

Video Article

Use of a Caspase Multiplexing Assay to Determine Apoptosis in a Hypothalamic Cell Model

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Abstract

The ability to multiplex assays in studies of complex cellular mechanisms eliminates the need for repetitive experiments, provides internal controls, and decreases waste in costs and reagents. Here we describe optimization of a multiplex assay to assess apoptosis following a palmitic acid (PA) challenge in an *in vitro* hypothalamic model, using both fluorescent and luminescent based assays to measure viable cell counts and caspase-3/7 activity in a 96-well microtiter plate format. Following PA challenge, viable cells were determined by a resazurin-based fluorescent assay. Caspase-3/7 activity was then determined using a luminescent substrate, DEVD, and normalized to cell number. This multiplexing assay is a useful technique for determining change in caspase activity following an apoptotic stimulus, such as saturated fatty acid challenge. The saturated fatty acid PA can increase hypothalamic oxidative stress and apoptosis, indicating the potential importance of assays such as that described here in studying the relationship between saturated fatty acids and neuronal function.

Video Link

The video component of this article can be found at <https://www.jove.com/video/51305/>

Introduction

Diets rich in saturated fatty acids such as palmitic acid (PA) have been linked to obesity and other comorbidities, including cardiovascular disease and diabetes^{1,2}. High fat diets have also been shown to increase oxidative stress, apoptosis, and neuronal degeneration in the hypothalamus, an important regulator of appetite and energy expenditure³⁻⁷. Understanding the mechanism through which high fat diet exposure induces hypothalamic dysregulation is thus important for development of pharmacological treatments for obesity. However, the cellular mechanisms through which dietary fat affects neuronal function remain unclear. A better understanding of how fats such as PA might trigger onset of hypothalamic apoptotic pathways is a necessary first step toward this aim. The goal of this article is to describe a multiplex assay for *in vitro* testing of neuronal response to PA exposure, developed for use in studies of hypothalamic neurodegeneration. We provide a detailed description of an *in vitro* 96-well format multiplex assay for measuring caspase 3/7 activity per total number of cells in a differentiated immortalized adult mouse hypothalamic cell line (designated A12) after oxidative challenge with PA⁸.

Briefly, cell viability is determined following PA challenge via a resazurin based assay. Resazurin is a cell permeable compound that undergoes enzymatic reduction in metabolically active cells, a process thought to occur via the mitochondria⁹. Viable cells continuously convert resazurin to resorufin, producing a fluorescent signal proportional to the number of viable cells. Caspase-3/7 activity is then analyzed using a DEVD-based luminescent assay. DEVD is an amino acid sequence (Asp-Glu-Val-Asp) cleaved by caspase-3. When this sequence is coupled to a luminescent substance, upon activation of intracellular caspase-3/7 and subsequent cleavage of DEVD substrate, the luminescent product is released. This reaction is proportional to caspase activity and thus to the induction of apoptosis. As dead cells cannot produce caspase, caspase-3/7 activity is by nature transient; therefore analysis should be completed between 30 min to 4 hr post-challenge, depending on effectiveness of cell stressor. Cell viability is inversely proportional to caspase-3/7 activity and can be used to determine mechanisms of cell death. For example, this method has previously been used to show that pretreatment with the peptide hormone orexin reduces apoptosis in hypothalamic cells challenged with hydrogen peroxide, suggesting that mechanisms affected by this treatment are important in protecting against oxidative damage¹⁰. It is important to note these assays are cell line- and tissue-dependent, as they rely upon mitochondrial activity to reduce assay reagents. This protocol has been optimized for adult mouse hypothalamic (A12) cells; however, methods described may be altered to fit within the scope of similar research.

Multiplexing assays from a single culture well provides an advantage over the traditional method of performing individual assays for several reasons. In addition to saving time, cell samples, and culture reagents, multiplexing assays can also provide knowledge of cell survival and death, provide internal controls, and eliminate the need for repetitive experiments^{11,12}.

Alternative methods have relied upon Western blots or ELISAs, which are reliable assays, but are expensive and time consuming (1-2 days), especially when using a 96-well format. Excluding the time it takes to culture cells for the multiplexing assay, the total time is less than 3 hr. While this protocol has been optimized for use with A12 cells, it may be altered for use in other models while keeping in mind factors that may influence cell integrity. Determining if this protocol would work for a series of experiments depends upon on the number of samples or experimental conditions and on planned downstream experiments.

