Published in final edited form as:

Free Radic Biol Med. 2009 April 1; 46(7): 905–913. doi:10.1016/j.freeradbiomed.2008.12.019.

Qualitative Determination of Superoxide Release at Both Sides of the Mitochondrial Inner Membrane by Capillary Electrophoretic Analysis of the Oxidation Products of Triphenylphosphonium Hydroethidine*

Xin Xu and Edgar A. Arriaga^a

Department of Chemistry, University of Minnesota, 207 Pleasant St. SE, Minneapolis, MN 55455

Abstract

Superoxide is released asymmetrically to both sides of the mitochondrial inner membrane. Since this membrane is impermeable to superoxide, two separate pools are formed at either side of the membrane, each with its own characteristics and potential biological effects. Here, we report an attomole-sensitive fast capillary electrophoretic method that can analyze superoxide in a single pool, either the matrix pool or that outside the mitochondria. The method uses triphenylphosphonium hydroethidine (TPP-HE) that reacts with the superoxide in both pools. Centrifugation is used to separate the mitochondria (i.e. matrix contents) from the supernatant (i.e. products released outside the mitochondria). Each fraction is then analyzed by a capillary electrophoresis with laser-induced fluorescence detection (CE-LIF) method that separates and detects hydroxytriphenylphosphonium ethidium (OH-TPP-E⁺), the fluorescent superoxide specific product. The separation takes < 3 min and the detection level is down to 3 attomole OH-TPP-E⁺. The method has proved to be effective detecting qualitatively superoxide release in the mitochondria of 143B cells, mouse liver, and rat skeletal muscle, both in the presence and absence of inhibitors. In addition, this study confirmed that Complex I releases superoxide only toward the matrix while Complex III releases superoxide toward both sides of the mitochondrial inner membrane. Furthermore, treatment with menadione induces superoxide release toward both sides of the mitochondrial inner membrane.

Keywords

Superoxide; mitochondria; triphenylphosphonium; liver; muscle; rotenone, antimycin A; menadione; capillary electrophoresis, laser-induced fluorescence; micellar electrokinetic capillary chromatography

Introduction

Superoxide is the first reactive oxygen species (ROS) generated in mitochondria [1] that upon transformation into other ROS may cause severe oxidative damage to mitochondrial DNA

*This work was supported by a grant from the National Institutes of Health (R01-AG-20866).

^aCorresponding author: Edgar A. Arriaga, Department of Chemistry, University of Minnesota, 207 Pleasant St. SE, Minneapolis, MN 55455, Tel: 612-624-8024; Fax: 612-626-7541; E-Mail: arriaga@umn.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

(mtDNA), lipids, and proteins [2]. The resulting oxidative stress has been associated with aging and age-related diseases, such as Alzheimer's and Parkinson's disease [3].

Superoxide produced by complexes I and III in the mitochondrial electron transport chain (ETC) is released asymmetrically to both sides of the mitochondrial inner membrane, which is superoxide impermeable, thereby forming two separate superoxide pools that have different fates and cause different cellular effects [4,5]. The first pool is made of superoxide released into the mitochondrial matrix where it is dismutated by manganese superoxide dismutase (Mn-SOD) to form hydrogen peroxide. The superoxide released outside the mitochondria creating the second pool is made of superoxide first released to the intermembrane space that then crosses the permeable outer mitochondrial membrane into the cytosol, where it may be dismutated by cytosolic copper/zinc superoxide dismutase (Cu/Zn-SOD) to form cytosolic hydrogen peroxide [6] (Figure 1). It can be seen that the subsequent transformation of superoxide into hydrogen peroxide and other ROS may play very different roles in both subcellular environments.

