SUPPORTING INFORMATION

Title: Chemical cytometry quantitates superoxide levels in the mitochondrial matrix of single myoblasts

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This supporting information includes the following sections:

I. Bulk analysis of OH-TPP-E^+ inside and outside mitochondria
II. Estimation of the amount of OH-TPP-E^+ in single myoblasts after CCCP treatment
III. Estimation of the amount of R123 in single myoblasts after CCCP treatment
IV. Fluorescence microscopy of myoblasts after TPP-HE loading
V. Fluorescence microscopy evaluation of mitochondrial membrane potential in single myoblasts

I. Bulk analysis of OH-TPP-E^+ inside and outside mitochondria

After incubation with TPP-HE, myoblasts were treated with 10 μg/mL digitonin at 4 °C for 20 min to selectively permeabilize their plasma membranes. When needed, before incubation with TPP-HE, cells were treated with either 5 μM rotenone or 5 μM antimycin A. The permeabilized cells were then centrifuged down at 600 g for 5 min. The supernatant was used to analyze the OH-TPP-E^+ released outside the mitochondrial inner membrane (i.e. the intermembrane space and cytosol). The cell pellet was dissolved in running buffer and treated with 2mg/mL proteinase K and 400U/mL DNase 1 prior to the analysis of the OH-TPP-E^+ in the mitochondrial matrix.[9]

Figure S-1 shows the electropherograms of TPP-HE oxidation products from the matrix and outside of the mitochondria prepared in bulk from ~ 1×10^6 myoblasts under basal conditions, upon treatment with rotenone, or upon treatment with antimycin A. According to equation 13 in the main manuscript, the amounts of OH-TPP-E^+ in the matrix and outside of the mitochondria of the myoblasts under basal conditions is ~ 7.1 amole/cell and ~0.34 amole/cell, respectively (Figure S-1A). Thus, the amount of superoxide product outside the mitochondria is ~ 4.8% of that in the matrix. Similarly, the amount of OH-TPP-E^+ outside the mitochondria is ~ 6.3% and 7.1% of that in the matrix of the myoblasts upon treatments with rotenone and antimycin A, respectively (Figure S-1B and C).