Protocol

1. Plating and Maintenance of Cell Culture

1. Warm Dulbecco's Modified Eagle Medium (DMEM) culture media (DMEM + 10% FBS + 1% Penicillin/Streptomycin/Neomycin) to 37 °C.
2. Obtain stock of frozen A12 cells from -80 °C.
3. Rapidly thaw cells in 37 °C water bath; once thawed, gently pipette cells to transfer to a 75 cm² vented culture flask and add 10 ml of culture media.
4. Incubate flask overnight in 5% CO₂ incubator at 37 °C. Aspirate media after 24 hr and replace with fresh media. Continue growing cells until 70-80% confluence is obtained (2-3 days growth).

2. Counting and Plating Cells

1. Warm DMEM and 1x-trypsin-EDTA solution to 37 °C.
2. Aspirate media from cells and wash two times with sterile phosphate buffered saline (PBS).
3. Add 500 µl of trypsin solution and incubate at 37 °C for 2-5 min.
4. Detach cells from flask using a scraper and suspend cells in 5 ml of media. Pass cells through strainer and pass through cell strainer into a clean 50 ml tube. May need to pass cells through strainer more than once, but do not repeat more than three times.
5. Count cells using a hemocytometer to determine amount of cells to culture to seed 6,000 cell/well in a 96-well clear bottom black or white walled plate in a final volume of 100 µl of media. Incubate plate in 5% CO₂ at 37 °C for 24 hr.

3. Determining Cell Viability and Caspase-3/7 Activity

1. Transfer PA to a sterile 5 ml glass vial and solubilize in 100% dimethyl sulfoxide (DMSO). Dilute stock PA solution 1:10 in DMSO and add it to prewarmed DMEM to obtain a final working concentration of 0.1 mM PA. For control media, add the same concentration of DMSO that is added to media containing PA.
2. Remove the media in each well and replace with 50 µl media +/- PA. Incubate the plate for 2 hr in 5% CO₂ at 37 °C. Then add 5 µl resazurin reagent and incubate for 10 min at room temperature.
3. Using a multimode microplate reader, record fluorescence (560_{EX}/590_{EM}) to measure cell viability. Results are presented as relative fluorescence units (RFU).
4. To the same plate, add 55 µl of caspase reagent and incubate at room temperature for 2 hr.
5. Again using the microplate reader, record luminescence to measure caspase-3/7 activity. Results are presented as relative luminescence units (RLU), with luminescence directly proportional to caspase-3/7 activity.
6. To normalize caspase-3/7 activity to cell count, divide cell viability (RFU) by caspase-3/7 activity (RLU).

Representative Results

The protocol above describes results in multiplexing two separate assays to determine mechanisms of cell death. **Figure 1** shows an overview of the protocol to determine cell viability and caspase-3/7 activity. Caspase activity was significantly increased in cells challenged with PA after 2 hr incubation (**Figure 2A** and **Table 1**). Loss of cell membrane integrity is a morphological change associated with the induction of apoptosis, which is apparent in cells after 2 hr exposure to PA (**Figure 2B**). **Figure 3** outlines the potential pathway in which PA induces apoptosis and demonstrates the importance of optimizing incubation time in the DEVD reagent.

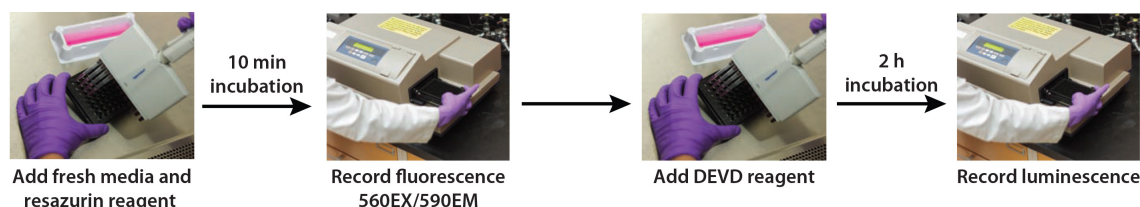


Figure 1. Experimental design for a multiplex assay to determine caspase-3/7 activity. Cells are seeded in a 96-well plate for 24 hr and then incubated in the presence or absence of PA. Incubation times in the presence of each reagent may vary depending on the model. [Click here to view larger image.](#)