The existing methods of detecting superoxide include the use of electron spin traps and assays based on reactions with aconitase, lucigenin, cytochrome c, or hydroethidine (HE) [5,7-12]. The cytochrome c and spin trap methods cannot detect superoxide in the matrix because neither of these molecules can cross the mitochondrial inner membrane and reach the matrix [11,13]. Methods based on aconitase and lucigenin are also limited in their ability to monitor superoxide in the mitochondrial matrix, either because of compromised specificity due to their inactivation by other ROS (i.e. aconitase method), or because of reagent-induced generation of superoxide in vivo (i.e. lucigenin method) [13,14]. HE is a membrane-permeable probe suitable for monitoring superoxide released outside the mitochondria where HE reacting with superoxide produces 2-hydroxy-ethidium [11,12,15]. Unfortunately, the reaction of superoxide with HE is 2-3 orders of magnitude slower compared to that with Mn-SOD (HE, $2 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$ vs. Mn-SOD, $1 \times 10^9 \text{ M}^{-1}\text{s}^{-1}$ [15]), resulting in no accumulation of the superoxide-specific hydroxyethidium in the matrix [16].

Recently, a novel HE derived probe, triphenylphosphonium hydroethidine (TPP-HE, a.k.a. MitoSOX red), was used to detect mitochondrial superoxide production by fluorescent microscopy and flow cytometry [15,17-19]. Due to the positively charged triphenylphosphonium group, the negative mitochondrial membrane potential (MP) of the mitochondria drives the accumulation of TPP-HE in the mitochondrial matrix [20]. Furthermore, TPP-HE has a faster reaction rate with superoxide than does HE [15] so that the structural and kinetic characteristics of TPP-HE favor its reaction with superoxide in the mitochondrial matrix, even in the presence of competing Mn-SOD.

TPP-HE forms hydroxyl-TPP-ethidium (OH-TPP-E⁺) upon reaction with superoxide or TPP-ethidium (TPP-E⁺) upon oxidation by other oxidative species, such as oxidases or cytochromes [15]. These products exhibit very similar fluorescence spectra with the maximal emission wavelength around 580 nm [11,15]. It has been suggested that such products can be specifically detected using different fluorescence excitation wavelengths [15].

An alternative to using different excitation wavelengths is to use capillary electrophoresis (CE) or high performance liquid chromatography (HPLC) to separate OH-TPP-E⁺ from TPP-E⁺, as done previously with the superoxide assay based on HE that produces both OH-E⁺ and E⁺ [11,16]. The HPLC method has femtomole (10^{-15} mole) limits of detection and separations are completed in 30 minutes [11]. In comparison, micellar electrokinetic capillary chromatography (MEKC), a mode of CE that uses a pseudo-stationary separation phase, when combined with laser-induced fluorescence detection (LIF) separates OH-E⁺ and E⁺ with high resolution, attomole (10^{-18} mole) limits of detection, and short separation times (~ 3 min) [16]. The use

of MEKC with LIF detection (MEKC-LIF) thus clearly is advantageous in instances where sensitivity and short separation times are indispensable.

In this report, we implemented an MEKC-LIF method to determine the abundances of OH-TPP-E⁺ and TPP-E⁺ that, similarly to the HE method, has high separation resolution, low limits of detection (i.e. attomole), and short separation times (i.e. < 3 min). Most importantly, this method is suitable for detecting superoxide being produced inside and outside mitochondria isolated from 143B cells, mouse liver tissue and rat skeletal muscle. By treating these mitochondria with rotenone, antimycin A and menadione, we determined that in all the mitochondria types investigated, Complex I only releases superoxide into the matrix of the mitochondria, Complex III releases superoxide to both sides of the inner mitochondrial membrane, and menadione induces the release of superoxide to both sides of the mitochondrial inner membrane.

Experimental procedures

Reagents

Mito-SOX red (TPP-HE) was purchased from Invitrogen-Molecular Probes (Eugene, OR). Sodium borate was purchased from EM Science (Gibbstown, NJ). Sucrose was purchased from Mallinckrodt (Paris, KY). Ethidium bromide was purchased from Bio-Rad (Hercules, CA). All the other reagents were obtained from Sigma-Aldrich (St. Louis, MO).