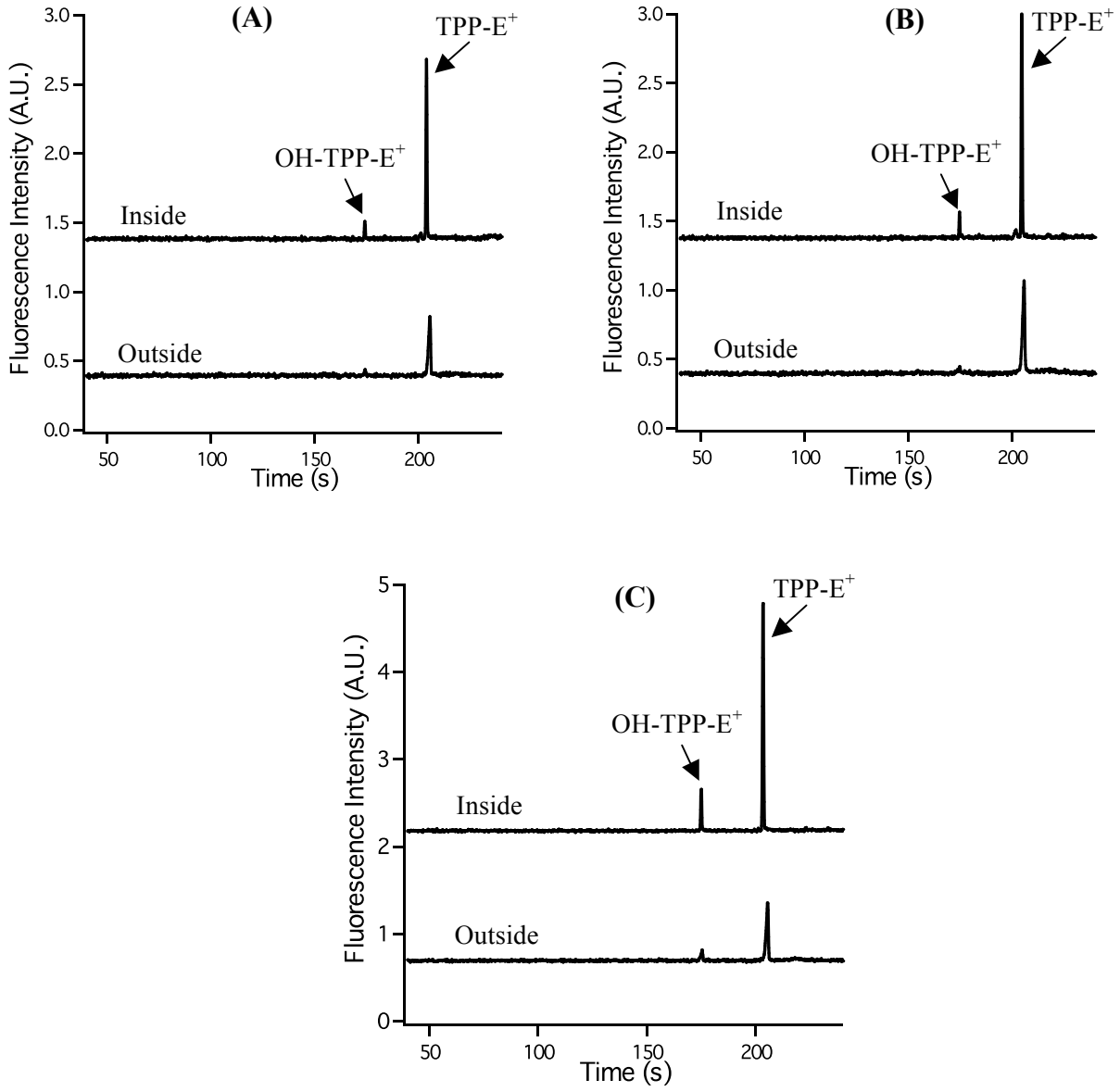

Figure S-1. Electropherograms of TPP-HE oxidation products inside and outside mitochondria isolated in bulk from myoblasts. Cells were either under basal conditions (A), treated with rotenone (B) or treated with antimycin A (C) prior to their analysis. The samples in the matrix and outside the mitochondria were diluted 20 and 5 times,

respectively, in running buffer before MEKC-LIF analysis. Separations and detection conditions were as described in Figure 1 in the main manuscript.

II. Estimation of the amount of OH-TPP-E^+ in single myoblasts after CCCP treatment

Based on the bulk analysis (Supporting Information, Part I), the amount of OH-TPP-E^+ in the mitochondrial matrix is estimated to be ~ 7.1 amol in a myoblast under basal conditions. If the same myoblast is treated with CCCP, its mitochondrial membrane potential is dissipated, decreasing the concentration of TPP-HE ~100-fold in the matrix relative to the basal conditions without CCCP treatment. Since the rate of OH-TPP-E^+ formation is proportional to the concentration of TPP-HE (c.f. equation 4 and 5), the formed OH-TPP-E^+ in the matrix with CCCP treatment is ~1% of that without CCCP treatment, which will be ~0.07 amol. On the other hand, CCCP would not affect the rate of formation of OH-TPP-E^+ outside the mitochondria. Thus, based on the bulk analysis the amount of OH-TPP-E^+ outside the mitochondria is ~ 0.34 amol (Supporting Information, Part I). Therefore the total amount of OH-TPP-E^+ in a CCCP-treated myoblast is ~0.41 amol. This amount is lower than the detection limit of OH-TPP-E^+ of the MEKC-LIF method described here (i.e. ~ 2 amol; signal/noise = 3), which explains the absence of the OH-TPP-E^+ peak in electropherograms of single myoblasts with CCCP treatment (c.f. Figure 2 in the main manuscript).

