Investigation of alternative stress responses of mammalian cells under mild hyperthermic conditions

Summary of the Ph.D. Thesis

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Introduction

Eukaryotic cells have two evolutionarily highly conserved systems to cope with environmental or pathophysiological stress conditions: the heat shock response (HSR) and the unfolded protein response (UPR) (Almanza et al., 2019; Chen et al., 2023; Ron and Walter, 2007). The effects of stress depend on the stress dose such as intensity and duration. By characterizing its effect on Chinese Hamster Ovary (CHO) cells, we have previously classified heat stress into three distinct categories, namely: severe, involving induction of heat shock proteins (Hsps) together with major macromolecular damage or even cell death; moderate (negligible protein denaturation with less intense Hsp induction), and mild, eliciting 'eustress' (without Hsp induction) (Peksel et al., 2017). We also showed that mild heat triggers a distinct, dose-dependent remodeling of the cellular lipidome followed by the expression of Hsps only at higher temperatures. A significant elevation in the relative concentration of saturated membrane lipid species and specific lysophosphatidylinositol and sphingolipid species suggests rapid membrane microdomain reorganization and an overall time-dependent increase in membrane rigidity in response to fluidizing heat. Our RNAseq experiments revealed that mild heat initiated stress-related signaling cascades in the endoplasmic reticulum (ER) in agreement with previous reports (Bettaieb and Averill-Bates 2015; Tiszlavicz et al. 2022; Xu et al. 2011) resulting in dose-dependent lipid rearrangement and an elevated resistance to membrane fluidization by benzyl alcohol (Tiszlavicz et al. 2022). To protect cells against lethal, protein-denaturing high temperatures, the classical HSP response was evolved. The presence of distinct layers of stress response elicited by different heat dosages highlight the capability of cells to utilize multiple tools to resist and survive potentially lethal stress conditions.

The ER consists of a dynamic membrane network that is required for the synthesis and modification of proteins and lipids. The accumulation of unfolded proteins in the ER lumen activates an adaptive unfolded protein response, UPR, as a mechanism to restore homeostasis. The UPR is mediated by three ER-localized transmembrane proteins: inositol-requiring 1a (IRE1a), PKR-like endoplasmic reticulum kinase (PERK), and activating transcription factor 6 (ATF6) (Malhotra and Kaufman 2007; Ron and Walter 2007; Yoshida 2007). Interestingly, recent findings show that lipid perturbation is also a direct activator of the UPR, independent of protein

unfolding. Although the mechanism of the UPR process has been extensively investigated, the relationship between heat stress, especially mild hyperthermia, and the ER homeostatic response remains unclear.

Our previous observation that mild, non-protein-denaturing heat induced a type of UPR in mammalian cells (Tiszlavicz et al. 2022), initiated our studies on intracellular heat production. At the subcellular level, the mitochondrion is the organelle best-known for thermogenesis (Beignon et al. 2022). Using a temperature-sensitive fluorescent indicator targeted to mitochondria, it has been shown that mitochondria are physiologically maintained at close to 50°C (Chrétien et al. 2018). The ER has also gained attention as another kind of organelle responsible for heat production, mediated by a Ca2+-ATPase (SERCA) pump (Bal et al. 2012). Although these studies were not the first suggesting that mammalian mitochondria and the ER could be warmer than their surroundings, such discoveries fostered attention and discussion, especially considering the thermal physics of the phenomenon (Macherel et al. 2021). While there is still continuing debate on sizzling organelles, we aimed to investigate the intriguing possibility that the high metabolic demand of the cellular stress response could generate thermogenesis in the ER, further elevating its temperature and ultimately leading to a heat-shock response.

Objectives

Based on our previous findings, we observed not only elevated Hsp gene induction but also an increase in the activity of inflammation-related signaling pathways following heat treatment at 42.5 °C. In contrast, mild heat treatment at 40 °C resulted in increased expression of genes related to the unfolded protein response in CHO cells (Tiszlavicz et al. 2022). Building on these observations, we aim to investigate the relationship between the heat shock response (HSR) and the unfolded protein response (UPR) in various mammalian cells exposed to mild fever-like temperatures.