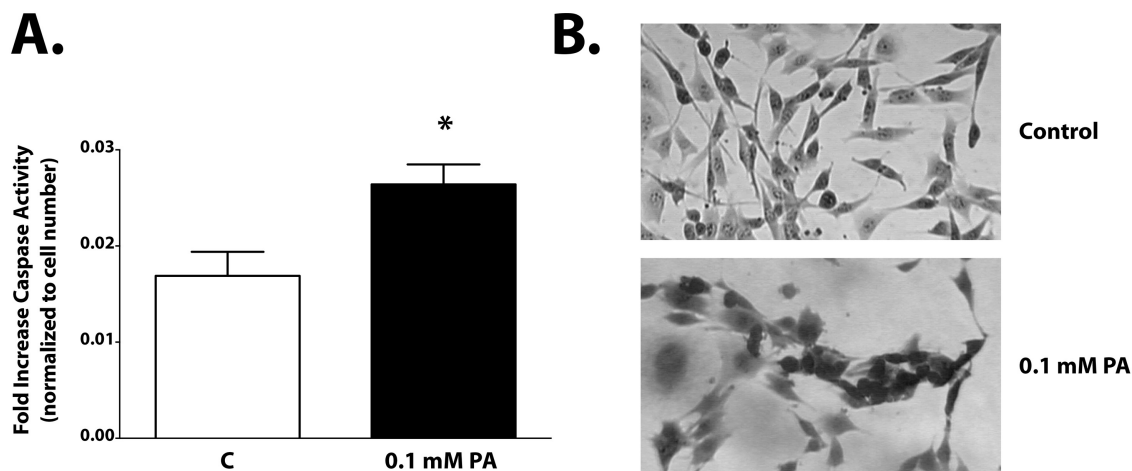


Figure 2. Caspase-3/7 activity is increased following 2 hr PA challenge. Hypothalamic A12 cells were treated in the presence or absence of 0.1 mM PA for 2 hr. Caspase-3/7 activity was significantly increased in cells treated with PA ($p < 0.01$). Cell membrane integrity is visibly lost in the presence of PA. [Click here to view larger image.](#)

