Application Note

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Thapsigargin-Induced Cellular Stress Response and Inhibition of Gq-dependent Calcium Signaling

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Abstract

In this application note, a novel endoplasmic reticulum stress sensor was used, along with an automated imaging-based approach to evaluate chemically induced cell stress by thapsigargin, a potent SERCA pump inhibitor, while simultaneously measuring its effects on Gq-mediated cell signaling. These results reveal that cell stress induced by thapsigargin is detected an order of magnitude prior to any changes in cellular proliferation. Kinetic monitoring of cell stress levels demonstrates the reversible nature of the stress sensor. Stress levels peak after 6 to 7 hours of treatment, but cells recover naturally from this stress after 22 hours. Gq-dependent Ca^{2+} signaling was reduced in a dose-dependent manner at both 6 and 24 hours during thapsigargin treatment, indicating that the effects of ER stress on Gq-mediated Ca^{2+} signaling remain even after the cell has shut down the ER stress response mechanisms.

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.[1-3] ER stress has been implicated in a wide range of diseases including neurodegenerative diseases, cancers, diabetes and ischemia,[2,4-6] and is also an area of emerging interest for drug development and toxicity testing.[7-10] 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.[11,12]

The UPR consists of three arms, each activating a unique mechanism to regulate the cellular response to ER stress.[1] Methods to detect activation of ER stress and the UPR range from PCR-based assays to fluorescent indicators.[13,14] However, few current ER stress detectors are irreversible indicators, optimized to detect both chemically and genetically induced stress. This application note demonstrates the use of a 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 its effects on Gq-mediated cell signaling (Figure 1). Importantly, high-content analysis of ER stress using an 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. The combination of live-cell detection of cell stress and signaling with kinetic high-content monitoring allows precise detection of changes in cellular stress levels and its effects on cellular signaling events.