Buffers

The MEKC running buffer contained 10 mM borate and 1 mM cetyltrimethylammonium bromide (CTAB) (pH=9.4). The buffer for producing superoxide with the xanthine and xanthine oxidase (X/XO) reaction contained 50 mM potassium phosphate and 0.1 mM EDTA (pH 7.4). The buffer for mitochondrial isolation from 143B cells and liver tissue contained 5 mM HEPES, 210 mM mannitol, 70 mM sucrose and 5 mM EDTA (pH 7.4). The buffer for mitochondrial isolation from muscle tissue contained 50 mM Tris-HCl, 100 mM sucrose, 100 mM KCl, 1 mM KH₂PO₄, 0.1 mM EGTA and 0.2% (w/v) BSA (pH 7.4). The respiration buffer was made of 10 mM HEPES, 125 mM KCl, 5 mM MgCl₂ and 2 mM K₂HPO₄ (pH 7.4). The mitochondrial lysis buffer contained 40 mM Tris HCl, and 6 mM MgCl₂, and 0.5% w/v Triton X-100 (pH=9.4). All buffers were made with deionized water and filtered through a 0.22- μ m filter before use.

Cell culture

The human osteosarcoma 143B cell line was obtained from the American Tissue Culture Collection (Manassas, VA). The cells were cultured in minimum essential medium containing 10% (v/v) calf serum and 10 μ g/mL gentamicin at 37°C and 5% CO₂. The cells were maintained by splitting them every 3–4 days.

Mitochondrial preparation

Mitochondria were isolated from either 143B cells, BALB/c mouse liver, or Fischer 344 rat skeletal muscle following previously reported procedures [16,21,22]. Respiratory control ratio (RCR) and mitochondrial membrane potential (MP) were used to assess the quality of the isolated mitochondria. Oxygen consumption was measured using a FOXY-R Oxygen Sensor (Ocean Optics Inc. Dunedin, FL) and RCR was calculated as the ratio of the rates of oxygen consumption in State 3 to those in State 2. The RCR values for freshly isolated mitochondria from cells and tissues were ~ 3 and 5, respectively.

The membrane potential ($\Delta\psi_m$) of isolated mitochondria was measured using a TPP⁺-selective electrode (World Precision Instruments, Sarasota, FL). The effect of TPP⁺ binding to the mitochondrial membrane was corrected using the equation reported by Labajova, et al [23].

$$\Delta\psi_m = \frac{RT}{F} \ln \frac{V_0[TPP^+]_0/[TPP^+]_t - V_t - K_0P}{V_mP + K_iP} \quad (1)$$

Where $[TPP^+]_0$ and $[TPP^+]_t$ are the concentrations of TPP⁺ before and after mitochondria addition, V_0 and V_t are the volumes of the medium before and after mitochondria addition, V_m is the volume of the mitochondrial matrix (1 μ L/mg protein [23]), P is the mitochondrial protein content (mg), K_0 is the apparent external partition coefficient TPP⁺ (14.3 μ L/mg), K_i is the apparent internal partition coefficient of TPP⁺ (7.9 μ L/mg), and R , T and F have their conventional meanings [23].

The membrane potential of mitochondria isolated from the 143B cell line was ~120 mV, and mitochondria from skeletal muscle and liver showed higher membrane potentials (i.e. ~140 mV), similar to previously reported values [24]. These data indicated that the freshly isolated mitochondria used in the experiments described below were actively respiring.

Sample preparations of superoxide oxidation products for CE analysis

Isolated mitochondria were incubated with 5 μ M TPP-HE and substrates (10 mM succinate or 5 mM pyruvate/malate) in 100 μ L of respiration buffer at 37°C for 30 min. When needed, prior to incubation in the presence of TPP-HE, samples were treated with rotenone, antimycin A or menadione at concentrations varying from 1 to 10 μ M. For rotenone and menadione treatments, a mixed substrate composed of 5mM pyruvate and 5mM malate was used; for antimycin A treatment, the substrate was 10 mM succinate, and 10 μ M rotenone was added to stop reverse electron transfer to complex I. After incubation, mitochondria were centrifuged down at 12,000g for 10 min. The supernatant was used to analyze the TPP-E oxidation products released outside the mitochondrial inner membrane by MEKC-LIF. The mitochondrial pellet was resuspended in respiration buffer and washed twice. The pellet was then dissolved, aided by sonication for 3 minutes, in 100 μ L mitochondrial lysis buffer.

