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Analysis of Superoxide Production in Single Skeletal Muscle Fibers

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Abstract

Due to their high energetic profile skeletal muscle fibers are prone to damage by endogenous reactive oxygen species (ROS), thereby causing alterations in muscle function. Unfortunately, the complexity of skeletal muscle makes it difficult to measure and understand ROS production by fibers since other components (e.g. extracellular collagen and vascular vessels) may also generate ROS. Single cell imaging techniques are promising approaches to monitor ROS production in single muscle fibers, but usually the detection schemes for ROS are not specific. Single cell analysis by capillary electrophoresis (a.k.a. chemical cytometry) has the potential to separate and detect specific ROS reporters, but the approach is only suitable for small spherical cells that fit within the capillary lumen. Here, we report a novel method for the analysis of superoxide in single fibers maintained in culture for up to 48 hours. Cultured muscle fibers in individual nanoliter-volume wells were treated with triphenylphosphonium hydroethidine (TPP-HE), which forms the superoxide specific reporter hydroxytriphenylphosphonium ethidium (OH-TPP-E^+). After lysis of each fiber in their corresponding nano-well, the contents of each well were processed and analyzed by micellar electrokinetic capillary chromatography with laser-induced fluorescence detection (MEKC-LIF) making it possible to detect superoxide found in single fibers. Superoxide basal levels as well as changes due to fiber treatment with the scavenger, tiron, and the inducer, antimycin A were easily monitored demonstrating the feasibility of the method. Future uses of the method include parallel single-fiber measurements aiming at comparing pharmacological treatments on the same set of fibers and investigating ROS production in response to muscle disease, disuse, exercise and aging.

Keywords

Single fiber; skeletal muscle; superoxide; tiron; antimycin A; micellar electrokinetic capillary chromatography; capillary electrophoresis; laser-induced fluorescence; soleus

INTRODUCTION

Reactive oxygen species (ROS) are one of the hallmarks of multiple diseases and aging.[1–2] Investigating ROS production and their effects on skeletal muscle fibers is difficult due to the heterogeneous and complex nature of skeletal muscle.[3] This tissue comprises of different muscle fiber types with distinctive metabolic properties,[3] nerves, endothelial and blood cells,[4] and extracellular matrix, making it difficult to distinguish the unique patterns of ROS production in each specific fiber type.[5–6]

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Single cell analysis techniques are usually adequate to meet bioanalytical demands imposed by sample heterogeneity and complexity in biological systems.[7] Among these, chemical cytometry has been used to electrophoretically separate the contents of individual cells.[8–11] When using these techniques, small spherical cells (or their contents) are completely injected into a narrow-bore capillary for analysis.[12–13] These techniques cannot be used to analyze muscle fibers due to their morphological properties (cylindrical and elongated) and dimensions (i.e. ~100 μm diameter and ~2.8 nL volume for isolated soleus muscle fibers). One approach to analyze non-spherical cells is the use of small vials (150 nL to 750 μL volume).[14–16] This approach has been used in the analysis of single neurons contents.[15–16] Unfortunately, skeletal muscle fibers are smaller than neurons (e.g. ~300 μm diameter, ~14.1 nL volume),[15] making it difficult to handle them and place them into vials directly for processing. To analyze samples smaller than the neurons, several studies have reported the use of picoliter wells in the analysis of single mammalian cells.[17–18] In this study, we apply similar strategies for single skeletal muscle fiber analysis by using 60-nL nanowells.

Another complication arises when single cells are taken from a bulk preparation and are analyzed sequentially. Those cells analyzed last may display compromised cellular functions. In order to address this issue, several studies have reported the use of cultured single skeletal muscle fibers.[19–20] These studies make it simpler to select specific types of fibers, avoid non-myogenic cells, and maintain an environment in which the fibers remain functional.[21–22] Although such a single muscle fiber preparation has been used to characterize properties such as the activation of satellite cells,[23] adhesion of nerve cells,[24] and the production of nitric oxide, [22] monitoring of these properties is performed directly by fluorescence microscopy. A salient report described the detection of ROS in single muscle fibers by fluorescence microscopy.[21] The measurement of ROS was, however, based on the oxidation of a non-specific probe, chloromethyl dichlorodihydrofluorescin (CM-H_2DCF), which is non-specific and reacts with a variety of ROS and reactive nitrogen species.[21,25] Sensitive methodologies to monitor specific ROS, such as the superoxide, have been recently developed based on the use of fluorogenic hydroethidine-based probes or amperometry.[26–31] Amperometric probes, while adequate to monitor superoxide released from single fibers, cannot report on intracellular superoxide levels. In this report we used one of these fluorogenic probes, triphenylphosphonium hydroethidine (TPP-HE), [30–32] which is membrane permeant and can also accumulate in mitochondria.