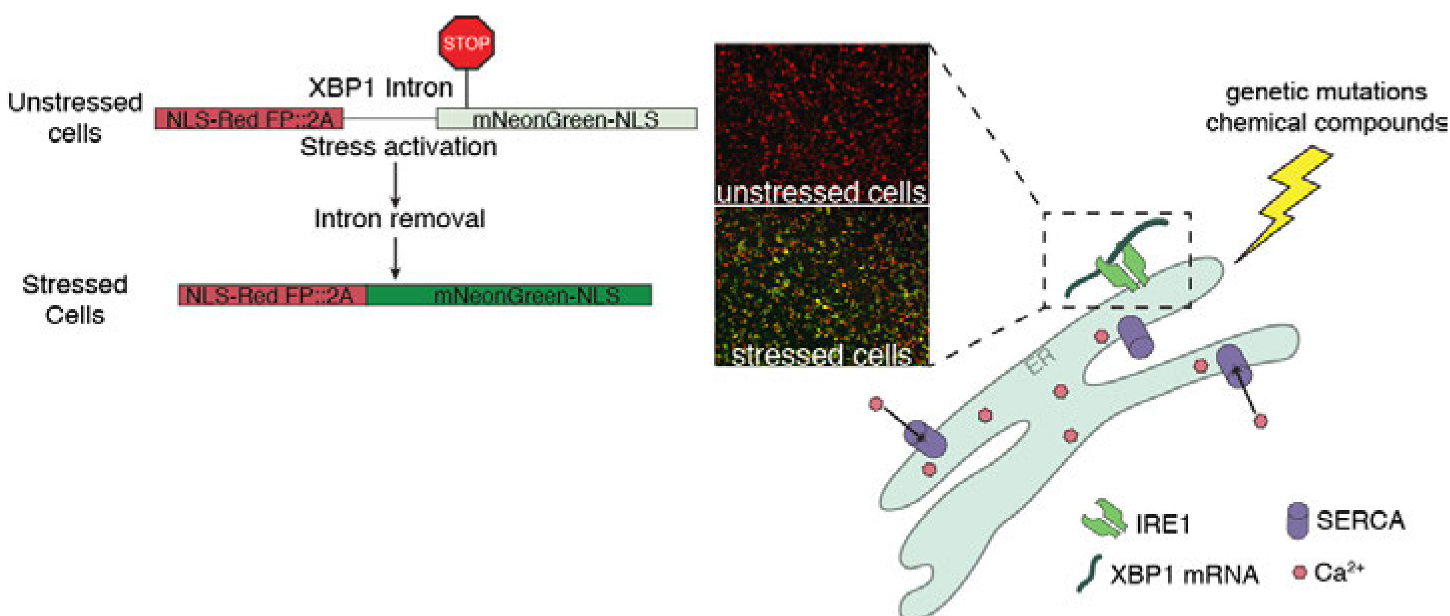

Figure 1. A genetically encoded fluorescent biosensor to detect ER stress. ER stress activates a unique splicing activty in the IRE1 protein, which sits in the ER membrane. IRE1 carries out an unconventional splicing event that removes an intron within the XBP1 mRNA, to produce a functional transcription factor to upregulate stress response genes. This system was coopted to create a genetically encoded fluorescent biosensor to detect ER stress. This sensor expresses a constitutively expressed nuclear localized red fluorescent protein to mark transduced cells. Upon cell stress activation, the XBP1 intron encoded into the biosensor is removed and a nuclear localized bright green fluorescent protein, mNeon Green, also becomes expressed. Quantifying the number of cells within a population that contain green fluorescence along with the constitutively expressed red fluorescence serves as a quantifcation of ER stress levels. For this study, the SERCA pump inhibitor thapsigargin was used to stress cells by disrupting Ca^{2+} dynamics within the ER.

Materials and methods

Agilent BioTek Lionheart FX automated microscope

The Lionheart FX is an all-inclusive system, optimized for live-cell imaging with up to 100x air and oil immersion magnification (Figure 2). Brightfield, color brightfield, phase contrast, and fluorescence channels offer maximum support for a wide range of imaging applications. A unique environmental control cover provides incubation to 40 °C and effective containment for CO_2/O_2 control. The humidity chamber and reagent injector add a greater level of environment optimization for live-cell imaging workflows. Agilent BioTek Gen5 3.0 microplate reader and imager software provides automated image capture, processing and analysis, plus annotation and movie maker functions. Gen5 3.0 offers ease and simplicity across a broad range of live and fixed cell applications.

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

Cell stress sensor

The cell stress sensor is a genetically encoded fluorescent biosensor that produces very bright fluorescence when the cell endures endoplasmic reticulum (ER) stress or undergoes the unfolded protein response (UPR). The biosensor is a two-color biosensor, allowing ratiometric detection of cellular stress and toxicity. The fluorescence ratio between the constitutively expressed, nuclear localized, red fluorescent protein and the stress induced, nuclear localized, green fluorescent protein is calculated to determine the level of cellular stress. Alternatively, comparing the cells in a population that express both the green and red fluorescence to those solely expressing red fluorescence can be used to determine the percentage of stressed cells within that population. A broad host of both chemical compounds and genetic mutations can induce ER stress. The UPR is one of the major stress pathways within the cell, which allows the stress sensor to detect a wide range of stress-induced stimuli, some whose primary target is not the ER.

R-GECO

The red fluorescent, genetically encoded Ca^{2+} indicator (R-GECO) increases in fluorescent intensity in response to increasing levels of intracellular calcium.[15] The optimal R-GECO excitation wavelength is 590 nm and the emission wavelength spans 600 to 700 nm. R-GECO can be paired with other GPCR biosensors from Montana Molecular, including cADDis, a green fluorescent cAMP sensor, or the green fluorescent upward and downward diacylglycerol (DAG) sensors to simultaneously detect both Ca^{2+} and cAMP or Ca^{2+} and DAG in living cells. The BacMam delivery system enables consistent and controllable expression in a wide variety of cell types including iPSC-derived cardiomyocytes.