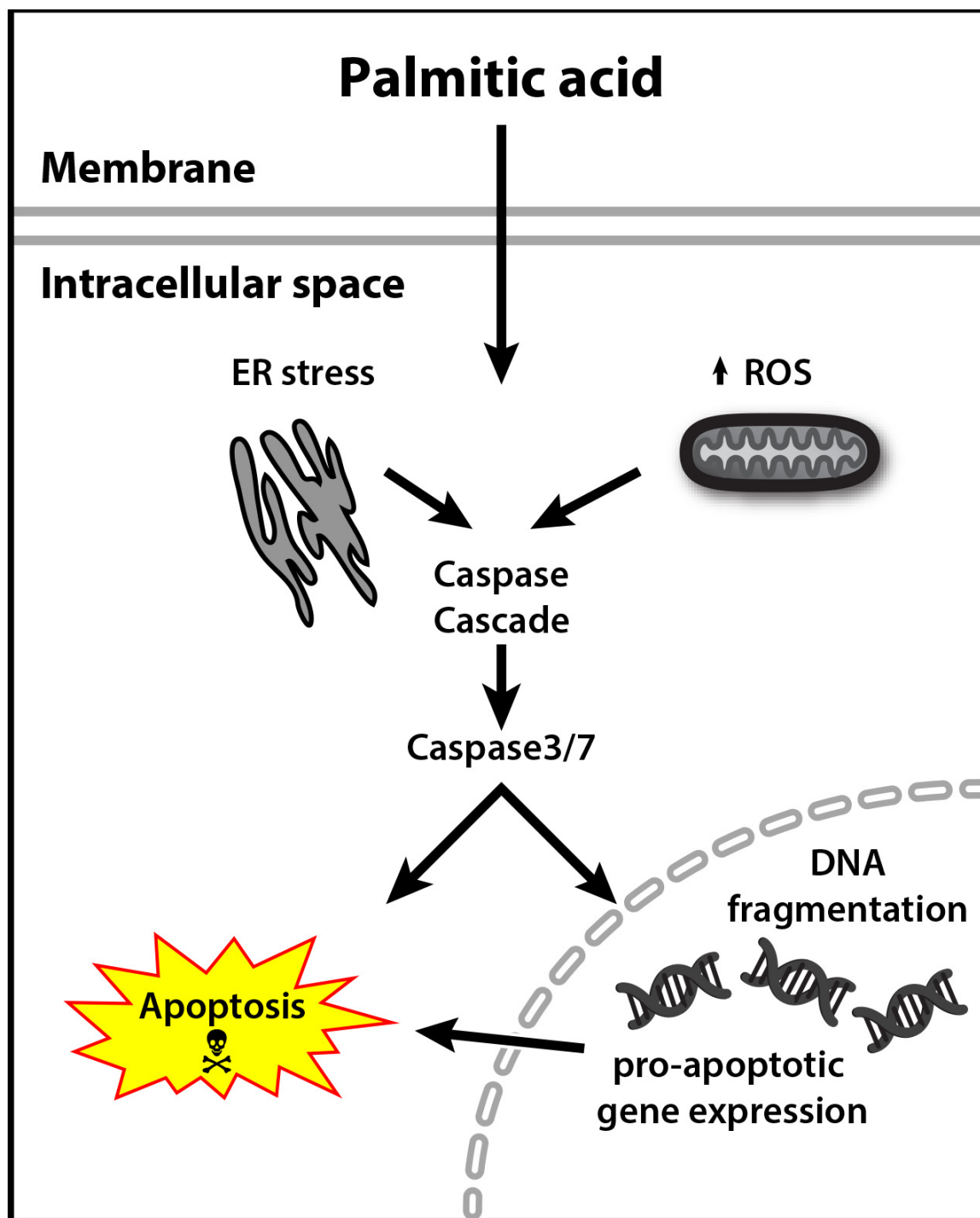


Figure 3. Potential pathway of PA induced cell death. [Click here to view larger image.](#)

Control			0.1 mM PA		
Viable Cells	Caspase 3/7	ratio	Viable Cells	Caspase 3/7	ratio
Fluorescence (560 _{EX} /590 _{EM})	Luminescence 650 nm peak	Caspase/ Viable Cells	Fluorescence (560 _{EX} /590 _{EM})	Luminescence 650 nm peak	Caspase/ Viable Cells
1951.8	45.488	0.023305667	808.78	16.731	0.020686713
1647.0	46.011	0.027936248	788.84	22.080	0.027990467
1911.0	34.508	0.018057561	777.68	28.234	0.036305421
1792.5	14.183	0.007912413	807.45	20.457	0.025335315

1965.7	19.868	0.010107341	829.14	17.777	0.021440288
1803.0	20.229	0.011219634	742.35	29.280	0.039442312
1804.8	27.188	0.015064273	761.77	17.777	0.023336440
1890.1	40.7825	0.021576901	756.66	20.914	0.027639891

Table 1. Example of collected data and calculated ratios.

Discussion

The multiplex assay is a well-accepted technique that has been used by scientists in numerous applications such as PCR microarrays, immunodetection, and other protein-based detection methods^{13,14}. Recently, multiplexing assays have become increasingly utilized in *in vitro* plate-based experiments and have been validated as an accurate method of assessing cytotoxicity and viability¹². In the above protocol, we demonstrate the effectiveness of multiplexing fluorescent and luminescent assays to determine caspase-3/7 activity and cell viability in A12 cells following PA challenge. Cell viability was determined within 10 min of initial PA insult, allowing for a rapid and reliable representation of viable cells. It should be noted that the rezasurin-based reagent is a live cell assay that allows for further endpoints to be measured; however, optimizing the incubation time to determine caspase-3/7 activity is essential to obtain usable results¹⁵.

Apoptosis can be activated by external stimulations, internal cell signals, and surface receptors resulting in distinct morphological and biochemical events. Increased caspase 3 activity, DNA fragmentation, loss of cell membrane integrity, chromatin condensation, and poly (ADP-ribose) polymerase (PARP) cleavage are some specific markers of apoptosis that can be investigated¹⁶. Activation of caspase 3 and 7 are primary modulators of apoptosis, making them essential markers to assess in pharmacological manipulations aimed at reducing apoptotic damage¹⁷. Cells undergoing prolonged apoptosis will eventually undergo secondary necrosis, observable by cell lysis, underscoring the importance of choosing an appropriate time frame to determine caspase-3/7 activity¹¹. As previously mentioned, caspase-3/7 activity is transient; therefore, depending on effectiveness of cell stressor, determining the appropriate timing to assay caspase activity can take some effort. Additionally, the main limitations for our multiplexing protocol are that caspase-3/7 activity can be determined but not quantified, and the resulting lysate cannot be used for downstream assays (*i.e.* Western blots or gene expression).

Understanding characteristics of the cell cycle of the chosen model and how it might respond to designated stressors when applying this technique is critical to obtain valid results. Additional considerations when planning studies that will evaluate apoptotic pathways are: 1) determining the appropriate density of cells that are seeded per well; 2) concentration and incubation of the stressor or drug used; and 3) the appropriate incubation time for the reagents used in an assay. The lack of standardization or of preliminary studies that address these considerations will generally result in erroneous data. The time and effort used to standardize the multiplexing assay we describe here provides a valuable, reliable, and timesaving technique for apoptotic studies¹⁰.

Disclosures

The authors have no conflicts to disclose.

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