III. Estimation of the amount of R123 in single myoblasts after CCCP treatment

The diameter of L6 myoblasts imaged in Figure S-2A is ~ 20.1 ± 4.1 μm (average ± Std. Dev.; n=16). When a myoblast is treated with CCCP, the mitochondrial membrane potential is dissipated, thus $[R123]_{inside} = [R123]_{outside}$ = 50 nM. Therefore, a CCCP-treated cell has ~ 0.21 ± 0.01 amol R123. This amount is lower than the limit of detection of R123 of the MEKC-LIF method described here (i.e. ~ 0.2 amol; signal/noise = 3). This explains the absence of this feature in electropherograms of single myoblasts treated with CCCP (c.f. Figure 2 in the main manuscript).

IV. Fluorescence microscopy of myoblasts after TPP-HE loading

An Olympus IX-81 inverted microscope (Melville, NY) equipped with a 10X objective, a TRITC filter set (ex. 510–560 nm, 565 nm dichroic, em. 570–650 nm) and a C9100-01 CCD camera (Hamamatsu, Bridgewater, NJ) was used to image myoblasts treated with TPP-HE. SimplePCI 5.3 software (Compix, Cranberry Township, PA) was used to control the hardware and capture the images.

Fluorescence detected in each cell results from the addition of the fluorescence produced by both OH-TPP-E^+ and TPP-E^+ (Figure S-2, Part B).

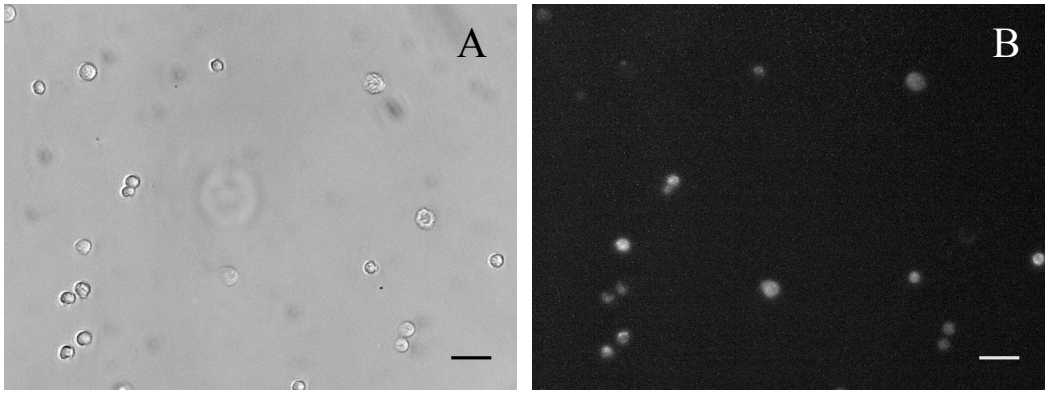

Figure S-2. Imaging of TPP-HE oxidation products in L6 myoblasts. (A) Bright-field image of the myoblasts in PBS. (B) Fluorescence image of the same cells after loading with TPP-HE. Scale bar = 50 μm. Exposure time = 100 ms.

V. Fluorescence microscopy evaluation of mitochondrial membrane potential in single myoblasts

The cultured myoblasts were labeled with 50 nM TMRM in medium for 30 min at 37 °C. After labeling, the cells were washed twice with PBS and imaged by epi-fluorescence microscopy. Then 50 μM carbonyl cyanide m-chlorophenylhydrazone (CCCP) was added to disrupt the mitochondrial membrane potential for 10 min, and the same cells were imaged again after CCCP treatment. The relative TMRM fluorescence was calculated for each cell as shown in Equation S-1, which is a relative indicator of the mitochondrial membrane potential of individual cells.[36]

$$\text{Relative TMRM fluorescence} = \frac{[(F_{before} - F_{background}) - (F_{after} - F_{background})]}{(F_{after} - F_{background})} \quad \text{(S-1)}$$

where F_{before} and F_{after} are the average fluorescence intensity values of each myoblast before and after CCCP treatment, and $F_{backgound}$ is the average fluorescence intensity of the image background.

