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THE DAMPENING OF LIPID DROPLET OSCILLATORY MOVEMENT IN NITROGEN STARVED FILAMENTOUS FUNGI BY A LOW DOSE OF MITOCHONDRIAL RESPIRATION INHIBITOR

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Abstract:

Lipid droplets (LDs) are small mobile organelles conserved in all eukaryotic cells. We wanted to test if the LD movement can be muffled by an incomplete inhibition of mitochondrial respiration, induced by treating hyphae of filamentous fungus *Phycomyces blakesleeanus* with 0.5 mM sodium azide. Nitrogen starved hyphae were used, in order to obtain LDs in larger sizes and numbers. The data obtained unequivocally showed: 1. Sodium azide treatment dramatically reduces the LD velocity and the distances LDs travel; 2. LDs in both controls and in azide-treated hyphae oscillate in a small confined space instead of travelling through the cell; 3. Azide-treated LDs oscillate less frequently and in smaller confinement than controls.

Key words: Sodium azide, *Phycomyces blakesleeanus*, *In vivo* microscopy, Nile Red

1. Introduction

Lipid droplets (LDs) are ubiquitous, highly phylogenetically conserved and seemingly simple organelles that are increasingly getting recognized as central coordinators of lipid metabolism between various cellular organelles [1]. LDs are often small and fairly mobile, found to form functional interactions with other cellular compartments [2]. During our filamentous fungi live microscopy investigations of LDs, the need has arisen to define a set of experimental conditions that will result in slowing down the LD movement just enough, to be able to perform colocalization studies *in vivo*. We present the results obtained by using a low concentration of sodium azide, the inhibitor of mitochondrial respiration. The azide effect and dose response on the model system fungi used in this study were previously measured in our lab [3].

2. Materials and Methods

Model organism was wild-type strain of the unicellular oleaginous filamentous fungus *Phycomyces blakesleeanus* (Burgeff) (NRRL 1555(−)), grown in lighted stationary plates from the spore stock as previously described [3]. In order to get larger LDs and possibly easer observe the movement of LDs, fungi were grown in nitrogen starvation conditions with the amino acids omitted from the standard medium. Live hyphae were stained with Nile Red. A drop of fungi culture was placed in custom built closed cover glass holder to be fitted on the microscope stage. The hyphae in the azide treated (0.5 mM) group were imaged by Two Photon Excitation (TPEF) microscopy with the time interval of 60 s. The

TPEF of Nile Red dye was excited by 1040 nm, 200 fs pulses from Yb KGW laser and the signal was collected through 400-700 nm band pass filter with the use of 40x1.3 objective. The details of the Nonlinear Laser Scanning Microscopy set-up can be found in [4]. The control group was imaged from independent hypha batch, cultured in the same conditions as the azide-treated group. Images for controls were taken on Zeiss Axiovert fluorescent microscope equipped with camera (Xenon lamp illuminated, DsRed filter, 20 X 0.8 NA objective), with image acquisition time interval: 1 s. LD movement was measured by the mTrack plugin of ImageJ freeware [5] from time lapse Nile Red image sequences. All parameters quantified were readily available from mTrack analysis, except mean frequency which was derived in the following way: for each LD tracked, the total path length was divided by the mean displacement from the starting point. The results are reported as mean ± SE at 95% confidence level. One way ANOVA or nonparametric rank sum (Mann Whitney) test was used for statistical testing.

2. Results and Discussion

Nile Red -stained LDs were imaged from nitrogen-starved hyphae with and without 0.5 mM azide (azide-treated and control group, respectively). The representative starting images of the time sequences are shown in Figure 1.A. It can be seen that images obtained by TPEF are much clearer, and probably represent true optical section of hypha, while in fluorescent microscope image, the various fainter structures around LDs are present, degrading the contrast of the image as expected. Since we were able to reliably identify LDs from both types of images, the LD movement analysis was not undermined with those differences in the image quality. The second methodological issue that could decrease the accuracy of the measurement is the possible occurrence of substantial bleaching of the dye, caused by the repeated illumination during the acquisition of image sequences.

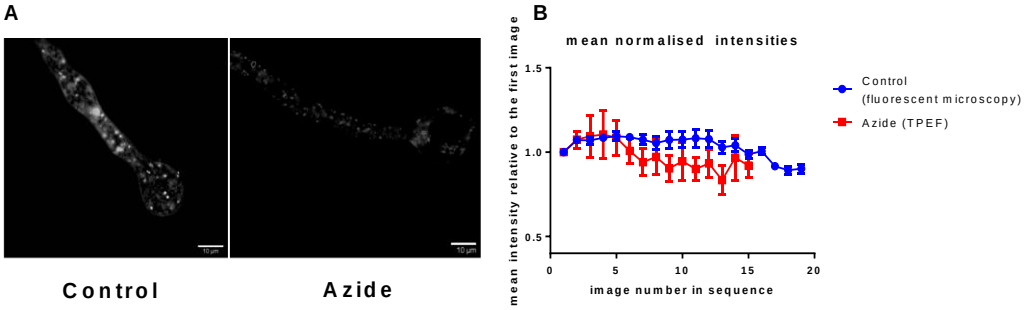

Fig. 1. Images of Nile Red -stained hyphae. A. Fluorescent microscopy image of hypha from the control group (left panel) and TPEF microscopy image of hypha from the azide-treatment group (right panel). The brightest small round features, visible on both images, represent the lipid droplets. B. Stability of Nile Red signal in images acquired in time sequence, quantified as mean normalized (to the intensity in first image of the sequence), intensity of randomly selected bright features in control and azide-treated group (n = 3 and n = 7 respectively).

