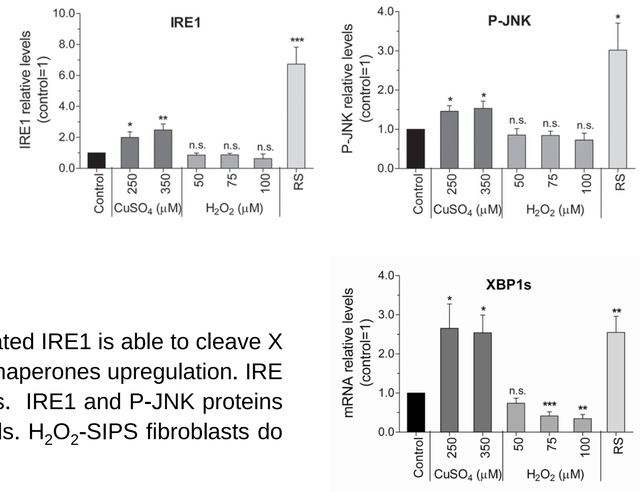


BiP, calnexin, PDI and Ero1 protein levels were assessed by *western blotting* in the different senescence models. BiP protein was found significantly increased both in RS and $CuSO_4$-SIPS cells. Both calnexin and PDI protein levels were reduced in the three cellular models of senescence, whereas Ero1 exhibited an augmentation comparatively to control cells' levels,

IRE1 signalling: XBP1 splicing and JNK phosphorylation

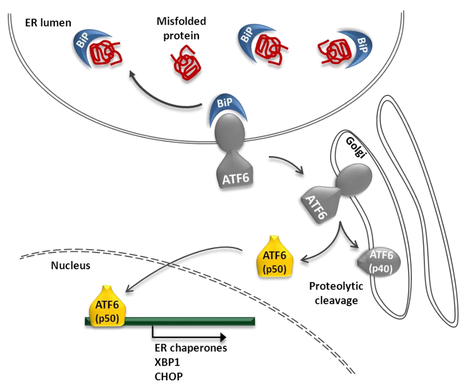


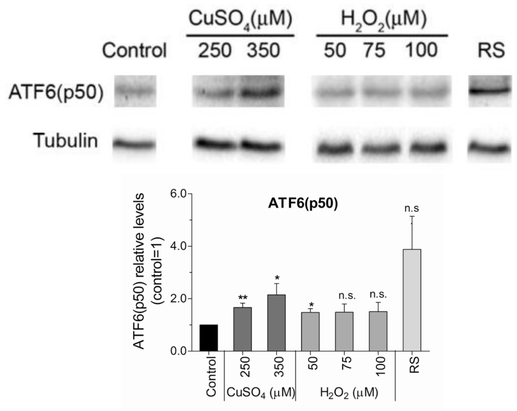




In ER stress conditions, BiP is released from IRE1 and leads to the activation of IRE1 signaling pathway. Activated IRE1 is able to cleave X Box-binding protein 1 (XBP1) mRNA generating an mRNA spliced variant (XBP1s), that ultimately lead to ER chaperones upregulation. IRE acivation can also lead to the phosphorylation of JNK, which may contribute either to cell survival or apoptosis. IRE1 and P-JNK proteins and XBP1s mRNA were found significantly increased in RS and $CuSO_4$-SIPS cells when compared to controls. H_2O_2-SIPS fibroblasts do not seem to have this pathway activated.

ATF6 activation by proteolytic cleavage

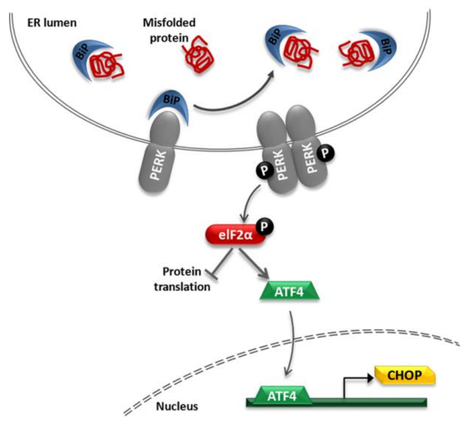


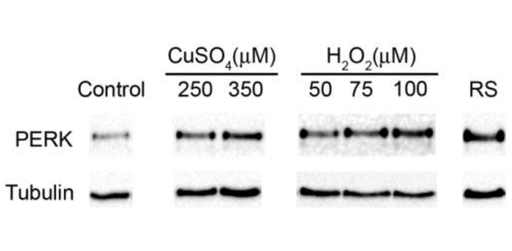


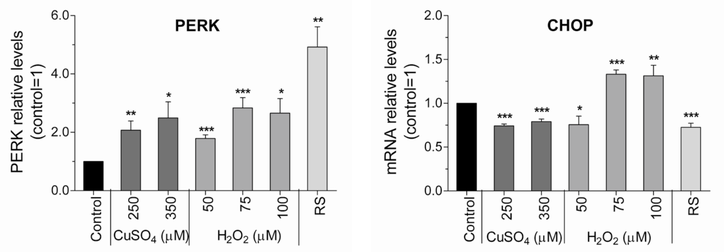
During ER stress, BiP dissociates from ATF6 and allows its transport to the Golgi compartment, where it is activated by proteolytic cleavage. This cleaved form – ATF6(p50) – translocates to the nucleus where it acts as a transcription factor and induces the transcription of ER chaperones, XBP1 and CCAAT/enhancer-binding protein homologous protein (CHOP).

The active ATF6(p50) protein was detected by *western blotting* and was found increased both in RS and $CuSO_4$-SIPS cells. H_2O_2-SIPS cells did not show significant variations in ATF6(p50) levels when compared to controls.

PERK pathway: induction of CHOP







In ER stress conditions, BiP dissociates from PERK. Activated PERK phosphorylates eukaryotic translation initiation factor 2α (eIF2α), leading to the inhibition of general protein translation and the selective translation of activating transcription factor 4 (ATF4), a transcription factor that promotes CHOP expression. CHOP has long been associated with ER stress-induced apoptosis but recently was shown as being able to activate autophagy, leading to cell survival. PERK protein levels were analyzed by *western blotting* and were found increased in the three cellular models of senescence. However, CHOP mRNA was found increased for the cells treated with hydrogen peroxide. Actually, in RS and $CuSO_4$-SIPS cells CHOP transcript levels were significantly reduced when compared to controls.

Conclusions

- **ER molecular changes are similar in RS and $CuSO_4$-SIPS**
  - BiP, calnexin, PDI and Ero1 levels are adjusted to restore proteostasis
  - Protective ER stress responses mediated by IRE1 and ATF6 are activated to promote cell survival
- **H_2O_2-SIPS cellular model does not exhibit IRE1 and ATF6 pathways activation, but a PERK-mediated upregulation of CHOP**
- **Since the disruption of copper homeostasis has been implicated in age-related diseases, as Alzheimer's and Parkinson's, we are convinced that the $CuSO_4$-SIPS model is a valuable tool for their study.**