The Relative TMRM fluorescence values for individual myoblasts (n=15) are summarized in Figure S-3, Part C. These results demonstrate that there is considerable variation in mitochondrial membrane potential among myoblasts in the same culture.

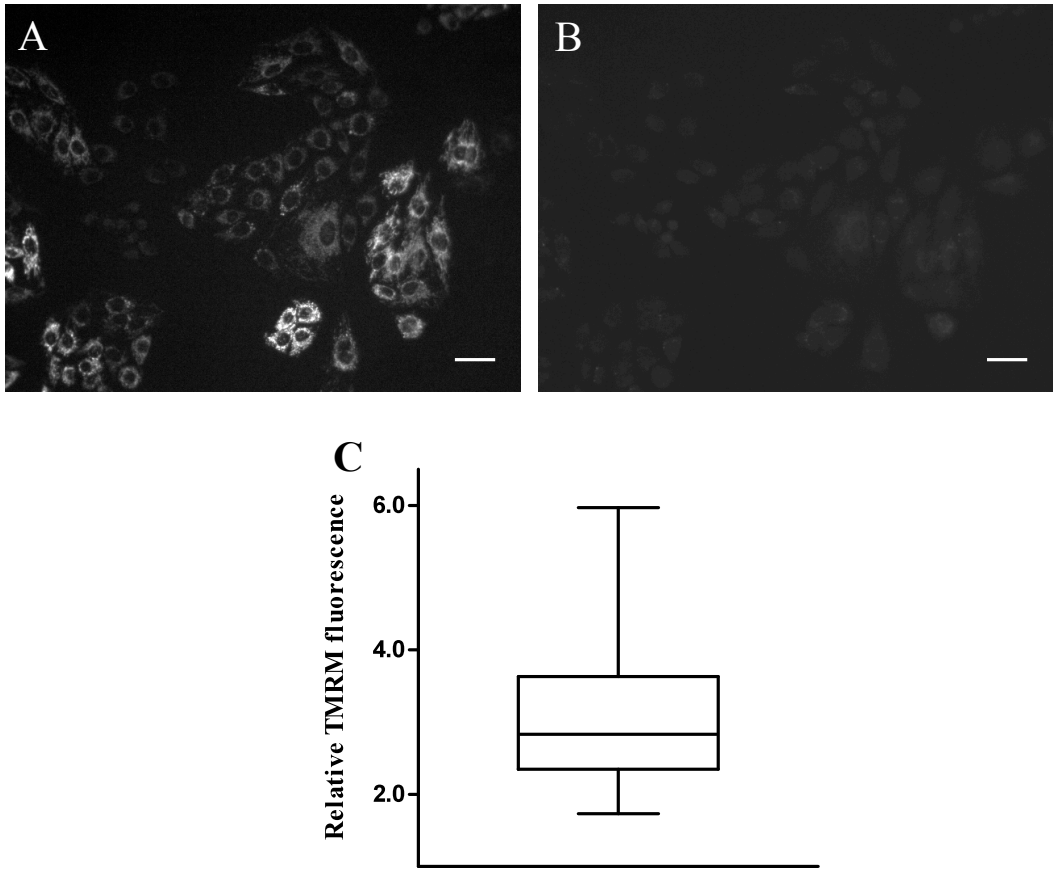

Figure S-3. Mitochondrial membrane potential in single skeletal muscle myoblasts. TMRM fluorescence images of the myoblasts before (A) and after (B) CCCP treatment**.** Scale bar = 50 μm. Other conditions were as in Figure S-2. (C) Box-plot presentation of relative TMRM fluorescence values of single myoblasts, n = 15.