Monitoring Cellular Stress Response and Inhibition of GPCR-Dependent Calcium Signaling Using Expressed Biosensors

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Introduction

Endoplasmic reticulum (ER) mediated cell stress and the unfolded protein response (UPR) serve as a major stress pathway in eukaryotic cells. ER stress and the UPR can be triggered by a number of cellular perturbations including misregulation of ER Ca^{2+}, accumulation of misfolded proteins within the lumen of the ER, and reactive oxygen species (Grootjans et al., 2016; Scheper and Hoozemans, 2015; Zeeshan et al., 2016). ER stress has been implicated in a wide range of diseases including neurodegenerative diseases, cancers, diabetes and ischemia (Auf et al., 2010; Kammoun et al., 2009; Kudo et al., 2007; Scheper and Hoozemans, 2015), and is also an area of emerging interest for drug development and toxicity testing (Huang et al., 2016; Kim et al., 2017; Plate et al., 2016; Roth et al., 2014). Activation of proteostatic pathways, such as the UPR and ER stress serves as an early indicator of cell stress or toxicity, which is a useful mechanism to detect drug or compound induced cell stress (Deavall et al., 2012; Foufelle and Fromenty, 2016).

The UPR consists of three arms, each activating a unique mechanism to regulate the cellular response to ER stress (Grootjans et al., 2016). Methods to detect activation of ER stress and the UPR range from PCR-based assays to fluorescent indicators (Iwawaki et al., 2004; Rong et al., 2015). However, few current detectors of ER stress are optimized for detecting both chemical as well as genetically induced stress, and are irreversible indicators. Demonstrated here is the use of a new and commercially available ER stress sensor developed by Montana Molecular to detect chemically induced cell stress by thapsigargin, a potent SERCA pump inhibitor, and simultaneously detect the effects on Gq-mediated cell signaling (Figure 1). Importantly, high content analysis of ER stress using the Agilent BioTek Lionheart FX automated microscope allows the detection of both the onset and recovery from thapsigargin-induced cell stress as well as simultaneous detection of Gq-mediated cell signaling over a 24-hour period. In sum, combining live cell detection of cell stress and signaling with kinetic high content monitoring allows precise detection of changes in cellular stress levels and its implications on cellular signaling events.

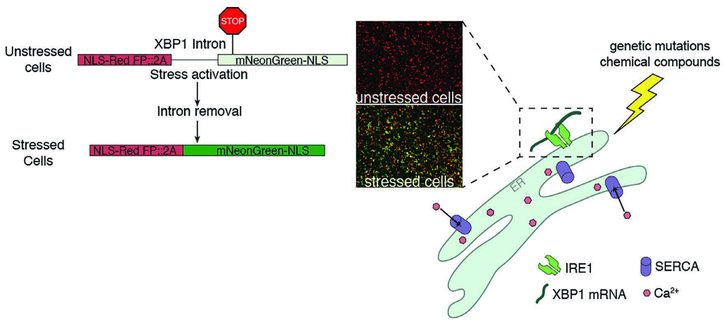

Figure 1. A genetically encoded fluorescent biosensor to detect ER stress.

Experimental

Agilent BioTek instrumentation

Agilent BioTek FX automated microscope with dual reagent injector module and augmented microscopy

All inclusive microscopy system: Optimized for live-cell imaging with brightfield, color brightfield, phase contrast and fluorescence channels. Up to 100x air and oil immersion magnification.

Up to 20 frames per second (fps) image capture and dual in-line reagent injectors: Enables characterization of rapid cellular events and addition of reagents with uninterrupted monitoring of cellular response.

Integrated environmental control: Incubation up to 40 °C with CO_2/O_2 and available humidity control provides optimal conditions for long-term imaging of live cells.

Powerful Agilent BioTek Gen5 microplate reader and imager software: Automated image capture, processing, and analysis tools, including dual masking for cell counting and subpopulation analysis, plus annotation and movie maker functions.

Figure 2. Agilent BioTek Lionheart FX automated microscope with dual reagent injector.