Cell transduction and plating

HEK293 cells were cultured in Advanced DMEM with 10% fetal bovine serum and penicillin-streptomycin in 5% CO_2 at 37 °C. Cultures were routinely trypsinized (0.05% Trypsin-EDTA) at 80% confluence. Cell transductions were done following Montana Molecular protocols with volumes optimized for cell density and viral titer, and desired number of samples. Briefly, viral transduction reaction (25 µL R-GECO sensor BacMam, 10 µL Cell stress sensor BacMam, 5 µL 300 mM valproic acid, 1 µL of hM1 receptor, and 9 µL of FluoroBrite DMEM (Thermo Fisher) plus 10% FBS and pen/strep) was added to 100 µL of a 150,000 cells/mL FluoroBrite DMEM cell suspension. After mixing gently, this 150 µL of mix was seeded per well in a Costar 3904 96-well microplate, which was then covered with aluminum foil to protect it from light and incubated in a tissue culture hood for 30 minutes. Cells were then transferred to a 37 °C incubator and cultured under normal growth conditions for 24 hours to ensure optimal sensor expression.

Imaging procedure

Twenty-four hours after plating transduced cells, the growth medium was replaced with fresh FluoroBrite DMEM containing thapsigargin (3.0 to 0.001 µM). Immediately after addition of thapsigargin, the plate was transferred to an Agilent BioTek Lionheart FX automated microscope with aligned reagent injectors primed with FluoroBrite DMEM plus 180 µM (6x final) carbachol.

Receptor activation experiments were performed at room temperature with the 10x objective. R-GECO sensors were imaged using the RFP 531 Ex, 593 Em LED filter cube. The cell stress sensor was imaged using the GFP 469 Ex, 525 Em LED filter cube set. RFP channel exposure settings were optimized

to visualize cells with baseline R-GECO fluorescence levels, while low enough to accommodate a considerable increase in R-GECO fluorescence following agonist addition. GFP channel exposure settings were fixed such that baseline cell stress sensor fluorescence in the cell population was dim but visible. Focus was maintained using the laser autofocus cube. A 0.5 frame per second (FPS) capture rate was used for each channel. Images were acquired for a total of 85 seconds with 20 µL of FluoroBrite DMEM plus 180 µM (6x final) carbachol dispensed at time equals 13 seconds.

Images for the kinetic cell stress experiments were acquired using the RFP and GFP channels as described above, as well as by phase contrast with the 10x phase objective. Images were acquired every hour over a 24-hour time course. Environmental growth conditions (5% CO_2, 37 °C, and ~80% humidity) were maintained within the Lionheart FX throughout the experiments.

Results and discussion

Ratiometric detection of cellular stress and toxicity

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 (Figure 3).

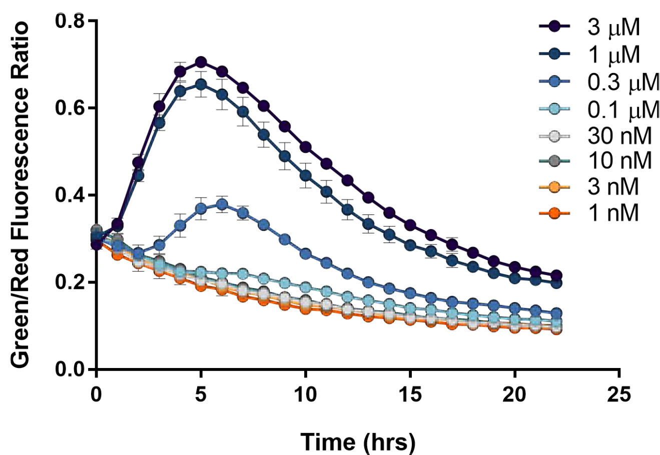

Figure 3. Kinetic profile of the ratio of green to red fluorescence intensity. The ratio of green (cell stress sensor) to red (nuclear marker) fluorescence intensity indicates that thapsigargin-induced cellular stress peaks in the HEK293 population approximately 6 hours after treatment, before returning to near baseline.