In this study, we introduce a technology to analyze superoxide within cultured single skeletal muscle fibers. We plated isolated single skeletal muscle fibers in nanoliter-volume wells. The fibers were functional (i.e., presence of mitochondrial membrane potential and absence of externalized phosphatidylserine) up to 48 hours. For superoxide analysis, the fibers were incubated in their respective nanovial with TPP-HE and lysed by freeze-thawing. The nanoliter volume wells prevent excessive dilution of the fiber contents after lysis and require small amounts of reagents. Subsequently, the contents released from each individual muscle fiber were analyzed by micellar electrokinetic capillary chromatography with laser-induced fluorescence detection (MEKC-LIF).[32] Detection of the superoxide specific product, OH-TPP-E^+, from each individual soleus skeletal muscle fiber demonstrated the feasibility of superoxide detection. Further single fiber experiments in which the fibers were treated with tiron (superoxide dismutase mimetic) and antimycin A (superoxide production inducer), validated the suitability of this approach for superoxide detection from individual fibers. This technology has potential to be used in studies comparing superoxide production in different skeletal muscle fiber types as a function of muscle performance, disease, and aging.

EXPERIMENTAL SECTION

Chemicals and Reagents

Tetramethylrhodamine methyl ester (TMRM), Alexa Fluor 488 annexin V, propidium iodide and MitoSOX Red (TPP-HE) were obtained from Invitrogen–Molecular Probes (Eugene, OR). Polydimethylsiloxane (PDMS) prepolymer (Sylgard 184) was obtained from Dow Corning (Midland, MI). All the other reagents were purchased from Sigma–Aldrich (St. Louis, MO). The annexin-binding buffer contained 10 mM HEPES, 140 mM NaCl and 2.5 mM $CaCl_2$ (pH 7.4). The lysis buffer contained 10 mM sodium borate, 1% w/v Triton X-100 and 50% w/v glycerol (pH 9.4). Glycerol prevents the evaporation from the small volume of lysis buffer (~ 50-nL) during the disruption step.[17] The MEKC running buffer contained 10 mM sodium borate and 2 mM cetyltrimethylammonium bromide (CTAB) (pH 9.3). All buffers were prepared using Milli-Q deionized water and filtered through 0.22-μm filters before use. Collagen (type I) was dissolved in culture medium (0.4 mg/mL). For collagen coating, each 35-mm petri dish (Sarstedt Inc., part # 831800) or 24-mm PDMS plate was coated with 120 μL collagen solution for 15 min on ice. The excess collagen solution on the top of the PDMS plate was scraped away using a glass coverslip before use.

Isolation of Single Soleus Skeletal Muscle Fibers

Muscle fiber segments, referred herein as single muscle fibers,[19] were isolated from the soleus skeletal muscle of male Fischer 344 rats (11 months old) following previously reported procedures.[21–22] Briefly, the soleus muscles were dissected and immediately transferred to minimum essential medium (MEM) containing 0.4% (w/v) collagenase (type H) for 2 hours at 37°C. The muscles were constantly shaken during the incubation period. Then a wide-bore pipette with a 1.5-mm diameter tip was used to gently triturate the digested muscle bundles and release the fibers. The obtained muscle fiber suspension was centrifuged at 600g for 30 seconds to remove the muscle debris in the suspension. The pelleted fibers was then resuspended in MEM and washed twice more.

For fluorescence microscopy evaluation of mitochondrial membrane potential and phosphatidylserine externalization, the fibers were plated onto 35-mm collagen-coated petri dishes. For superoxide analysis by MEKC-LIF, the fibers were plated onto collagen-coated PDMS plates and allowed to settle into its 60-nL volume wells. In both instances, fibers were cultured in MEM at 37°C in 5% CO_2.

Evaluation of Mitochondrial Membrane Potential

This property was evaluated to assess the viability and respiratory activity of single skeletal muscle fibers after isolation. An Olympus IX-81 inverted microscope (Melville, NY) equipped with 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 evaluate the mitochondrial membrane potential.[33] SimplePCI 5.3 software (Compix, Cranberry Township, PA) was used to control the hardware and capture all the images.