Methods

Live-cell imaging of cell stress and GPCR activity using Montana Molecular biosensors

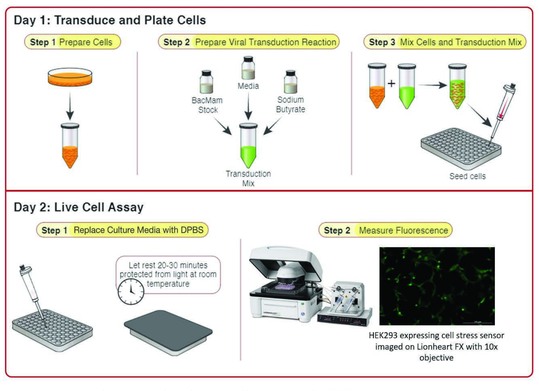

Figure 3. A simple protocol to detect cell stress and GPCR activity.

Results and discussion

Automated imaging-based quantification of treatment-induced cellular stress

Ratiometric detection of cellular stress and toxicity using expressed biosensor

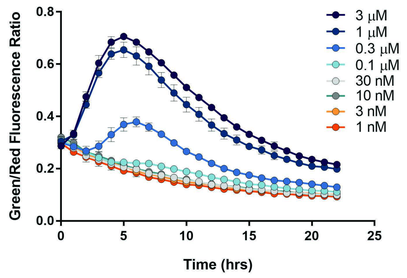

Figure 4. Thapsigargin-induced cellular stress was monitored using the cell stress sensor. The ratio of green (cell stress sensor) to red (nuclear marker) fluorescence intensity was calculated to account for variations in cell number across different conditions. A kinetic profile of the green-to-red fluorescence ratio revealed that peak cellular stress was reached approximately 6 hours after thapsigargin treatment, with a greater than two-fold increase in the green-to-red fluorescence ratio observed at the highest concentrations of thapsigargin.

Although ratiometric detection of cellular stress provides a valuable end point metric for evaluating the level of induced stress in a cell population, changes in the expression level of the nuclear marker over time can lead to kinetic profiles that are difficult to interpret. This shows that stress levels appear to drop below baseline due to a steady increase in the expression level of nuclear marker over the 24-hour period.

Kinetic cell counting using red nuclear marker and automated image analysis indicates inhibition of cellular proliferation by thapsigargin

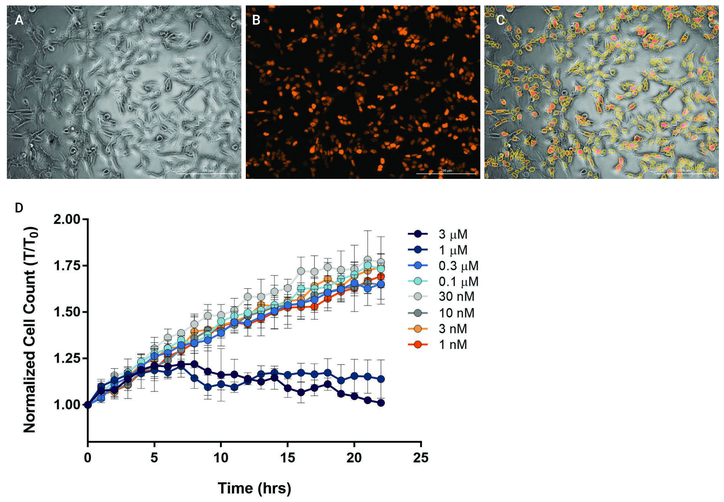

Figure 5. Automated determination of cell number using Agilent BioTek Gen5 image analysis software and the cell stress sensor expressed red nuclear label. Gen5 image analysis was used to determine the number of cells present in each well to more accurately account for changes in population size throughout the experiments when evaluating cell stress levels. The Gen5 software obtained cell counts by placing object masks around each labeled nuclei for every time point. The resulting kinetic profile indicates that the number of cells began to decrease within 6 hours of the 3.0 µM and 1.0 µM thapsigargin treatment, while all other treatment conditions resulted in robust cell proliferation over the 24-hour period. (A) 10x phase contrast image of cells. (B) Corresponding RFP image of the red nuclear label. (C) Object masks are placed around each nuclei using the RFP channel. (D) Accurate kinetic cell counts were generated for evaluating cell proliferation and viability.