We aimed to:

- Investigate the effects of dose-dependent heat treatments on the expression of genes involved in different stress responses.
- Investigate the origin and relationship between the HSR and the UPR under mild heat stress conditions.
    - Identification of the activated UPR signalling pathways:
        - *Assessing UPR activation triggered by fever-like temperatures.*
        - *Investigating signaling pathways responsible for UPR activation during mild heat stress.*
    - Exploration of the activated HSR:
        - *Determining HSR activation in response to mild heat stress.*
    - Mapping cellular thermogenesis:
        - *Investigating whether intracellular thermogenesis during stress responses induces Hsp gene expression.*
        - *Assessing the involvement of mitochondria and endoplasmic reticulum in intracellular heating during mild heat stress.*
        - *Studying the impact of thermogenesis on the heat shock response.*

Experimental methods

Cell culture:

- Routine cell culturing (U2OS wild type, U2OS mEOS, U2OS XBP1, U2OS ATF4, U2OS B-gTEMP and MEF cells)
- Generation of U2OS cell line expressing the fluorescently labeled XBP1 (mNeonGreen) or ATF4 (mScarlet) UPR sensors using pLHCX-XBP1 mNeonGreen NLS plasmid (Addgene plasmid #115971; http://n2t. net/addgene:115971; RRID:Addgene_11595971) or pLHCX-ATF4 mScarlet NLS plasmid (Addgene plasmid # 115970 ; http://n2t.net/addgene:115970; RRID:Addgene_115970).
- Generation of U2OS B-gTEMP and MEF B-gTEMP cell lines by transient or stable transfection with B-gTEMP/pcDNA3 plasmid (Addgene plasmid # 188447; http://n2t.net/addgene:188447; RRID: Addgene_188447).

Labeling with fluorescent dyes:

- ER thermo and Mito thermo yellow, Hoechst 33342, MitoTracker Red CMXRos and DeepRed, Mag-fluo-4 ER Ca2+ indicator

Heat treatment or treatment with ER stress inducing agents (tunicamycin, thapsigargin)

RNA isolation and real-time quantitative polymerase chain reaction (RT-qPCR)

Measurement of XBP1 and ATF4 protein levels using flow cytometry

ER Ca^{2+} level measurement using flow cytometry

ER temperature measurement of ER thermo yellow-labeled cells using fluorescence microscopy

Measurement of mitochondrial temperature of Mito thermo yellow-labeled cells using spectrofluorometry

Measurement of mitochondrial membrane potential using fluorescence microscopy

Measurement of IRE1 clustering using superresolution microscopy

Intracellular temperature measurement using confocal microscopy

Results

The focus of my PhD thesis is on investigating heat sensing in living cells and their response to heat stress, with a particular emphasis on the role of membranes in heat sensation and adaptation. Our previous studies on mammalian cells have revealed a mild, fever-like temperature range (39.5-40°C), where the cells are able to acquire thermo tolerance in the absence of heat shock protein (Hsp) synthesis (Peksel et al. 2018). Our results suggest that the stress response to heat can vary significantly between cell types. Our qPCR results revealed that in MEF (mouse embryonic fibroblast) cells, the induction of Hsp genes (*Hsp25, Hsp70*) is already notable following 40 °C heat treatment, whereas in U2OS (human osteosarcoma) cells, this induction is considerably less pronounced. Interestingly, in U2OS cells, the expression of the unfolded protein response (UPR) genes (*XBP1, CHOP*) is increased concomitantly with Hsp gene induction, whereas in MEF cells the classical Hsp response seems to repress the UPR response. To examine the contribution of different UPR pathways, we used dSTORM super-resolution microscopy to detect IRE1 clustering in U2OS cells in response to mild heat treatment, which serves as an initial indicator of IRE1 pathway activation. By flow cytometry, we measured an increase in the induction of IRE1-dependent XBP1 protein and the PERK-dependent ATF4 protein. Using B-gTEMP plasmid we observed that the temperature of the endoplasmic reticulum (ER) and the mitochondria can differ significantly from the ambient temperature or the temperature of the whole cell. Using an ER thermo yellow temperature-sensitive ER-specific fluorescent assay, we detected temperature-dependent thermogenesis (potential SERCA-Ca^{2+}-ATPase uncoupling) in the ER of MEF cells, whereas this was not observed in U2OS cells. Our observations suggest that even mild heat stress (40°C) can induce a high local temperature (~48°C) in the ER of MEF cells that results in protein denaturation and, in its defense, increased Hsp production. At the same time, we measured a concomitant decrease in ER Ca^{2+} levels in MEF cells, which is presumably due to the uncoupling of the SERCA pump by mild heat. Mito thermo yellow-labeled U2OS and MEF cells were measured by spectrofluorometry to determine different mitochondrial temperatures following heat treatment at 40 °C. For U2OS cells, the mitochondrial temperature of oligomycin-treated and control cells increased to the same extent after 40 °C heat treatment, whereas for MEF cells, a higher mitochondrial temperature was measured in control cells

compared to oligomycin-treated cells. Concurrent with the increased mitochondrial temperature, an increase in mitochondrial membrane potential was observed in MEF cells.