First, the muscle fibers were treated with 50 μg/mL saponin at 4°C for 20 min to selectively permeabilize their plasma membrane,[34] therefore eliminating the effect of the plasma membrane potential. Then the cultured fibers were labeled with 50 nM TMRM in MEM for 30 min at 37 °C. After labeling, the fibers were washed twice with PBS and imaged by epi-fluorescence microscopy. Then 100 μM carbonyl cyanide m-chlorophenylhydrazone (CCCP) was added to disrupt the mitochondrial membrane potential for 10 minutes and the same fibers were imaged again after CCCP treatment. The remaining fluorescence is attributed to the non-specific binding of TMRM to the mitochondrial membranes.[33,35] Thus a value of relative

TMRM fluorescence was calculated for each muscle fiber as shown in Equation 1, which is indicative of the relative mitochondrial membrane potential of individual muscle fibers.[33]

$$\text{Rel.flouresence}=[(F_{before}-F_{background})-(F_{after}-F_{background})]/(F_{after}-F_{background}) \quad \text{Eq. (1)}$$

where F_{before} and F_{after} are average fluorescence intensity values of each muscle fiber before and after CCCP treatment, and $F_{backgound}$ is the average fluorescence intensity of the background in the image. Mitochondrial membrane potential measurements were performed at 24, 48 and 72 hours after plating of the single muscle fibers.

Treatment and Lysis of Isolated Single Skeletal Muscle Fibers in Nanoliter-volume Wells

In order to prepare a substrate with 100 nanowells, a silicon master was fabricated based on previously reported methods.[36] Briefly, a mixture of PDMS prepolymer was casted onto the silicon master and cured at 65 °C for 1 hour.[37] The PDMS plate was then peeled from the master and cleaned by 30-min sonication in ethanol. The cleaned PDMS plate was then oxidized in a 0.35 mbar oxygen plasma for 30 seconds,[38] which was carried out in the Nanofabrication Center at the University of Minnesota. The plasma treated PDMS plate was stored in Milli-Q deionized water prior to collagen coating. The final device had 100 wells with 650 μm in diameter and 200 μm in depth. The wells are spaced at intervals of 1.5 mm from each other.

For fiber analysis, the isolated muscle fibers were plated onto a collagen-coated PDMS plate and allowed to settle into its 60-nL volume wells at 37 °C for 30 min. The unattached fibers and those in the medium were removed by aspiration and scraping with a coverslip. Those attached fibers on the bottom of the wells were not affected by the use of the coverslip since they were not touched by the flat edge of the coverslip during the scrape. Subsequently, MEM was added and the plate (attached fibers in the wells) was maintained at 37°C in 5% CO_2 (within 48 hours of culture) (Figure 1, Part i). To measure superoxide production, the entire PDMS plate was incubated with 5 μM TPP-HE in MEM for 30 min at 37 °C (Figure 1, Part ii). These incubation conditions have been previously used for whole cells and isolated mitochondria.[32] For control experiments, before incubation in the presence of TPP-HE, the entire plate was treated with 1 mM tiron,[22,25] or 5 μM antimycin A at 37°C for 30 min.[32]

After washing the plate with PBS, wells containing one muscle fiber were localized with the aid of an inverted microscope (TE300, Nikon, Melville, NY) using bright field imaging. For delivering, a 150-μm-o.d., 50-μm-i.d. fused silica capillary (Polymicro Technologies, Phoenix, AZ) was fully filled with the lysis buffer (~ 800 nL). 50 nL of the lysis buffer containing 50% w/v glycerol was then delivered into each individual well hydrodynamically at 7.3 kPa for 120 s by this capillary (Figure 1, Part iii). Five freeze-thaw cycles (−80 and +37 °C, the plate held at each temperature for 20 min in each cycle) were applied to the entire plate to completely disrupt individual muscle fibers and release their contents (Figure 1, Part iv). The efficiency of fiber lysis was assessed by comparing the fluorescence intensity of the muscle fiber before and after its disruption and the removal of the lysate in the well. After lysis, the whole single-fiber lysate was suctioned into a capillary by applying − 10.8 kPa for 81 s, and then delivered into a microvial with 5 μL lysis buffer containing 2 mg/mL protease K and 400 U/mL DNase I to remove the DNA (Figure 1, Part v). DNA digestion is important because variable amounts of DNA in the samples may cause inconsistent fluorescence enhancement of OH-TPP-E^+ due to the DNA intercalation with OH-TPP-E^+.[32] The transfer of the single fiber lysate to a separate vial for DNA digestion and CE analysis is necessary because of the following reasons: (i) the volume of protease K and DNase I buffer needed to digest single-fiber DNA exceed the capacity of each individual nanowell; (ii) high concentration of glycerol (50% w/v) in the 50-nL single