Responder rates determined using image-based cellular analysis provide accurate and biologically relevant quantification of thapsigargin-induced cellular stress

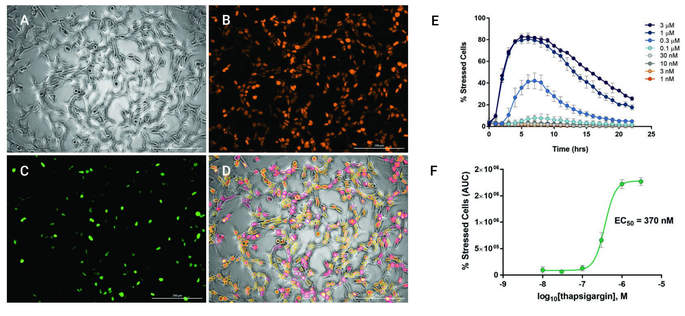

Figure 6. Quantifying cell stress sensor fluorescence within each cell using Agilent BioTek Gen5 object masking provides a more detailed evaluation of stress response. The fluorescence intensity of the cell stress sensor within each cell was measured and used to define the total number of stressed cells in the population. The ratio of stressed cells to the total number of cells was calculated and reported as percent stressed cells. (A) 10x phase contrast images captured throughout the experiment enable qualitative evaluation of cell morphology over time. (B) Images captured in the RFP channel are used to generate automated cell counts using the red fluorescent-labeled nuclei and Gen5 object masks. (C) The corresponding fluorescence intensity of the cell stress sensor within each cell is then measured using the GFP channel. (D) Stressed cells (fuchsia masks) and nonstressed cells (yellow masks) are defined using Gen5 subpopulation analysis. (E) Kinetic profiles of the percent stressed cells provide a more detailed and accurate evaluation of the cellular stress response over time resulting from thapsigargin treatment. (F) Quantitative analysis of the dose-dependent effect of thapsigargin treatment on cellular stress response over time was conducted using the area under the curve (AUC) from each responder rate profile.

Measuring effects of cellular stress on GPCR signaling

Monitoring Gq-dependent Ca^{2+} release using R-GECO biosensor and automated image analysis

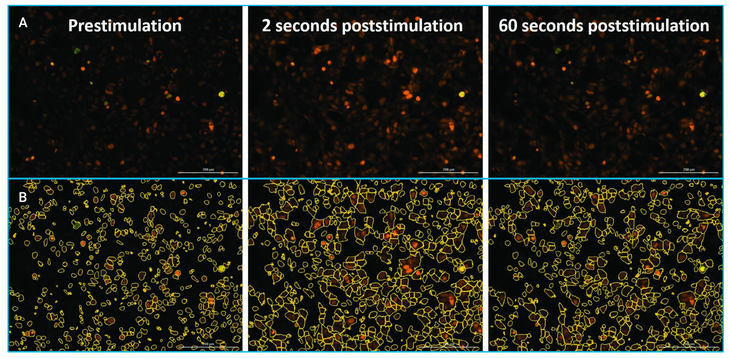

Figure 7. HEK293 expressing the R-GECO calcium biosensor were used to measure release of intracellular calcium resulting from Gq-coupled hM1 receptor stimulation. Image panel of untreated HEK293 cells (A) with Agilent BioTek Gen5 placed masks around cells containing R-GECO fluorescence (B). R-GECO fluorescence – which increases with increasing levels of Ca^{2+} – is initially low prestimulation. After stimulation of Gq-coupled hM1 receptors by injection of 30 µM (final) carbachol, rapid intracellular mobilization of Ca^{2+} and a corresponding increase in R-GECO fluorescence is observed, followed by a gradual decrease in cytoplasmic Ca^{2+} levels.

