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THE EFFECTS OF SELENITE ON FILAMENTOUS FUNGI LIPID DROPLETS MONITORED *IN VIVO* LABEL FREE USING ADVANCED NONLINEAR MICROSCOPY TECHNIQUE

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Abstract:

Third Harmonic Generation (THG) microscopy was employed as a method of choice for lipid droplet (LD) measurements and quantification of the effect of selenite on LDs.

Nonlinear laser scanning microscopy (NLSM) employs ultra-short laser pulses for imaging. THG microscopy is the modality of NLSM. Strong THG signals can only be observed from regions with non-uniformities with respect to their refractive index. Such regions in biological samples are lipid-water interfaces, and by far the brightest features in cells are LDs. For that reason, THG microscopy is the appropriate method for imaging of LDs from live unfixed cells, without the need for additional labeling.

The biological effects of spore- to- end- of- exponential- phase duration (27 - 30 h) of exposure to 1 mM selenite were monitored *in vivo* on the cells of filamentous fungi in liquid culture. We measured the lipid droplet density and size distribution in a model fungi *Phycomyces blakesleeanus*. The in-house built microscope frame complemented with Yb KGW laser (1040 nm, 200 fs pulses) was used, while detection was enabled in the transmission arm by PMT through the Hoya glass UV filter (peak at 340 nm).

From THG images of control and Se^{+4}–treated hyphae, LD size and number were measured, showing that LD density was increased by more than 60% in Se^{+4}–treated hyphae, compared to control. The average LD size distribution seemed slightly changed by Se^{+4} -treatment. The obtained results suggest that 1 mM selenite treatment probably induces cellular stress response in filamentous fungi.

Keywords: Lipid droplets, Selenite, Third Harmonic Generation Microscopy, *Phycomyces blakesleeanus*

1. Introduction

Selenium is an essential trace element for humans and animals, while it is not necessary for plants and fungi. The availability and biological activity of selenium depend on its dose and chemical form [1]. In trace amounts, selenium enhances antioxidant capacity in a number of selenoproteins while at higher concentrations, selenium is toxic due to its prooxidative effects like oxidation of protein thiols and reactive oxygen species generation [2]. Since the oxidative stress is among the main intracellular signals sustaining autophagy [3], and lipid droplet (LD) biogenesis seems to be a general cellular response to high autophagic flux according to recent studies [4], we hypothesized that increased LD formation could be an immediate cellular response to a high selenium exposure.

In order to reliably monitor LDs *in vivo* by imaging, and measure the effects of selenite-induced oxidative stress- mediated cellular changes, it would be necessary to employ the imaging method that causes minimal additional phototoxicity. Otherwise, the oxidative stress induced by, for example, confocal imaging of labeled LDs, could potentially interfere with the processes underlying the measurements. For those reasons, Third Harmonic Generation (THG) microscopy was employed as a method of choice for LD measurements and quantification of the effect of selenite on LD number and sizes. THG microscopy is one of the modalities of nonlinear laser scanning microscopy (NLSM). In NLSM, the high laser intensity, and low average power due to employing ultra-short laser pulses, allow for the generation of nonlinear imaging signals. Although THG microscopy is not chemically specific, strong THG signals can only be observed from regions with non-uniformities with respect to their refractive index. Such regions in biological samples are lipid-water interfaces, and by far the brightest features in live cells are LDs [5]. For that reason, THG microscopy is especially appropriate method for LD imaging from live unfixed cells, without the need for additional labeling.

The biological effects (on the lipid droplet density and size distribution) of exposure to 1mM selenite were monitored *in vivo* on the cells of filamentous fungi in liquid culture. Filamentous fungi are one of the main pathways for selenium entrance into ecosystems and able to concentrate selenium in the mycelia [6]. Additionally, fungi are simple to manipulate, unicellular model system, that is naturally without selenoproteins encoded in genome [7]. Therefore, in fungi the prooxidative effects of selenium can be observed unhindered with simultaneous beneficial selenium-mediated effects.

2. Materials and Methods

Model organism was unicellular wild-type strain of the oleaginous filamentous fungus *Phycomyces blakesleeanus* (Burgeff 1925) (NRRL 1555(−)), grown in lighted stationary plates from the spore stock as previously described [8]. To observe the effect of treatment with 1mM sodium selenite, the prepared fungi activated spore culture volume was divided to control culture and treatment culture (same as control, with addition of sodium selenite in final concentration 1mM). The experiments were performed in triplicate.