fiber lysate can deteriorate the electrophoretic separations if injected directly for MEKC analysis (data not shown); and (iii) this approach allows to carry out several injections from the same fiber lysate, if needed. After the DNA digestion, the sample was then analyzed by MEKC-LIF to determine the amount of OH-TPP-E^+ (Figure 1, Part vi).

MEKC-LIF Analysis

The capillary electrophoresis instrument used for the MEKC-LIF analysis of single fiber contents has been previously described.[12,32] The 488-nm line (12 mW) of an Argon-ion laser (Melles Griot, Irvine, CA) was used for excitation and fluorescence was detected in the range of 607–662 nm range using a bandpass filter (Omega Optical, Brattleboro, VT). The separations were carried out using 150-μm-o.d., 50-μm-i.d. fused silica capillaries (Polymicro Technologies, Phoenix, AZ) at − 400 V/cm in MEKC running buffer. Samples were injected hydrodynamically for 1 s at 10.8 kPa, which introduced 3.7 nL sample into the capillary. The capillary was washed for 5 min with 0.1 M NaOH and 5 min with running buffer between each run. The limit of detection (signal/noise = 3) for ethidium standard was ~0.5 amol.

Data Analysis

Data were presented as mean ± standard deviation (SD). All the images were analyzed using Image J 1.42 software (NIH, Bethesda, MD). The MEKC electropherograms were analyzed using Igor Pro 5.0 software (Wavemetrics, Lake Oswego, OR) and the peak area of OH-TPP-E^+ was normalized to the volume of the analyzed muscle fiber, which was estimated according to the fiber's radius and length (Table S1). Statistical significance of the data was determined by Student's t test, with P values of < 0.05 considered significant.

RESULTS AND DISCUSSION

Function of Single Skeletal Muscle Fibers in Culture after Isolation

Muscle tissue is heterogeneous in nature, which complicates the analysis of the properties attributed to individual muscle fibers. In this report we describe an approach to analyze the contents of individual viable single fibers.

Single fibers were cultured in MEM immediately after the isolation,[21–22] thus making it possible to have more functional fibers available for different treatments and evaluation of superoxide levels. The function of the isolated muscle fibers was initially assessed by monitoring the externalization of phosphatidylserine up to 72 hours. The translocation of phosphatidylserine from the inner to the outside leaflet of the plasma membrane occurs during apoptosis.[39] By monitoring the levels of phosphatidylserine on the outside leaflet of the plasma membrane, we established that fibers were not apoptotic up to 48 hours after isolation (Supporting Information, Figure S-1).

The mitochondrial membrane potential plays a vital role in the production of ATP in soleus muscle, which is predominantly composed of type I fibers and has abundant mitochondria.[40] Moreover, the mitochondrial membrane potential drives the accumulation of the superoxide probe TPP-HE into the mitochondria within the muscle fiber,[30] thus membrane potential is a critical parameter to effectively monitor superoxide levels. To monitor the mitochondrial membrane potential of single muscle fibers during culture, we labeled the fibers with TMRM, a membrane potential-sensitive cationic dye.[35,41] TMRM is nontoxic to mitochondria at the low concentrations used here and has low non-specific binding to mitochondria compared to other dyes such as rhodamine 123. Figure 2A shows the bright-field image of a single soleus skeletal muscle fiber in culture for 24 hours with the characteristic striations.[21] After the permeabilization of the plasma membrane and TMRM staining, the muscle fiber displays bright fluorescence due to the accumulation of TMRM within the fiber's mitochondria (Figure 2B).