The detection of superoxide within the mitochondrial matrix using the TPP-HE probe may be complicated by the uncontrolled enhancement of the OH-TPP-E⁺ and TPP-E⁺ fluorescent responses upon intercalation with mtDNA [15,17,25]. As in a previous study in which HE was used as a probe [16], we removed mtDNA in the mitochondrial samples through digestion with DNase 1, thereby successfully eliminating the uncontrolled fluorescence enhancement of OH-TPP-E⁺ and TPP-E⁺. In order to digest mtDNA, the dissolved mitochondrial sample was treated with 2 mg/mL proteinase K for 45 min, and subsequently with 400 U/mL DNase 1 for 15 min at 37 °C.

MEKC-LIF analysis

The CE instrument used in these studies has been described previously [16,26]. Briefly, the 488-nm line of an Argon-ion laser (Melles Griot, Irvine, CA) was used for excitation. The fluorescence of interest was selected with a bandpass filter (Omega Optical, Brattleboro, VT) transmitting in the 607 - 662 nm range, and was measured with a photomultiplier tube (R1477, Hamamatsu, Bridgewater, BJ) biased at 1000V. The output from the photomultiplier tube was collected at 10 Hz using a NiDaq I/O board (National Instruments, Austin, TX) and saved as binary files. The detector response was maximized by aligning the optics while 10⁻⁷ M ethidium solution was being continuously delivered through the capillary at - 400 V/cm. The limit of detection (signal/noise = 3) for OH-TPP-E⁺ was ~ 3 amol.

Mitochondrial lysates were analyzed by MEKC-LIF, with each sample analyzed in triplicate. The separations were carried out using 50- μm i.d., 150- μm o.d. fused silica capillaries (Polymicro Technologies Inc., Phoenix, AZ) at - 400 V/cm in MEKC running buffer. Samples were injected using a 1-second hydrodynamic injection at 10.7 kPa, which introduced 3.8 nL of the sample into the capillary. A 5-min wash with running buffer was performed between runs.

Data analysis

The data from the CE peaks were analyzed using Igor Pro software (Wavemetrics, Lake Oswego, OR). The electrophoretic mobilities of each peak were calculated by dividing the capillary length by the migration time of the respective peak and the electric field used for the separation. In the “untreated” control, small peaks of OH-TPP-E⁺ and TPP-E⁺ are consistently present as minor oxidized impurities in TPP-HE (due to prolonged storage, exposure to air, etc.), and their amounts may vary in different preparations of TPP-HE [11,12,15]. Therefore, all the reported peak areas were corrected by subtracting those of the blanks (i.e. the reagent alone) and then normalized to 1 mg·mL⁻¹ protein concentrations. Ratios of OH-TPP-E⁺ to TPP-E⁺ were also calculated using their respective corrected peak areas, where “0 μM ” referred to conditions for isolated mitochondria that had been only energized by substrates without the addition of rotenone, antimycin A or menadione. The resulting values are presented as means $\pm$ standard deviation (SD). The student’s t test was performed to test the statistical significance of the data, with P values of < 0.05 considered significant.

Results

Separation and detection of TPP-HE oxidation products of the X/XO reaction by MEKC-LIF

The X/XO reaction has been widely used to generate superoxide in *in vitro* superoxide studies [27,28]. Here, we used this reaction to generate superoxide so that it would then react with TPP-HE to form its oxidation products. The reaction mixture was analyzed by MEKC-LIF. Figure 2A shows electropherograms in which the peaks of the TPP-HE oxidation products, OH-TPP-E⁺ and TPP-E⁺, are separated with a resolution of 5.9 in ~ 3 min. The respective net electrophoretic mobilities were $(1.20 \pm 0.02) \times 10^{-4}$ and $(2.50 \pm 0.03) \times 10^{-4} \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$ (n=3 runs).

The OH-TPP-E⁺ peak area increased with reaction time, as was to be expected from the reaction of superoxide with TPP-HE with its continuous accumulation of OH-TPP-E⁺ as the X/XO reaction proceeds. In addition, when SOD was added to the X/XO reaction mixture, OH-TPP-E⁺ did not accumulate, confirming that this oxidation product is associated with superoxide. However, the TPP-E⁺ peak area remained almost unchanged at the various X/XO reaction times used here; the relative standard deviation (RSD) of peak area was ~ 6.0 % (n=3), which is typical in the analysis of pure compounds by MEKC-LIF. Lastly, the ratio of the peak areas of OH-TPP-E⁺ to TPP-E⁺ as a function of the X/XO reaction time (Figure 2B) confirms that OH-TPP-E⁺ is the specific product of TPP-HE oxidation by superoxide.

