Closing scientific grant report from 1st September 2014. to 31st August 2018.

Introduction and aims of the project

The lumen of the endoplasmic reticulum (ER) is the first compartment of the eukaryotic secretory pathway. Its characteristic microenvironment ensures proper protein folding which is a vital requirement for the cell. The most characteristic features of the lumen are the high Ca^{2+} concentration and an oxidizing redox balance. Either the depletion of luminal Ca^{2+} or the alteration of the redox balance can lead to ER stress, a dangerous accumulation of unfolded proteins in the ER lumen. ER stress triggers an adaptive program of signal transduction pathways, called the Unfolded Protein Response (UPR). Unresolved ER stress can finally result in programmed cell death.

Recently, several groups reported a reducing shift of the ER luminal redox balance upon Ca^{2+} depletion. Prevention of Ca^{2+} uptake by SERCA pump inhibiting thapsigargine (TG) or hormones inducing ER Ca^{2+} release caused immediate reduction of the ER lumen detected by redox sensitive fluorescent proteins. The rapid reductive shift can be explained by a quick change of the local concentrations of redox active compounds, either by the uptake of reducing or by the release of oxidizing molecules. A further explanation was published after the beginning of the research grant describing the role of calreticulin (CRT) (Avezov et al, 2015).

In this research project, we further examined the underlying mechanism of Ca^{2+} sensitive reduction of the ER lumen by real time measurements using Grx1-roGFP1-iE_{ER} and HyPer-ER and found evidence for a Ca^{2+}-depletion driven GSH transport process through the ER membrane. Any disturbance in redox or calcium balance leads to accumulation of misfolded polypeptides, and activates a signal transduction pathway, the unfolded protein response (UPR) which can lead to cell death. We also wished to examine the details of ER stress induced signaling pathways.

Fluorometric measurements on HyPer-ER sensor were performed in the laboratory of Dr. Miklós Geiszt, Budapest, whereas Grx1-roGFP1-iE_{ER} monitoring was carried out in the laboratory of Christian Appenzeller-Herczog, Basel, Switzerland.

Results

ER luminal redox poise is predominantly determined by the GSH/GSSG ratio; therefore first we measured how the calcium depletion induced reductive shift is influenced by cellular glutathione levels. We depleted cellular GSH levels by overnight treatment with Buthionine sulfoximine (BSO) a GSH synthesis which resulted in a 75 % drop of total glutathione concentration in HEK293 cells. This treatment did not change the steady state of Grx1-roGFP1-iE_{ER} or HyPer-ER; however upon addition of TG the reductive transition was completely abolished.

The possible release of oxidizing molecules from the ER was measured by the redox state of the cytosolic Grx-roGFP2 upon ER calcium depletion. We found that cytosolic redox poise was not

measurably disturbed in response to TG, excluding the possibility of an oxidizing molecule efflux.
Permeabilization of the plasma membrane by digitonin, thus diluting the cellular cytosolic content did not seem to influence the steady-state redox state of HyPer-ER sensor but completely abolished the TG induced lumen reduction in Hela cells.
To assess the changes in intraluminal glutathione we used a recently published calculation method that combines the experimental values of $[GSH]^2$:[GSSG] and [GSH]:[GSSG] (Montero et al, 2013), in collaboration with the laboratory of Christian Appenzeller-Herczog, Basel. We found that Ca^{2+} release drastically increased the total glutathione levels in the ER. Together these results strongly suggested that that cytosolic GSH enters the ER lumen upon ER luminal Ca^{2+} depletion.

Our preliminary data showed that opening the translocon peptide channel by puromycin also causes a reductive shift in ER lumen. This reducing shift could be prevented by anisomycin pretreatment or GSH depletion by BSO. The permeability of the translocon was proved to be regulated by BiP, the calcium binding Hsp70 chaperone of the ER lumen. Therefore we examined whether lowering BiP levels by silencing or specific proteolysis would impinge on the reductive shift upon calcium depletion. HEK cells stably expressing Grx1-roGFP1-iEER were treated with siRNA against BiP for 48 hours before imaging. Although BiP levels were effectively decreased, as shown on western-blots, the TG induced reductive shift did not changed. Next we examined the effect of treatment with AB5-subtilase cytotoxin, which inactivates BiP by specific proteolysis. Monitoring the ER redox state for up to 3 hours after the toxin treatment didn't show change in Grx1-roGFP1-iEER fluorescence or the TG induced reductive shift, therefore we concluded that BiP does not participate in this process.
To confirm the role translocon peptide channel in the possible GSH transport we silenced Sec61 protein which was successful as verified by western-blot. However calcium depletion dependent hypo-oxidation was as prominent in Sec61 lacking cells as in non-silenced cells. These data argues that the Sec61 translocon doesn't participate in the calcium depletion induced redox shift other than being a calcium leakage channel.
We also checked if a nonselective transporter would open in the ER membrane by measuring activity of intraluminal enzymes with high latency. UDP-glucoronyl-transferase and Mannose-6-phosphatase and 11beta-hydroxysteroid-dehydrogenase type 1 were examined, but we could not find decrease in their latency upon calcium release. These results suggest opening of a specific GSH transporter instead of a non-selective pore.