Upon treatment with the mitochondrial uncoupler, CCCP, the mitochondrial membrane potential is dissipated.[33] The remaining fluorescence is thus independent from the mitochondrial membrane potential (Figure 2C).[33,35] The relative TMRM fluorescence, before and after CCCP treatment (see Equation 1), is then a relative indicator of the mitochondrial membrane potential of individual muscle fibers. As shown in Figure 2D, the relative TMRM fluorescence was unchanged between single muscle fibers cultured for 24 and 48 hours, whereas there was a significant decrease in the fibers cultured for 72 hours. These results show that isolated soleus skeletal muscle fibers in culture are viable for up to 48 hours after isolation and have adequate mitochondrial membrane potentials to maintain their production of reactive oxygen species (i.e. superoxide production).

Handling of Isolated Single Skeletal Muscle Fibers in Individual Wells

Single muscle fibers were plated into 60-nL volume PDMS wells (Figure 3A and B) with the subsequent probe staining and lysis steps carried out in the same wells. Figure 3C shows the bright-field image of a single soleus muscle fiber cultured in the center of a nanoliter-volume well for 24 hours, which displays normal skeletal fiber morphology. The muscle fiber emitted bright fluorescence after it was loaded with TPP-HE (Figure 3D), which is expected to arise from intracellular TPP-HE oxidation products including both OH-TPP-E^+ and TPP-E^+. Then 50 nL lysis buffer was then added to each well that contained a muscle fiber and five freeze-thaw cycles were applied to disrupt the fiber and release the fiber contents (Figure 3E). Figure 3D shows the fluorescence intensity before lysis. Figure 4F shows the fluorescence after lysis and removal of the lysate. Based on these images, the lysis process and the transfer of the contents for MEKC-LIF analysis are fairly effective because, after the removal of the whole single-fiber lysate, the fluorescence intensity was minimal (~ 5% of original intensity).

Analysis of Superoxide Products in Isolated Single Skeletal Muscle Fibers

TPP-HE is oxidized by superoxide to form OH-TPP-E^+ and by intracellular species, such as cytochromes and oxidase,[30] to form TPP-E^+. The fluorescence emission spectra of these two products overlap strongly, which makes it extremely difficult to completely resolve the fluorescence signal of OH-TPP-E^+ from TPP-E^+ by standard fluorescence microscopy and flow cytometry.[30] The MEKC-LIF method used here has been effective at resolving OH-TPP-E^+ and TPP-E^+ found in mitochondria isolated from cells, liver and muscle tissues.[32] In this study, MEKC-LIF was also adequate to resolve OH-TPP-E^+ from TPP-E^+ formed in single muscle fibers under basal conditions and upon fiber treatments with antimycin A or tiron (Figure 4A). Under basal conditions, the OH-TPP-E^+ detected in single muscle fibers is attributed to the reaction of TPP-HE with superoxide generated from various intracellular sources, including mitochondria, NAD(P)H oxidase and phospholipase A2 enzymes.[42] When single fibers were treated with tiron, the OH-TPP-E^+ peak area (corrected for fiber size) decreased significantly compared to those fibers under basal conditions (Figure 4B). Tiron is a membrane-permeable superoxide dismutase (SOD) mimetic, which has been reported to scavenge the intracellular generated superoxide in cultured myotubes and mice skeletal muscle fibers.[22,25] In the presence of tiron, the small amount of OH-TPP-E^+ represents an impurity (due to prolonged storage, exposure to air and light, etc)[32] and is also found when the TPP-HE alone is analyzed by MEKC-LIF (Supporting Information, Figure S-2). The difference in the OH-TPP-E^+ peak area with and without tiron treatment indicates that the method is adequate for detection of intracellular superoxide production in single soleus skeletal muscle fibers.

The detection of superoxide production in single muscle fibers was further confirmed by treatment with antimycin A, a respiratory inhibitor.[43] Mitochondria have been reported to be one of the main sites of superoxide generation in skeletal muscle, where superoxide is released by complex I and III in the mitochondrial electron transport chain (ETC).[43] As an inhibitor of complex III, antimycin A blocks the electron transfer through the ETC and stimulates

superoxide production.[43–44] In the muscle fibers treated with antimycin A, the peak area of OH-TPP-E^+ increased by ca. 50% compared to the basal levels.