Our findings may have implications in the development of therapeutic strategies to combat disease in which the UPR was shown to play an important role, like cancer, neurodegenerative and metabolic diseases. Knowledge of cell-to-cell variation in response to hyperthermia could also be relevant for preventing heat-stress caused by global warming.

Summary of results

Our research aimed to investigate the possible relationship between membrane changes during mild, fever-like heat treatment (39-40°C) and the cellular stress response. The study of the stress response is crucial to understand how cells respond and adapt to various changes in their environment. We investigated the effects of different doses of heat on mammalian cell lines.

The results presented in the thesis revealed the following cellular events:

1. Different cell types show different dose-dependent stress transcriptome profiles following heat treatment:
    - In MEF (mouse embryonic fibroblast) cells, increased Hsp gene (*Hsp25, Hsp70*) induction was observed already following heat treatment at 40°C, while in U2OS (human osteosarcoma) cells the elevated expression of HSP25 could only be observed at higher (41-42°C) temperatures.
    - In U2OS cells, Hsp gene induction is accompanied by increased expression of the unfolded protein response (UPR) genes (XBP1, CHOP), whereas in MEF cells the classical Hsp response seems to repress the UPR.
2. Both the IRE1 and PERK branches of the UPR signalling pathway are activated under mild heat stress conditions:
    - Using dSTORM super-resolution microscopy, we detected IRE1 clustering upon fever-like heat treatment, which is one of the first signs of activation of the IRE1 pathway.
    - Significant upregulation of IRE1-dependent XBP1 protein and PERK-dependent ATF4 protein induction was detected using flow cytometry.
3. Heat shock response (HSR) can also be activated by mild heat stress:
    - In the ER of MEF cells, even mild heat stress (40°C) could result in high local temperatures (~48°C) that may lead to local protein denaturation and increased heat shock protein production.

4. The temperature of the endoplasmic reticulum (ER) and the mitochondria differ significantly from the temperature of the whole cell and the environment.
    - Using B-gTEMP intracellular thermometer and confocal microscopy, we showed that the perinuclear region of MEF cells is warmer than the rest of the cell.
5. Cell type-dependent cellular thermogenesis was detected after fever-like heat treatment:
    - Using ER-thermo-yellow, a temperature-sensitive ER-specific fluorescent probe, temperature-dependent thermogenesis (potential SERCA-Ca^{2+}-ATPase uncoupling) was detected in the ER of MEF cells, whereas this was not observed in U2OS cells.
    - Up to 48°C can be measured in the ER of MEF cells after fever-like heat treatment.
    - Concurrently, decrease in ER Ca^{2+} levels in MEF cells was measured, which is presumably due to the heat-induced uncoupling of the SERCA pump.
    - Using Mito thermo yellow probe and spectrofluorimetry, different mitochondrial temperatures were measured in U2OS and MEF cells following heat treatment at 40°C. Mitochondrial temperature of oligomycin-treated and control U2OS cells increased to the same extent following heat treatment at 40°C, whereas a higher mitochondrial temperature was measured in control MEF cells compared to oligomycin-treated cells.
6. Increase in mitochondrial membrane potential was observed in MEF cells following fever-like heat treatment.