As demonstrated in a graph shown in Figure 1B, the Nile Red signal intensity was stable during the time needed for sequence acquisition. At the end of the image sequence, the intensity was at 90 ± 2% from the starting values in the control group sequences, and at 92 ± 7% in the azide group, under the conditions used.

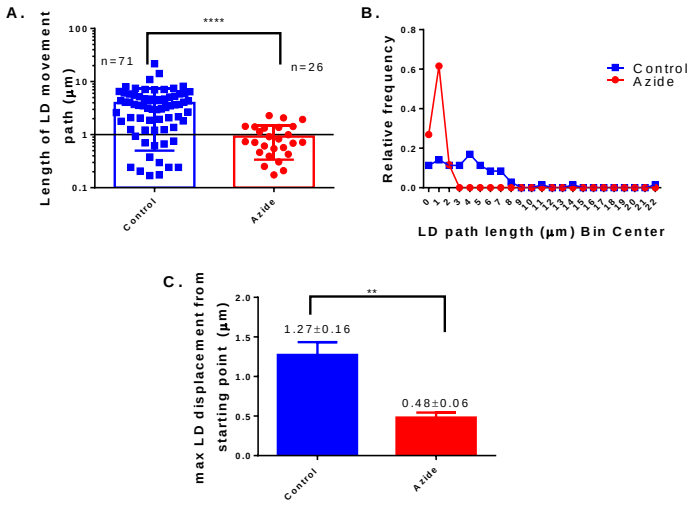

Fig. 2. The movement parameters obtained from LD tracking image analysis. A. The length of LD paths (µm). All obtained values from control and azide-treated hyphae are displayed; B. Distribution of LD path lengths, presented as a relative frequency histogram. C. Maximal displacement of LDs from the starting point, showing that most of LDs stayed close to the starting point throughout the imaging time.

All obtained LD path length values from control hyphae (mean: 4 ± 3 µm), from 12 s image sequences and azide-treated (mean: 0.9 ± 0.6 µm) from 11 min, are displayed in Figure 2A. Even measured on such different time frames, the control total path length values (movement in 12 s) were significantly larger than from azide-treated (movement in 11 min); Mann-Whitney test, $p< 0.0001$. Literally all of the visible LDs (in both groups) displayed oscillatory movement and were not transported further from the location occupied at the beginning of the image series. Maximal displacement from the starting point for each LD was pulled and mean value calculated for each experimental group: $maxD2S_{control}= 1.3 \pm 0.2$; $maxD2S_{azid} = 0.5 \pm 0.1$ (in µm); $p = 0.004$.

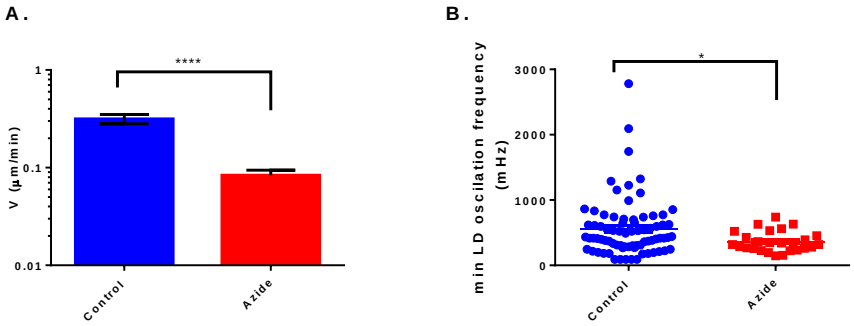

Fig. 3. The speed and frequency of LD movement were significantly lowered by azide treatment compared to control A. The mean speed of LD movement (µm / min) ($p< 0.0001$). B. The frequencies of LD oscillatory movement (mHz) ($p = 0.0333$).

Therefore, it can be concluded that LDs, under the conditions used, travelled just a few microns or less during acquisition. The average speed of LD movement (in µm / min) was dramatically reduced by azide-treatment: $V_{control} = 0.3155 \pm 0.034$; $V_{azide} = 0.084 \pm 0.01$. $p< 0.0001$ (Figure 3A).

Since the vast majority of the imaged LDs displayed oscillatory movement during the observation time, we expressed the LD movement as minimal oscillation frequency, in order to acknowledge the possibility of the presence of higher frequencies, outside of the frequency range of our observations. The minimal frequency of LD oscillatory movement (Figure 3B) was significantly lowered by azide treatment: $f_{control} = 558 \pm 52$ mHz compared to $f_{azid} = 363 \pm 31$ mHz ($p = 0.0333$).

It is possible that, due to the repeated illumination, LD transport was slowed down due to phototoxicity. Since the transport to more remote locations could not be observed even in the starting portions of image sequences, before the eventual photoxicity could take place, we concluded that

phototoxicity does not seem to be of importance for the phenomena observed.

Recent imaging study with extended spatial and temporal resolution of LDs in mammalian cells under control conditions also have shown that during the experiment LDs displayed mainly oscillatory movements, with short intermittent contacts with mitochondria [6]. There are two main types of LD movement described in the literature: oscillatory in confined space [7] and directional in short or long trajectories [8]. In addition, it would be expected that during autophagy processes, the periods of contact between LDs and mitochondria would be more frequent and/or of longer duration [9], possibly causing somewhat stationary LD behavior that we have observed.

4. Conclusions

LD movement in sodium azide-treated hyphae is significantly slowed down. The *in vivo* colocalization studies are feasible, if conditions used are replicated. The purely oscillatory movement and relative immobilization of LDs in controls should be investigated further.

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