It is important to bear in mind that we are reporting qualitative superoxide levels in single fibers. Assessing superoxide levels in a biological system is complicated due to its natural dismutation, its enzymatic transformation into other species, and its chemical reactions with other molecules.[43,45–46] These factors are usually unknown and impede quantitation with calibration curves prepared with superoxide generating systems such as the xanthine/xanthine oxidase (X/XOD) system, in which the concentration of steady state superoxide can be estimated from the concentration of the added XOD.[28–29] Furthermore, attempts to match the conditions of the X/XOD system with those found within single fibers to obtain a reliable quantitation scheme require knowledge of other factors such as pH, ionic strength and trace metal concentration that affect superoxide generation in the X/XOD system.[28–29] Another factor complicating superoxide quantitation in single fibers arises from the TPP-HE accumulation in both the extra-mitochondrial and mitochondrial matrix of single fibers.[30,32] Each of these two environments presents different conditions for superoxide production.[44] Particularly, since TPP-HE accumulation in mitochondrial matrix is dependent on the mitochondrial membrane potential, a variation in membrane potentials between the fibers could cause different accumulation of TPP-HE in each fiber and thus affect the production of OH-TPP-E^+.[32,46–47] Despite the caveats associated with the lack of the control or predictability of the intracellular environment, the clear trends in superoxide levels observed upon treatment of single fibers with tiron and Antimycin A (c.f. Figure 4B) point to the utility of the superoxide single fiber analysis reported here. Indeed future refinement of the current methodology may include the use of mitochondrial membrane potential probes (e.g. TMRE) to assess and correct for the mitochondrial membrane potential status of the fiber under investigation.

While superoxide itself is not usually directly associated with biomolecular damage and oxidative stress, assessing superoxide levels in single fibers is clearly a surrogate for oxidative stress. Superoxide is commonly the first ROS produced in biological systems and can be transformed into other species, such as hydrogen peroxide and highly reactive hydroxyl radical.[43] An elevated superoxide level in muscle fibers under drug treatments or pathological conditions could lead to a higher production of these secondary species and cause severe damage to the proteins, nucleic acids and phospholipids, thereby leading to increased oxidative stress.[43,45] Thus, the methodology presented here has potential to be an early indicator of oxidative stress in individual muscle fibers.

CONCLUDING REMARKS

We reported a new approach to monitor superoxide production in single skeletal muscle fibers cultured in separate nanowells. These skeletal muscle fibers cannot be analyzed by conventional chemical cytometry techniques because their cylinder-like shape and large diameter (~ 100 μm), are not compatible with the required direct insertion into capillaries commonly used in chemical cytometry. After staining with superoxide probe TPP-HE, individual fibers were lysed in their corresponding wells and their lysates were effectively removed for MEKC-LIF analysis of the superoxide-specific product OH-TPP-E^+. The suitability of the method to monitor changes in superoxide production was confirmed by inhibiting and enhancing superoxide production with tiron and antimycin A, respectively.

Future developments may include the use of specific inhibitors and stimulants to monitor specifically superoxide generation by mitochondria, NAD(P)H oxidase and phospholipase A2 enzymes,[42] as well as other reactive oxygen species specific probes, further enhancing our understanding of the generation of reactive oxygen system in complex muscle tissues. Methods based on amperometry could also be coupled to this method and used to monitor the

extracellular superoxide released from cultured single fibers in future studies. In this study we focused only on the soleus muscle, however, the current methodology could be easily extended to investigate changes in superoxide production in other skeletal muscle fiber types.[48] Of particular interest would be to monitor changes in superoxide production associated with age and muscle disuse-induced dysfunction, and muscle degenerative diseases.[49–51]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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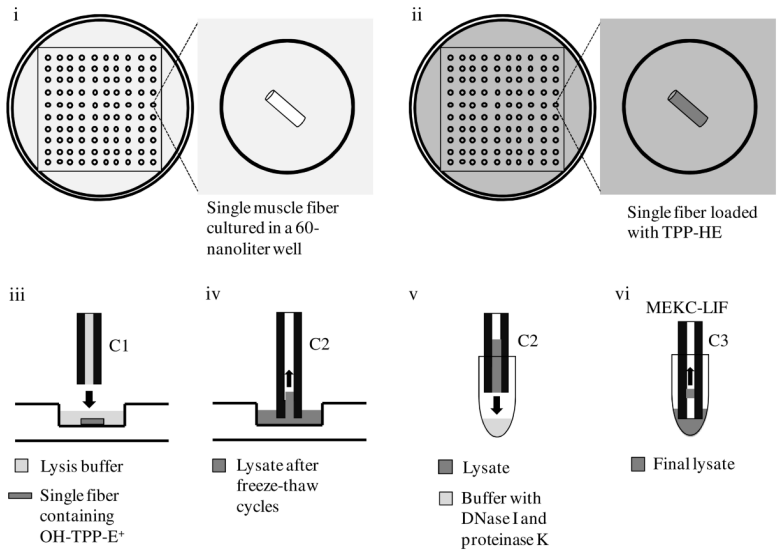

Figure 1.