Although ratiometric detection of cellular stress provides a valuable endpoint 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. It is shown 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 expressed red nuclear marker and automated image analysis

Gen5 microplate reader and imager software 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 automated software obtained cell counts by placing object masks around each labeled nuclei for every time point (Figure 4). The resulting kinetic profile indicates that the number of cells began to decrease within 6 hours of the 3.0 and 1.0 µM thapsigargin treatment, while all other treatment conditions resulted in robust cell proliferation over the 24-hour period (Figure 5).

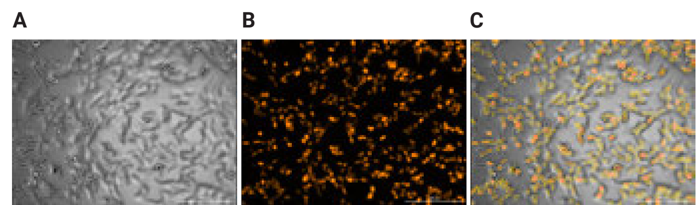

Figure 4. Automated determination of cell number using Agilent BioTek Gen5 image analysis software and the Cell Stress Sensor red nuclear label. (A) Phase contrast image of cells. (B) Corresponding RFP image of the red nuclear label. (C) Object masks are placed around each nuclei using the RFP channel, generating accurate kinetic cell counts for evaluating cell proliferation and viability.

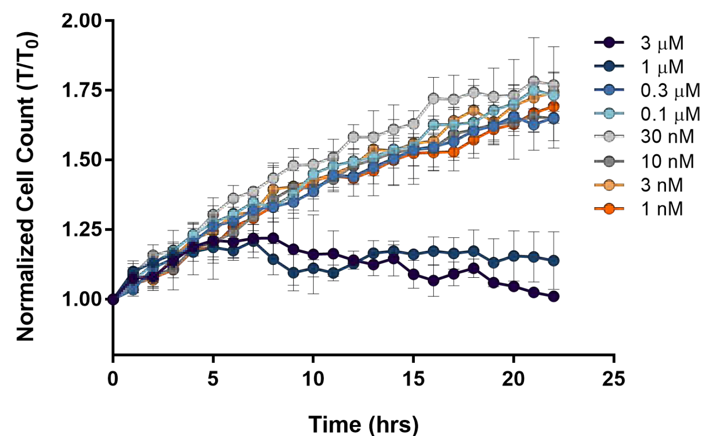

Figure 5. High concentrations of thapsigargin inhibit cell proliferation. Kinetic evaluation of HEK293 cell counts based on the red nuclear label reveal that the 3 µM and 1 µM concentrations of thapsigargin significantly inhibit cell counts. HEK293 grown under all other thapsigargin treatments exhibited robust cell growth.

Percentage of cells exhibiting stress response

The total number of cells per well were determined using the red nuclear marker as described above. The fluorescence intensity of the cell stress sensor within each cell was then measured and used to define the number of cells that were stressed (Figure 6). The ratio of stressed cells to the total number of cells was calculated and reported as percent stressed cells. Kinetic profiles of the percent stressed cells (Figure 7), in conjunction with the kinetic proliferation profiles, provide a more detailed and accurate evaluation of the cellular stress response resulting from thapsigargin treatment. 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 percent stressed profile, resulting in a calculated EC_{50} value of 370 nM (Figure 8).

