A novel assay evaluating mitophagy in postmitotic skeletal muscle cells

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Abstract

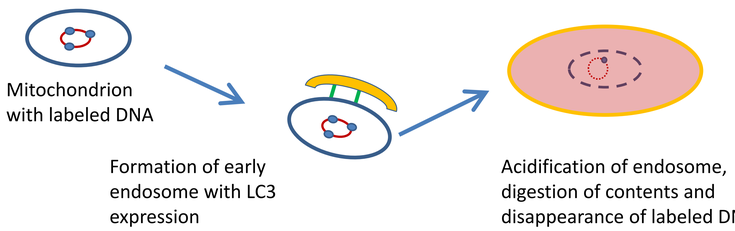
We developed a quantitative assay for mitophagy in postmitotic C2C12 muscle cells. The assay employs an ELISA readout and is simple and robust. Mitochondrial DNA synthesis was stimulated pharmacologically by an AMPK activator described by Xiao et al (2013) and referred to as compound-991. Cells were exposed to this compound (10uM) for two hours, following which they were allowed to incorporate the thymidine analogue bromodeoxyuridine (BrdU) into newly synthesised DNA for a further two hours. AMPK activation induced a 4 - 10 fold increase over background in the incorporation of BrdU. Microscopic examination confirmed that in these postmitotic cells, incorporated BrdU was localised to mitochondria and not nuclei. During subsequent culture after removal of BrdU, the level of label in cells declined with time to approximately 25% by 24 hours, and to background levels by 48 hours. The membrane depolariser carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) accelerated this process, such that at 6 hours, the BrdU signal was reduced to between 0-30% of initial levels. Pretreatment with bafilomycin, an inhibitor of early endosome acidification, for 24 or 48 hours, partially reversed the CCCP stimulated loss of BrdU. We confirmed by confocal microscopy that the BrdU in bafilomycin-treated cells colocalised with EEa1, a marker of early endosomes. Finally, we showed that treatment with 50 uM urolithin, a known promoter of mitophagy, accelerated BrdU signal loss such that at 6 hours, treated cells displayed about 50% of the untreated control value. This assay represents a robust and simple method to evaluate compounds that affect mitophagy in postmitotic muscle cells.

Introduction

Mitochondrial biogenesis and mitochondrial autophagy (mitophagy) regulate cellular adaptation in response to mitochondrial dysfunction. It has been recognised increasingly in recent years that defects in the dynamic mechanisms of biogenesis, fission / fusion, and mitophagy, underlie many different disease processes. Altered mitochondrial dynamics may be the cause of various pathologies not necessarily associated with genetic defects in mitochondrial DNA

Mitophagy has typically been studied by microscopic methods, either electron microscopy that can directly reveal the containment of mitochondria within phagosomal structures, or fluorescence microscopy, and these methods are relatively low throughput and not amenable to adaptation for drug discovery. In this study, we developed a simple readily quantitated ELISA assay for mitophagy applicable to differentiated skeletal muscle and potentially to other post-mitotic cells.

Figure 1: Schematic representation of assay

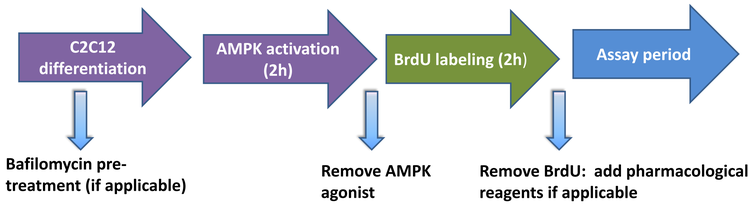


Materials and Methods

We synthesised the AMPK activator characterised and referred to as compound 991 by Xiao et al (2013); this compound was originally described in the patent literature (Giordanetto et al, 2012) as a cyclic benzimidazole derivative of compound A-769662 (Cool et al, 2006).

C2C12 cells were differentiated for 5 days as described by Kubo (1991) and then treated with compound 991 (10uM) for 2 hours, after which they were cultured with BrdU (10mM ; Roche BrdU labelling kit) for a further 2 hours. BrdU was removed and additional treatments applied as described. The membrane depolarising agent carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) was used at 20mM. At varying time points the cells were fixed and processed for BrdU ELISA detection using the Roche kit protocol; in some experiments, signals were amplified by using a one hour incubation with an intermediate biotinylated goat anti-mouse IgG to detect the kit-supplied mouse anti-BrdU antibody, followed by Streptavidin HRP for 1 hr (1-10,000 dilution). Signal was detected after washing using TMB substrate. For imaging , primary antibodies used overnight were anti-Brdu (1-50) and anti EEA1(1-200), detected with secondary FITC or Texas red labelled species specific antibody as appropriate. For cytometry, Hela cells overexpressing Parkin were treated as described. Live cells were analysed immediately after labeling with the membrane potential sensitive dye TMRE, or were fixed and stained with antibodies against cytochrome oxidase subunit 4 and then subjected to cytometry.