Separate pools of TPP-HE oxidation products of isolated mitochondria

Superoxide is released asymmetrically toward both sides of the mitochondrial inner membrane and the two resulting pools remain separated because the mitochondrial inner membrane is impermeable to superoxide. TPP-HE, on the other hand, permeates the inner membrane and can be found at either side of this membrane where it reacts with superoxide to form OH-TPP-E⁺. This oxidation product is practically retained at the side of the membrane where it is produced because its permeation through the inner membrane is extremely slow. For example, after 30 minutes of incubation of mitochondria OH-TPP-E⁺, there is no detectable accumulation of the oxidation product in the mitochondrial matrix (Supplementary Material,

Figure 1). This is consistent with the high activation energy to cross the membrane [20, 29, 30] resulting from the two positive charges on the OH-TPP-E⁺ molecule.

Since the exchange of TPP-HE oxidation products between the two sides of the mitochondrial membrane is negligible, differential centrifugation is suitable for isolating a mitochondrial pellet containing the matrix oxidation products from the supernatant containing the oxidation products found outside the mitochondria. After centrifugation, the pellet and the supernatant are solubilized separately and analyzed by MEKC-LIF. Thus, we are able to analyze the TPP-HE oxidation products found both inside and outside mitochondria of the same preparation.

Effects of rotenone, antimycin A, and menadione on the generation of superoxide in 143B cells

Complex I releases superoxide when mitochondria respire with an NADH-linked substrate (such as a mixture of pyruvate and malate) [4]. Treatment with rotenone, an inhibitor of complex I, increases superoxide production and release into the matrix [31]. As expected, the OH-TPP-E⁺ in the matrix increases as the concentration of rotenone increases (Figure 3). As shown in the control (Figure 3A, Mito + TPP-HE, without substrate), the two small peaks are impurities of OH-TPP-E⁺ and TPP-E⁺ found in TPP-HE. These peaks are used as corrections for the samples with substrates and rotenone. As shown in Figure 3C, the OH-TPP-E⁺ peak area increases 12-fold (10 μ M rotenone) compared to the control (0 μ M rotenone), while there is no obvious change for the non-superoxide specific product, TPP-E⁺. The fact that both the OH-TPP-E⁺ and TPP-E⁺ peak areas in the supernatant electropherograms (Figure 3B) are close to zero and remain almost unchanged as the rotenone concentration increases, confirms that rotenone induces a dose-dependent increase of superoxide accumulation only in the matrix, and has no apparent effect on non-superoxide specific oxidations.

Complex III is another important site of superoxide production in mitochondria when the substrate is succinate and this production is being enhanced by the presence of antimycin A [5]. Figure 4A shows changes in the peak areas of OH-TPP-E⁺ (black markers) and TPP-E⁺ (white markers) in both the matrix (solid line) and outside the mitochondria (dashed line) following antimycin A treatments. With increased concentrations of antimycin A (i.e. 1 to 10 μ M), both OH-TPP-E⁺ and TPP-E⁺ increase inside and outside the mitochondria. These results clearly demonstrate that antimycin A induces superoxide release to both sides of the mitochondrial inner membrane, and that it also causes nonspecific TPP-HE oxidation.

We also used another common superoxide generator, menadione, to stimulate mitochondrial superoxide production. Menadione is considered to be an anti-tumor prodrug that induces superoxide formation, leading to cellular apoptosis [32-34]. As expected, the OH-TPP-E⁺ peak area showed a menadione dose-dependent increase, both inside and outside mitochondria (Figure 4B). When menadione increased from 1 to 10 μ M, the respective increase in the OH-TPP-E⁺ matrix was 4 to 20-fold higher than in the control (i.e. 0 μ M menadione). Similarly, for the same menadione dosages, the OH-TPP-E⁺ outside the mitochondria was 2 to 4-fold higher in than the control. For TPP-E⁺ inside and outside the mitochondria, however, the peak area remained almost unchanged as the menadione concentration increased. These results demonstrate that menadione induced superoxide release to both sides of the inner membrane, but did not cause nonspecific oxidation of TPP-HE.