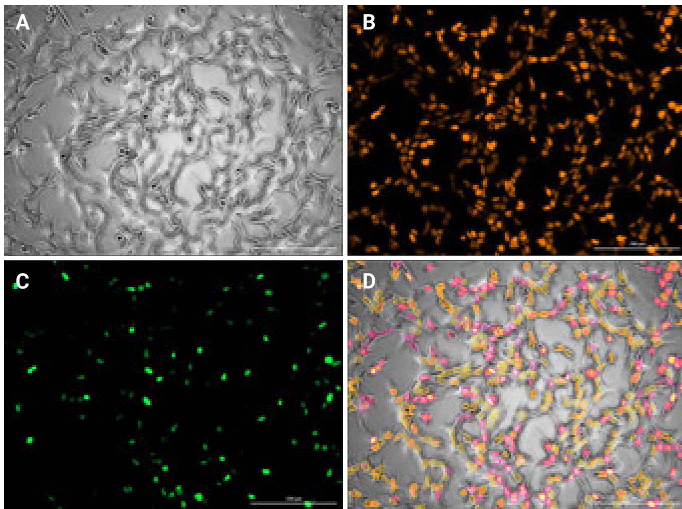

Figure 6. Quantifying Cell Stress Sensor fluorescence within each cell using Agilent BioTek Gen5 object masking provides a more detailed evaluation of stress response. (A) Phase contrast images captured throughout the experiment enable qualitative evaluation of cell morphology over time. (B) Images captured in the RFP channel are used to identify and count each cell 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.

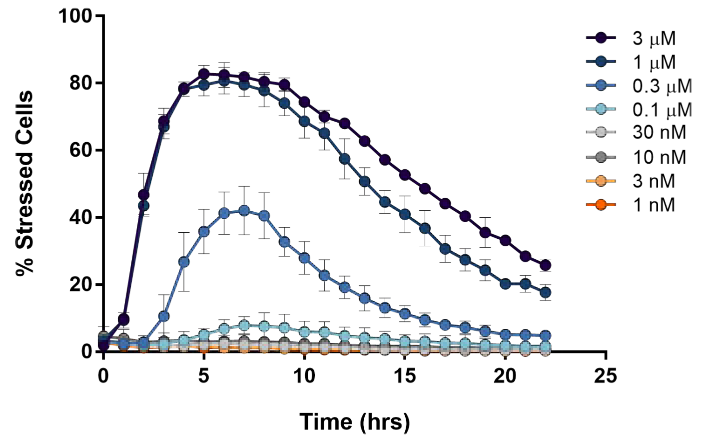

Figure 7. Kinetic profile of the ratio of stressed cells to the total number of cells Percent Stressed Cells is automatically calculated by Agilent BioTek Gen5 and provides a sensitive and biologically relevant metric for evaluating stress response.

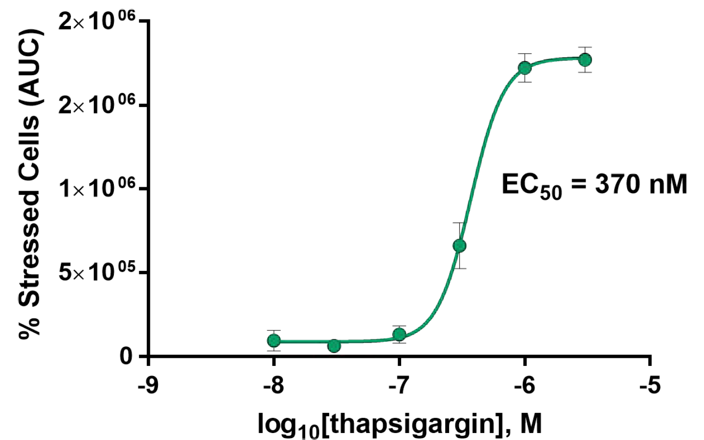

Figure 8. Quantifying dose-dependent effect of thapsigargin on cell stress levels. AUC of percent stressed cell profiles was used to calculate an EC_{50} value from the kinetic data.