As a method of choice for label free *in vivo* LD measurements, the application of THG microscopy was employed: 1040 nm, 200 fs pulses from Yb KGW laser were used; THG signal was detected by PMT in the transmission arm after passing through the Hoya glass UV filter with the peak at 340 nm. The obtained images were analyzed in ImageJ to quantify LDs number and size. The results are reported as mean ± Standard error (SE) and statistically tested by student t-test with 95% confidence level.

3. Results and Discussion

In THG images obtained from control and selenite (Se^{+4})-treated hyphae, LDs can be readily observed (Figure 1a).

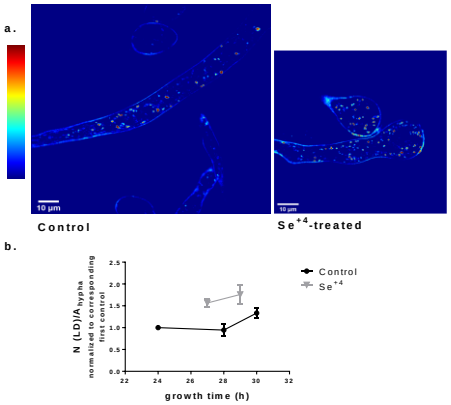

Figure 1. a.) THG images of: Control (28h), in the left panel; Se^{+4}–treated hypha (26h) in the right panel. Calibration bar is shown on the left (bottom: minimal; top: maximal intensity). Brightest spots represent LDs, and the faint cell wall THG signal can be seen as well. The increased LD density in treated group is visible. b.) LD density (LD number per unitary hypha area) was normalized for each independent experiment (n=3) to the LD

density value of the first control (24h) and plotted as a function of growth time. Control: black circles; Se^{+4}–treated: gray triangles.

From THG images of control and Se^{+4}–treated hyphae, LD number (Figure 1b) and size were measured (Figure 2). Fig. 1b. graph shows that average LD density increased by more than 60% in Se^{+4}-treated hyphae, compared to control. LD density was calculated as: (LD number in the hypha) / (Area of that hypha (μm^2)). Trend for slight increase of LD density in oldest controls is also evident, although it is less pronounced than in treated group (33 ± 16% increase in aged controls vs. 88 ± 26% in aged Se^{+4}-treated). Average LD size was unchanged (Fig.2b.), not supporting the expectation that the stress induces generation of new LDs. Distribution of LD sizes on the other hand, shows that Se^{+4}–treated LDs are more frequently small (around 1 µm) compared to LDs in controls. In addition, LD distribution in controls, but not in treated group, always had a "right side shoulder"-telltale sign that the separate population of LDs with diameters larger than group average is present. Same finding is more clearly seen in Fig 2c. graph, where the obtained parameters of Gauss function fits to the distribution of LD diameters are shown.

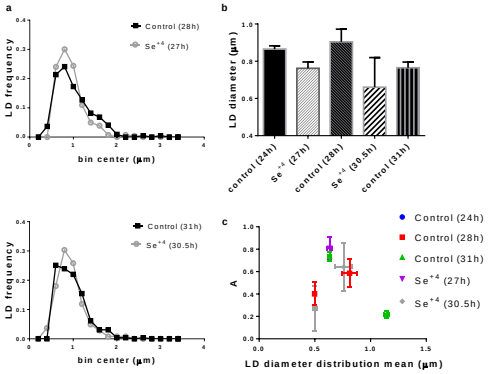

Figure 2. Size of LDs in the control and the Se^{+4}-treated group. a.) Distributions of LD size for the Se^{+4}-treated and for the age closest control. Top: 27 h treatment / 28 h control. Bottom: 30.5 h treatment / 31 h control. b.) Mean ± SD diameters of LDs in all groups (n = 200 - 400 LDs for each group). c.) Obtained parameters of the Gauss fit to LD size distribution. A - frequency of the component fitted. Some group distributions could be fitted with one normal distribution, but most often, two components were present.

LDs in the model fungus *Phycomyces blakesleeanus* are very small (mean diameters in all groups are less than 1 µm), while the resolution limit of the images presented is at around 0.4 µm. Smaller than average LDs were barely above the limit. Therefore, the close proximity to resolution limit is probably the cause for our inability to reliably detect the Se^{+4}–induced generation of the smallest LDs and subsequent lowering of the mean diameter.

3. Conclusions

We were able to measure shift of the distribution of sizes, and the increase of the number of LDs induced by 1 mM selenite treatment. THG modality of NLSM enabled *in vivo* and label free physiological study that provided data in support of the hypothesis presented. Based on our data, it can be concluded that selenite induces cellular stress leading to autophagy and subsequent LD formation.

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