Effect of thapsigargin treatment on Gq-dependent calcium signaling over 24-hour time course

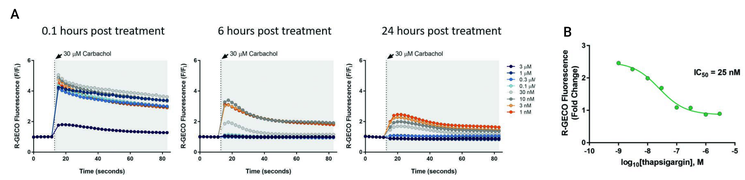

Figure 8. Kinetic monitoring of R-GECO fluorescence reveals calcium signaling kinetics are altered by thapsigargin in a dose- and treatment duration-dependent manner. HEK293 expressing hM1 receptor and the R-GECO calcium sensor were treated with thapsigargin, a potent SERCA pump inhibitor, to evaluate the long-term effects of thapsigargin on Gq-dependent calcium signaling. Carbachol (30 µM final) was added to the cells via the aligned reagent injectors to stimulate hM1 receptors at three different time points: 0.1, 6, and 24 hours post thapsigargin treatment. Independent wells were used for each time point (i.e. each well received a single injection of carbachol at either 0.1, 6, or 24 hours). (A) Kinetic profiles of R-GECO fluorescence were generated for each time point. (B) Quantitative analysis of the dose-dependent effect of thapsigargin on Gq-dependent calcium signaling 24 hours post treatment generated an IC_{50} value of 25 nM. Combined, these results indicate that thapsigargin inhibits Gq-mediated signaling in HEK293 at considerably lower concentrations, and for a longer period, compared to the induced cellular stress response.

Multiplexing cell stress and R-GECO biosensors to interrogate calcium signaling within stressed and unstressed cells within a population

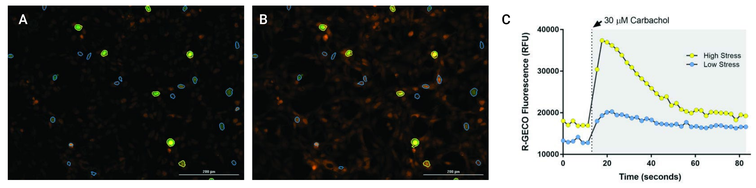

Figure 9. Evaluating of calcium signaling within cells exhibiting either low or high cellular stress using subpopulation analysis. The results from the cell stress studies revealed that increasing concentrations of thapsigargin lead to an increase in the number of cells in which ER stress or the UPR is activated. In order to correlate the effect of calcium signaling directly with cell stress levels, Agilent BioTek Gen5 image analysis tools were used to measure calcium-induced changes in R-GECO fluorescence only within cells exhibiting cellular stress after 6 hours of treatment with thapsigargin. Subpopulation analysis was used to define stressed cells as exhibiting either a high level of stress (yellow masks) or a low level of stress (blue masks). (A) Population of stressed cells 6 hours after addition of 0.1 µM thapsigargin before stimulation by carbachol and (B) 6 seconds after carbachol injection. (C) The resting levels of calcium within the nucleus, and the resulting increase in calcium levels following addition of carbachol, were measured for both subpopulations of cells.

Conclusion

- Demonstrated here is the ability to monitor cell stress in live cells and in real time using a novel expressed biosensor, while simultaneously examining the effects of cell stress on second messenger signaling.
- Cell stress induced by thapsigargin is detected an order of magnitude prior to any changes in cellular proliferation.
- Image-based cellular analysis enables unique quantitative evaluation of cell stress, including percentage of cells within the population exhibiting stress over time.
- Calcium signaling kinetics monitored using the R-GECO biosensor are altered by thapsigargin treatment in a dose- and time-dependent manner.
- Subpopulation analysis reveals that cells exhibiting high levels of stress response have considerably different prestimulation and poststimulation levels of calcium compared to cells exhibiting low levels of stress.

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RA44391.2008217593

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Printed in the USA, July 9, 2021
5994-3252EN