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

HEK293 cells expressing the Gq-coupled 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, 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, 6, or 24 hours). Kinetic profiles of R-GECO fluorescence were generated for each time point (Figure 9). Immediately after cells were treated with thapsigargin, Gq-mediated calcium signaling was reduced ~75% in wells containing 3 µM thapsigargin, while calcium signaling appeared to be unaffected at lower thapsigargin concentrations.

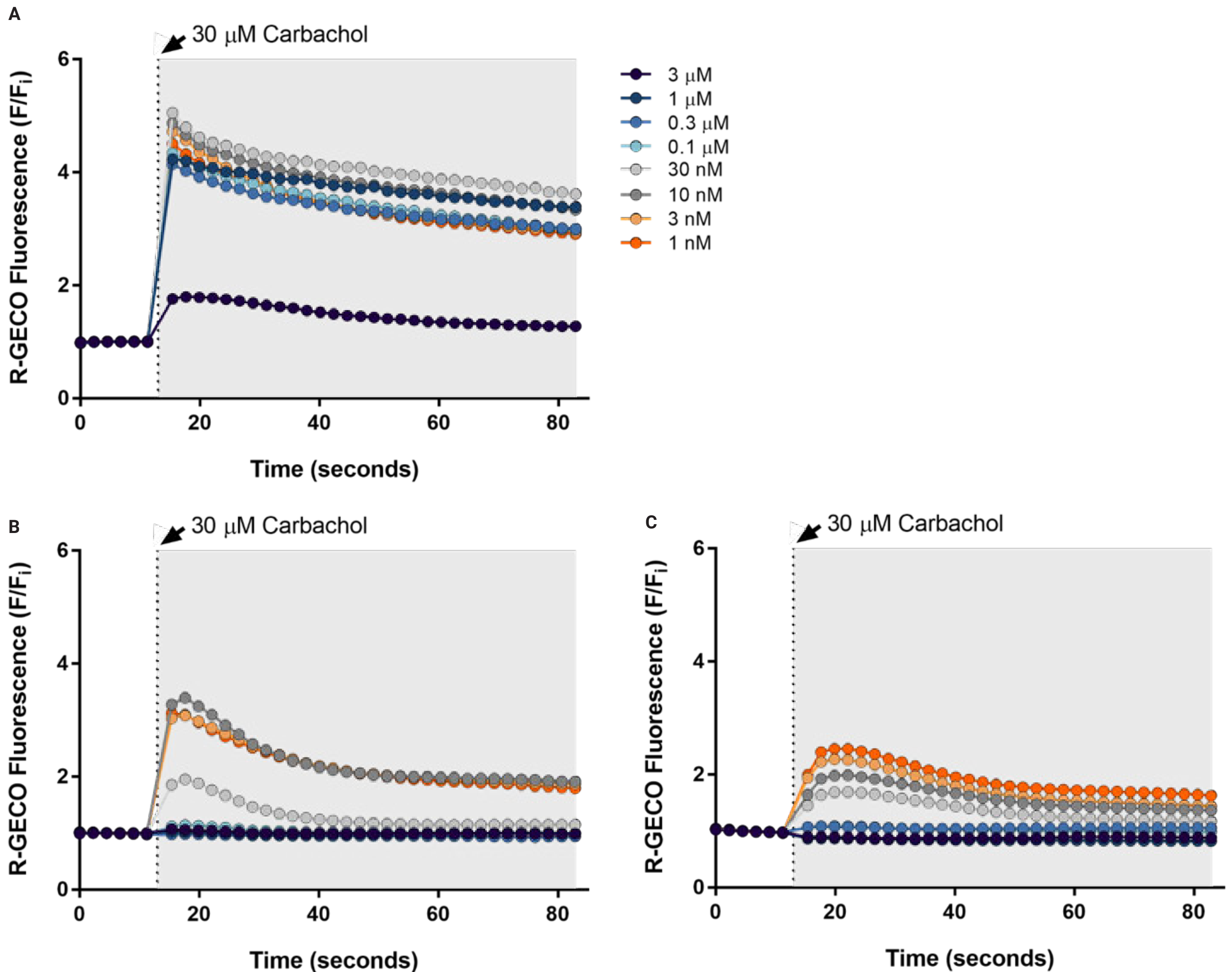

Figure 9. Evaluation of thapsigargin-induced inhibition of Gq-dependent calcium signaling over 24-hour time course. (A) Calcium mobilization within HEK 293 was triggered by the injection of 30 µM (final) carbachol, an hM1 agonist, into the growth medium immediately after treating cells with thapsigargin (B) 6 hours post thapsigargin treatment, and (C) 24 hours post thapsigargin treatment.

Six hours after addition of thapsigargin, calcium signaling was reduced across all treatment concentrations, with ~50% reduction at the lowest concentration of thapsigargin (1 nM), and down to undetectable levels in wells containing 3.0 to 0.1 µM thapsigargin. After 24 hours of treatment, Gq-mediated calcium signaling was further reduced, with 1.0 nM thapsigargin treatment conditions exhibiting ~75% lower calcium signaling compared to initial response.

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 (Figure 10). 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.

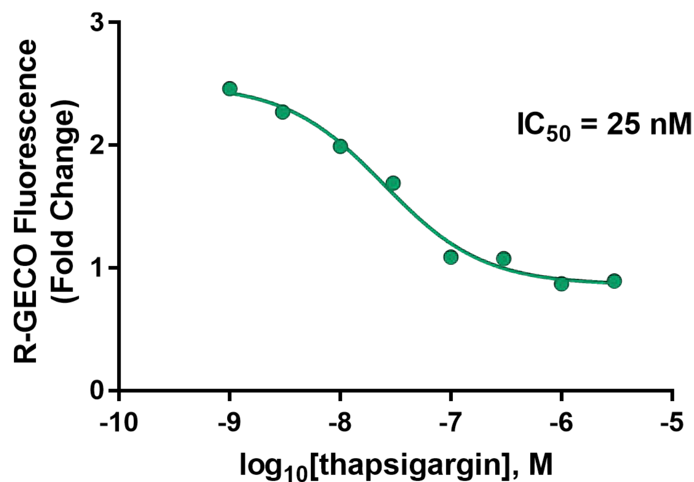