Handling of single skeletal muscle fibers. (i) Single muscle fibers are delivered onto a PDMS array of 100 wells held in a 35-mm petri dish; the fibers attach to the bottom of the wells; the attached fibers are cultured in MEM at 37°C in 5% CO_2. (ii) The PDMS array is incubated with TPP-HE, which leads to formation of the superoxide reporter, OH-TPP-E^+. (iii) The lysis buffer is delivered hydrodynamically by a capillary (C1) into individual wells that contain one muscle fiber. (iv) Five freeze-thaw cycles (−80 and +37 °C, respectively) are applied to lyse the single fibers in the wells; the whole single-fiber lysate is then aspirated into the capillary (C2). (v) The lysate within the capillary (C2) is delivered into a microvial containing protease K and DNase I. (vi) A sample of the final lysate is injected into a capillary (C3) and subsequently analyzed by MEKC-LIF.

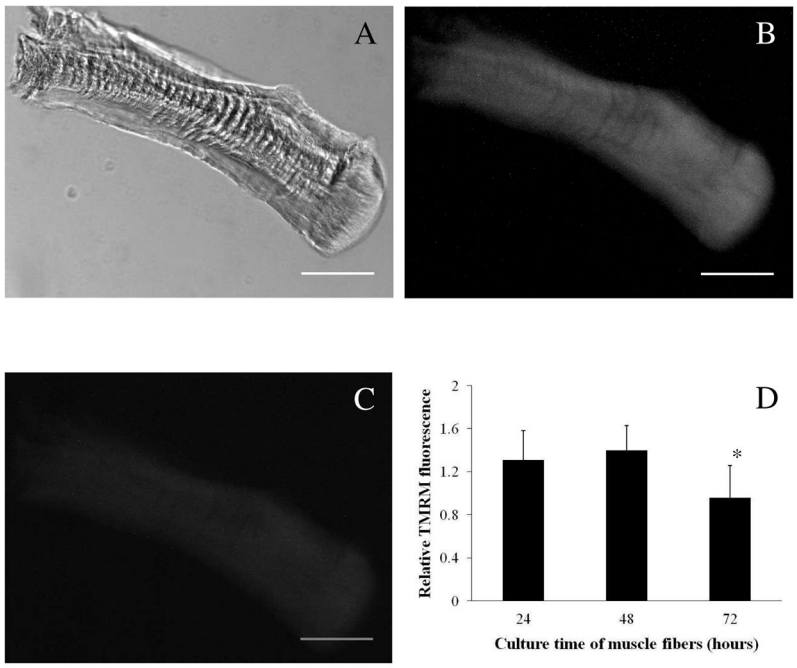

Figure 2.

Mitochondrial membrane potential in isolated single soleus muscle fibers. (A) Bright-field image of a single muscle fiber, 24 hours in culture. (B, C) TMRM fluorescence images of the same muscle fiber before and after CCCP treatment, respectively. Scale bar = 100 μm. (D) Relative TMRM fluorescence intensities at various culture times. 500 ms exposure time is used. Data are presented as means ± SD, n = 8–9 fibers. * $p < 0.05$ vs 24 hours.