Outline of Procedure



Results

Figure 2: Only mitochondria are labelled with BrdU in postmitotic skeletal muscle cells

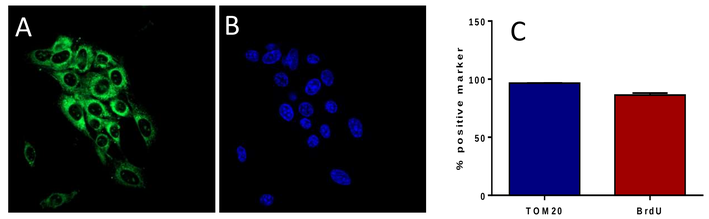


Figure 2: Imaging of BrdU (A) and DAPI (B) stain in C2C12 myoblasts. FACS analysis in Hela cells with Tom20 and BrdU (C). Partially differentiated C2C12 cells were labelled with BrdU and then fixed and stained. Prominent cytoplasmic labelling but no nuclear labelling, together with co-staining of BrdU and mitochondrial marker TOM20 in cytometry experiments confirm that only mitochondrial DNA is labelled under these conditions

Figure 3: Basal and stimulated mitophagy assayed by BrdU label attenuation

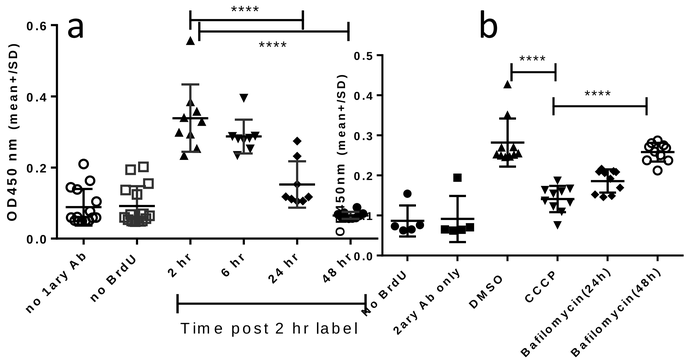


Figure 3: a: Unstimulated decay of mitochondrial BrdU signal. C2C12 myotubes were treated with AMPK agonist and labelled with BrdU. After removal of BrdU, the level of BrdU labeling remaining after varying times was quantitated. The rate of loss of BrdU represents the normal rate of unpotentiated mitophagy in these cells.

B: CCCP potentiation of decay rate and reversal by bafilomycin pretreatment. After AMPK stimulated labelling of mtDNA, CCCP potentiated the rate of signal loss, such that at 6 hrs only 20% remained vs untreated control. This corresponds to the loss of mitochondrial mass in the experiment shown in figure 4. Pretreatment with the endosome acidification inhibitor bafilomycin partially reversed this loss, confirming that the loss of labelled DNA is mediated through an endosomal mechanism.

Results

Figure 4: Flow cytometry confirms loss of membrane potential and mitochondrial mass on CCCP treatment

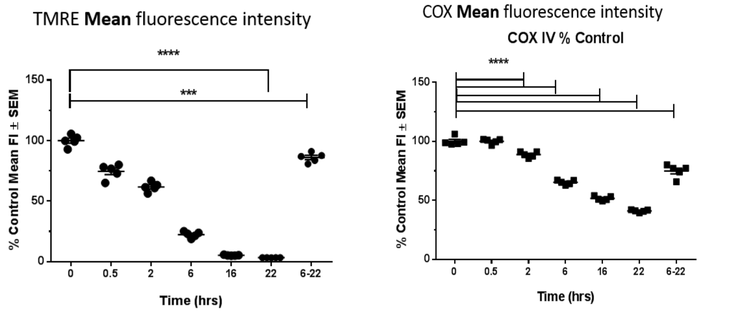


Figure 4: Effect of CCCP on mitochondrial membrane potential and mass. HeLa cells overexpressing Parkin were treated with CCCP (for varying periods and membrane potential and mitochondrial mass were analysed by flow cytometry using TMRE fluorescence, or immunofluorescence of cytochrome oxidase subunit 4 respectively. CCCP caused a loss of mitochondrial mass confirming the interpretation of BrdU signal loss in Fig.3. Removal of CCCP at 6 hrs resulted in recovery of membrane potential and partial recovery of mitochondrial mass

Figure 5: Endosomal localisation of incorporated BrdU and effect of urolithin

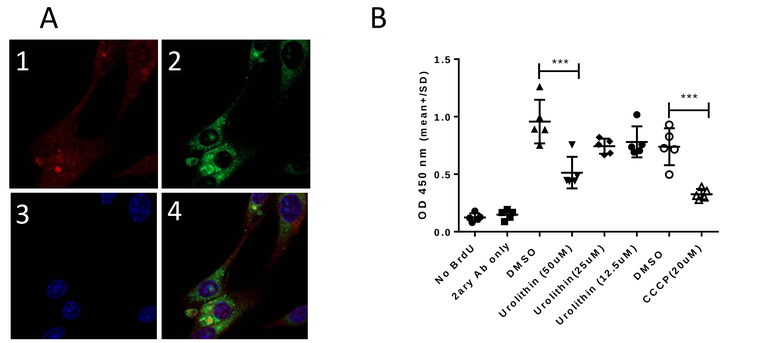


Figure 5: (a) C2C12 myotubes were pretreated with bafilomycin for 48 hrs and then mtDNA was labelled and the cells treated with CCCP for 6 hrs. BrdU was visualized with a green fluorescent antibody and the early endosomal marker EEa1 with a red fluorescent antibody. Colocalisation (panel 4) indicates that the BrdU is found in endosomes and that the assay represents authentic mitophagy.
(b) Mitophagy was induced by urolithin at high concentrations in agreement with a recent report (Ryu D et al et al (2016)

Conclusions and Future Directions

We describe a simple medium-throughput quantitative assay for studying mitophagy in a skeletal muscle cell line which does not require microscopy or cytometry. We are currently extending the assay to confirm its general applicability to all post mitotic cell types.

References

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