Összefoglaló

Doktori értekezésem fókuszában az élő sejtek hőérzékelése, valamint a hőstresszre adott válaszának vizsgálata áll, különös tekintettel a membránok hőérzékelésben és alkalmazkodásban betöltött szerepére. Emlős sejteken végzett korábbi vizsgálataink egy enyhe, lázszerű hőmérsékleti tartományt (39,5–40 °C) tártak fel, ahol a sejtek hőtűrő képessége a hősokkfehérje (Hsp) szintézis hiányában is kifejlődik (Peksel és mtsai 2018). Kísérleteink szerint a hőkezelésre adott stresszválasz sejttípusonként jelentősen eltérhet. qPCR eredményeink rámutattak, hogy MEF (egér embrionális fibroblaszt) sejtekben már a 40 °C-os hőkezelést követően fokozott Hsp gén (*Hsp25, Hsp70*) indukció figyelhető meg, míg U2OS (humán oszteoszarkóma) sejtekben ez csak kisebb mértékben jellemző. Érdekes módon U2OS sejtekben a Hsp génindukcióval egyidejűleg a selejtfehérje-válasz (UPR) gének (*XBP1, CHOP*) expressziója is megnő, míg MEF sejtekben úgy tűnik, a klasszikus Hsp válasz represszálja az UPR választ. A különböző UPR útvonalak hozzájárulásának vizsgálata érdekében U2OS sejtekben dSTORM szuperrezolúciós mikroszkópiával kimutattuk a lázszerű hőkezelés hatására bekövetkező IRE1 klaszterizálódást, ami az IRE1 útvonal aktiválódásának egyik első jele. Áramlási citometriával az IRE1-függő XBP1 fehérje és PERK-függő ATF4 fehérje indukciójának szigfinikáns emelkedését mértük. B-gTEMP plazmid használatával megfigyeltük, hogy az endoplazmatikus retikulum (ER) és a mitokondriumhálózat hőmérséklete jelentősen eltérhet a teljes sejt és a környezet hőmérsékletétől. ER-thermo-yellow hőmérséklet-érzékeny ER-specifikus fluoreszcens próbával a MEF sejtek ER-jában hőmérsékletfüggő termogenezist (potenciális SERCA-Ca^{2+}-ATPáz szétkapcsolódást) detektáltunk, míg U2OS sejteknél ez nem volt megfigyelhető. Megfigyeléseink arra engednek következtetni, hogy a MEF sejtek ER-jában már enyhe hőstressz (40 °C) esetén is kialakulhat olyan magas lokális hőmérséklet (~48 °C), ami fehérjedenaturációt és ennek védelmére fokozott hősokkfehérje-termelődést eredményez. Ezzel egyidejűleg az ER Ca^{2+}-szintjének csökkenését mértük MEF sejtekben, mely feltételezhetően a SERCA-pumpa hő általi szétkapcsolásának tulajdonítható. Mito thermo yellow-jelölt U2OS és MEF sejtek spektrofluorimetriás mérése során eltérő mitokondriumhőmérsékletet mértünk a 40 °C-os hőkezelést követően. U2OS sejtek esetén az oligomycin-kezelt és a kontrollsejtek mitokondriumhőmérséklete azonos mértékben nőtt a 40 °C-os hőkezelés hatására, míg MEF

sejtek esetén a kontrollsejtekben magasabb mitokondriumhőmérséklet volt mérhető az oligomycin-kezelt sejtekkel összehasonlítva. A megnövekedett mitokondroiumhőmérséklettel egyidejűleg, mitkondriális membrán-potenciál-növekedést (azaz mitokondriumaktivitás-növekedést) figyeltünk meg MEF sejtekben.

Eredményeink hozzájárulhatnak az UPR-t érintő betegségek, például a rák, neurodegeneratív és anyagcsere-betegségek terápiás stratégiáinak fejlesztéséhez, valamint a sejtek közötti eltérések a hipertermiára adott válaszokban is fontosak lehetnek a globális felmelegedés kontextusában.

Publications

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Publications related to the thesis:

1. B. Dukic, Z. Ruppert, M. E. Tóth., Á. Hunya, Á. Czibula, P. Bíró, Á. Tiszlavicz, M. Péter, G. Balogh, M. Erdélyi., G. Timinszky, L. Vígh, I. Gombos, Z. Török: Mild Hyperthermia-Induced Thermogenesis in the Endoplasmic Reticulum Defines Stress Response Mechanisms. Cells, 2024, 13, 1141., DOI: https://doi.org/10.3390/cells13131141 (IF2023-2024: 6,0; SJR indicator: Q1)

2. Á. Tiszlavicz, I. Gombos, M. Péter, Z. Hegedűs, Á. Hunya, B. Dukic, I. Nagy, B. Peksel, G. Balogh, I. Horváth, L. Vígh, Z. Török: Distinct Cellular Tools of Mild Hyperthermia-Induced Acquired Stress Tolerance in Chinese Hamster Ovary Cells, Biomedicines, 2022, DOI: https://doi.org/10.3390/biomedicines10051172 (IF2021-2022: 4,757)

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2. B. Dukic, Á. Tiszlavicz, I. Gombos, B. Peksel, M. Péter, G. Balogh, I. Horváth, L. Vígh, Z. Török: Investigation of fever-like hypethermia induced early cellular events, Interdisciplinary Doctoral Student Conference, 28 November 2020, online oral presentation

3. B. Dukic, Á. Tiszlavicz, I. Gombos, B. Peksel, M. Péter, G. Balogh, I. Horváth, L. Vígh, Z. Török: Investigation of fever-like hypethermia induced early cellular events, 'XIII. Tavaszi Szél' Conference, 16 October 2020., oral online presentation

4. K. Nagy, B. Dukic, Á. Ábrahám, P. Galajda: Rapid evolution of antibiotic resistance in heterogeneous environment, 26th Conference of Hungarian Biophysical Society, 22-25 August 2017, Szeged Hungary

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1. B. Dukic, Á. Tiszlavicz, P. Bíró, M. Péter, G. Balogh, V. Varga-Zsíros M. Erdélyi, L. Vígh, I. Gombos, Zs. Török: Enyhe stressz hatására felforrósodó organellumok, 14-17 May 2024, 53th Membrane Transport Conference, Sümeg, poster

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