Figure 10. Dose-dependent effect of thapsigargin on Gq-mediated calcium signaling. Fold change of R-GECO fluorescence 24 hours post treatment was used to calculate an IC_{50} value of 25 nM.

Monitoring calcium signaling only within cells identified as stressed

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. To correlate the effect of calcium signaling directly with cell stress levels, 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. Stressed cells were further categorized as having either high or low stress levels, and the kinetic profiles of Gq-mediated calcium signaling for each subpopulation were compared (Figure 11). Interestingly, cells exhibiting the highest level of stress appear to have a greater relative increase in cytoplasmic levels of calcium following activation by carbachol, compared to cells with lower stress levels. These results could in part be due to the more severely stressed cells exhibiting a rounded-up morphology, which is revealed in the associated high-contrast brightfield images.

The ability to monitor cell stress and toxicity and its resulting effects on cellular signaling is important for both understanding disease pathology and in the drug discovery and development processes. This study demonstrates the ability to monitor cell stress in live cells and in real time, while simultaneously examining the effects of cell stress on second messenger signaling. Using the cell stress assay from Montana Molecular and the Agilent BioTek Lionheart FX, a high content readout for detecting cell stress by monitoring the percentage of stressed cells in a population was developed. This assay can be used to determine both cell stress and proliferation readouts in response to the SERCA pump inhibitor thapsigargin.