Real time monitoring of Grx1-roGFP1-iEER in HEK293 cells revealed that cyclosporine A (CsA) addition alone surprisingly provoked the same immediate sensor reduction as it was seen after TG addition. This redox transition was also sensitive to cellular GSH content since BSO pretreatment hindered the effect of CsA. Determination of quantitative changes in GSH concentration using the method mentioned above, also verified that CsA treatment drastically increases the GSH content of the ER lumen. In further experiments we examined the known targets of CsA, such as calcineurin and cyclophillin proteins. We found that the CsA provoked lumen reduction was not due to the inhibition of cyclophilin A and B or of calcineurin.

It was suggested that the calcium depletion caused hypo-oxidizing state of ER lumen is a result of sequestration of thiol oxidizing enzyme, PDI1A by the calcium binding calreticulin (CRT) protein (Avezov et al., 2015). We examined this hypothesis in our experimental system using fluorescent probes in calreticulin knockout mouse embryonic fibroblasts. Thapsigargin, puromycin and CsA treatment as well provoked the same ER reduction on wild type and CRT -/- cells suggesting that calreticulin is not required for this phenomenon.

Based on these results we conclude that the influx of cytosolic glutathione rather than the inhibition of local oxidoreductases is responsible for the reductive shift upon Ca^{2+} mobilization and postulate the existence of a Ca^{2+}- and cyclosporine A-sensitive glutathione transporter in the ER membrane.

These results were summarized in a manuscript and under submission to BMC Biology.

We also started to investigate the role of calcium depletion caused redox change in protein homeostasis of the ER. We optimized the conditions for detecting events of the UPR and changes in cell viability due to ER stress.

These experiments were parts a publication (Márton M et al 2017).

Presentations

1.Poster presentation
Beáta Lizák, Gergely Kosztyi, Petra Ágoston, Julia Birk, Davide Montero, Richard Zimmermann, Christian Appenzeller Herzog, Gábor Bánhegyi: Endoplasmic reticulum Ca2+ depletion triggers import of reductants from the cytosol, The structure and function endoplasmic reticulum, EMBO meeting, Girona, Spain, 26-31 October 2014.

2. Selected oral presentation
Gergely Kosztyi, Beáta Lizák, Petra Ágoston, Julia Birk, Davide Montero, Richard Zimmermann, Christian Appenzeller Herzog, Gábor Bánhegyi: Depletion of endoplasmic reticulum Ca2+ provokes import of reductants from the cytosol, Molekuláris Élettudományi Konferencia, Eger 2015. március 27-29.

3.Oral preseantation awarded by first prize
Gergely Kosztyi, Beáta Lizák, Petra Ágoston, Julia Birk, Davide Montero, Richard Zimmermann, Christian Appenzeller Herzog, Gábor Bánhegyi: Depletion of endoplasmic reticulum Ca2+ provokes import of reductants from the cytosol, National Scientific Student's Research Conference, 31st March 2015, Budapest

Publications

1. Márton M, Kurucz A, Lizák B, Margittai É, Bánhegyi G, Kapuy O.

A Systems Biological View of Life-and-Death Decision with Respect to Endoplasmic Reticulum Stress-The Role of PERK Pathway. *Int J Mol Sci. 2017 Jan 5;18(1*).

2. Manusrcipt under submission to BMC Biology:

Beáta Lizák, Julia Birk, Melinda Zana, Gergely Kosztyi, Miklós Geiszt, Richard Zimmermann, Christian Appenzeller Herzog, Gábor Bánhegyi
Ca2+ mobilization dependent hypo-oxidation of the ER lumen is due to influx of cytosolic glutathione

The travel cost of OTKA-PD Grant was used to cover the travel and accommodation costs of Gergely Kosztyi at Molekuláris Élettudományi Konferencia, Eger 2015. március 27-29.

The project was paused from May of 2015 to June 2016 due to maternity leave.