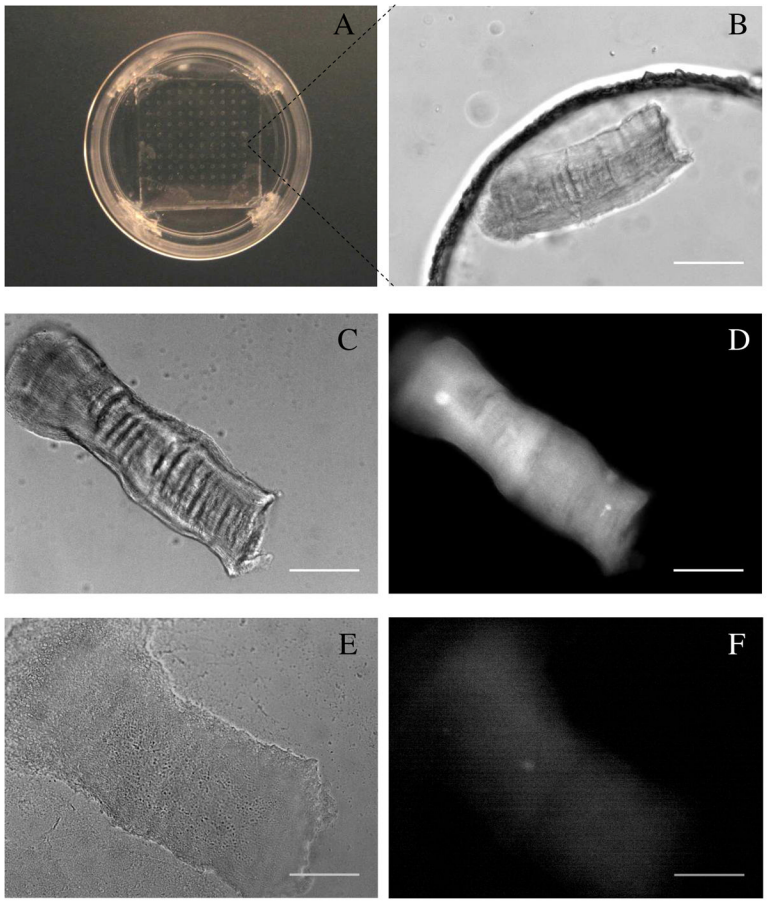

Figure 3.
Lysis of single soleus skeletal muscle fibers cultured in a nanoliter-volume well. (A) Photograph of a PDMS plate with the 100-well array in a 35-mm petri dish. (B) Bright-field image of a skeletal muscle fiber in one of the wells of the PDMS plate. (C) Bright-field images of a muscle fiber in the center of a well. (D) Fluorescence image of TPP-HE oxidation products in the muscle fiber shown in (C) before treatment. (E) The same muscle fiber in (C) after the lysis and removal of lysate for MEKC-LIF analysis. (F) Fluorescence image corresponding to the conditions described in (E). Scale bar = 100 μm.

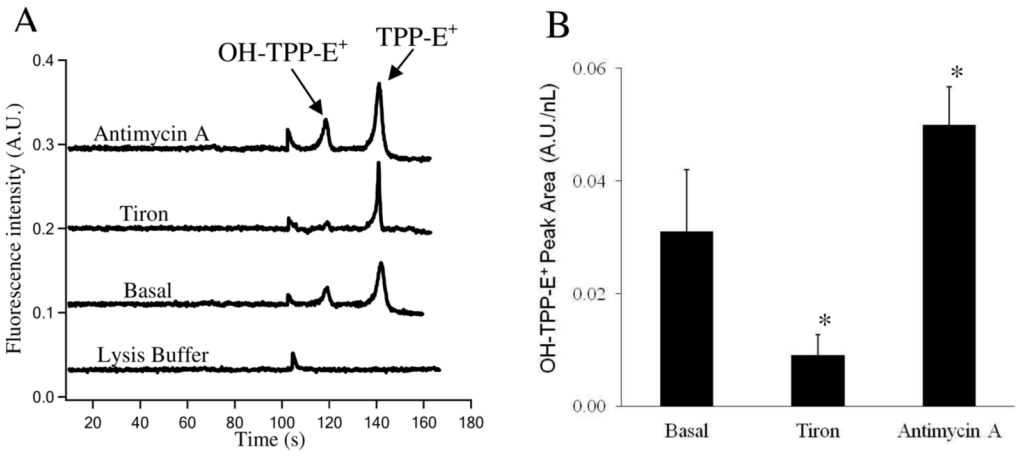

Figure 4.

TPP-HE oxidation products in isolated single soleus muscle fibers. (A) Electropherograms of the lysis buffer (blank) and the oxidation products, OH-TPP-E^+ and TPP-E^+, in the lysates of individual muscle fibers under basal conditions and upon treatments with tiron and antimycin A. Separations were performed in a 40-cm-long capillary at − 400 V/cm in MEKC running buffer. The 488-nm line of an argon-ion laser was used for excitation, and a 607–663 nm bandpass filter was used for detection. Traces have been offset vertically for clarity. (B) OH-TPP-E^+ peak area for single muscle fibers; areas are corrected for the respective fiber volume. Data are presented as means ± SD, n = 3–5 fibers. * $p < 0.05$ vs basal.