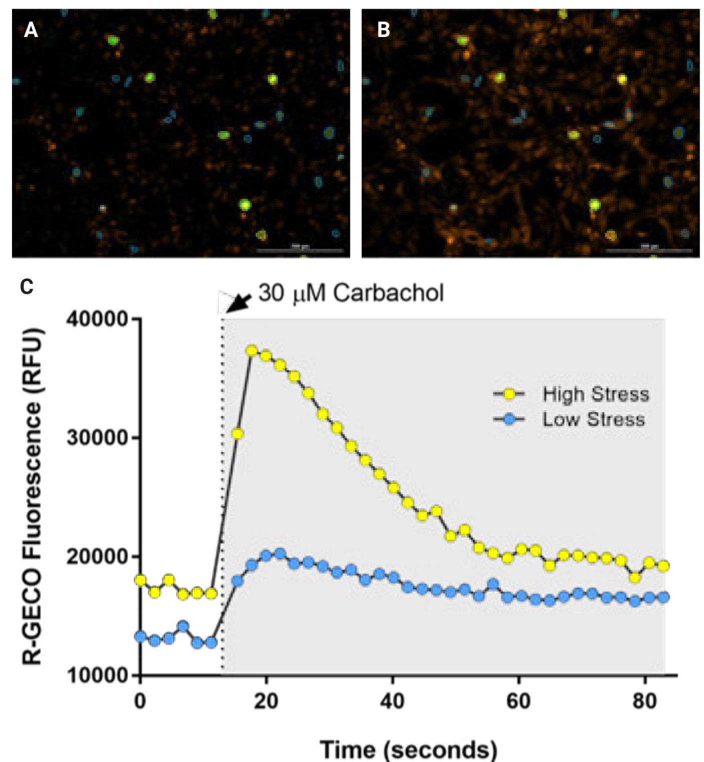

Figure 11. Subpopulation analysis for evaluating calcium signaling only within cells exhibiting cellular stress. Subpopulation analysis was used to define stressed cells as exhibiting either a high level of stress (yellow masks) or a low level of stress (blue masks). (A) Population of stressed cells 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

The results reveal that cell stress induced by thapsigargin is detected an order of magnitude prior to any changes in cellular proliferation. Kinetic monitoring of cell stress levels demonstrates the reversible nature of the stress sensor. Stress levels peak after 6 to 7 hours of treatment, but cells recovery naturally from this stress after 22 hours, matching previously reported cellular stress responses to thapsigargin treatment.[16] However, simultaneous analysis of Gq signaling revealed that thapsigargin treatment altered Gq-mediated Ca^{2+} signaling even after the ER stress response was alleviated. Gq-dependent Ca^{2+} signaling was reduced in a dose-dependent manner at both 6 and 24 hours during thapsigargin treatment, indicating that the effects of ER stress on Gq-mediated Ca^{2+} signaling remain even after the cell has shut down the ER stress response mechanisms. These data highlight an important feature of the Agilent BioTek Lionheart FX automated microscope, the ability to monitor both long-term and short-term kinetic responses, as well as demonstrate the importance of the multiplexing capabilities of the cell stress and Ca^{2+} biosensors on the Lionheart FX.

Lastly, a key feature of this assay is the ability to identify both stressed and unstressed cells within a population and interrogate those cells separately. The cell stress sensor was used to identify stressed and unstressed cells from within a population of cells treated with thapsigargin and monitor their unique Gq-mediated Ca^{2+} signaling responses. These types of analyses will be important for situations when only a subset of cells are experiencing stress and need to be isolated for analysis, as is the case for many neurodegenerative disorders such as Parkinson's or Huntington's disease. In conclusion, the high-content assay developed in this study to analyze cell stress using the Agilent BioTek Lionheart FX and the cell stress sensor from Montana Molecular is a rapid, reliable method to quantitatively measure cell stress and its effects on cellular signaling through second messengers.

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RA44216.4685069444

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Printed in the USA, February 1, 2021
5994-2587EN
AN100118_11