Because the mitochondrial membrane potential causes TPP-HE concentrations in the matrix to be several hundred fold higher than outside the mitochondria, [20], comparing the superoxide released to both sides of the inner mitochondrial membrane is complicated. On the other hand, TPP-HE concentrations in the medium decrease only ~1-2 % due to its preferential accumulation in the matrix (Supplementary Material). Thus, the somewhat constant TPP-HE

concentration outside the mitochondria makes it possible to use our method to detect superoxide in this subcellular region, albeit at a slower reaction rate.

The selectivity of OH-TPP-E⁺ over TPP-E⁺ formation has been previously investigated by measuring the [OH-TPP-E⁺]/[TPP-E⁺] ratio [12,15]. Here, we used this ratio to investigate the effects of rotenone, antimycin A, and menadione treatments on the selectivity of OH-TPP-E⁺ formation. Rotenone induced the formation of up to 40 times more OH-TPP-E⁺ than TPP-E⁺ in the matrix, suggesting that this Complex I inhibitor selectively induces superoxide production without significantly causing other superoxide-independent oxidations (Figure 5A, black squares). On the other hand, outside the mitochondria, the [OH-TPP-E⁺]/[TPP-E⁺] ratio was small and remained constant as the rotenone concentration increased (Figure 5B). In contrast to the case for rotenone, the [OH-TPP-E⁺]/[TPP-E⁺] ratios inside and outside the mitochondria remained constant as the antimycin A concentration changed in the treatment (open circles in Figures 5A and 5B, respectively). These results suggest that antimycin A oxidizes TPP-HE by more than one mechanism, which warns us that caution must be used when interpreting superoxide dependent effects induced by antimycin A. Lastly, menadione was the only compound that showed a selective increase in superoxide-dependent oxidations of TPP-HE both inside and outside the mitochondria (triangles in Figures 5A and 5B, respectively).

Superoxide release in the mitochondria of liver and skeletal muscle

In addition to the measurements done on the mitochondria of 143B cells, we investigated superoxide release in liver and skeletal muscle mitochondria. Figure 6 compares the accumulations of OH-TPP-E⁺ and TPP-E⁺ in 143B cells, liver and muscle mitochondria treated with rotenone, antimycin A and menadione. Clearly, the effects of these treatments in regard to the selectivity of OH-TPP-E⁺ versus TPP-E⁺ (Figure 6, up versus down bars, respectively) and superoxide release (i.e. inside and outside; Figures 6 A versus 6B, respectively) follow the same trends for the three mitochondria types. These trends have already been described above for the mitochondria taken from 143B cells (c.f. Figures 3 - 4). It is worth noting that the detected amounts of both OH-TPP-E⁺ and TPP-E⁺, inside and outside the mitochondria, consistently showed the trend muscle > liver > 143B cells (Figure 6 A and B). The differences between mitochondrial superoxide generation in the cell line, liver and muscle shown here reflect differences in the efficiency of mitochondrial respiration, or in the quality of these preparations.

The selectivity ratio of OH-TPP-E⁺ to TPP-E⁺ formation inside or outside the mitochondria of muscle and liver tissues was also determined (Figure 7, down and up bars, respectively). The higher levels of inhibitor-induced superoxide production for muscle and liver mitochondria are in agreement with the notion that these tissues have highly respiring mitochondria. In conclusion, a comparison of the ratios of the three mitochondrial sources investigated here suggests that there is a general trend in the superoxide-specific and non-specific oxidation of the TPP-HE probe regardless of the source.

Discussion

In this study, a method based on the superoxide probe TPP-HE and the separation of its oxidation products by MEKC-LIF was developed to monitor mitochondrial superoxide release. The general applicability of the method was confirmed by investigating the effects of different compounds inducing superoxide production in mitochondria isolated from 143B cells, liver and muscle. The method was successful in evaluating superoxide released into the mitochondrial matrix and the intermembrane space, as well as in evaluating superoxide-specific and non-specific oxidation of the probe.

TPP-HE is a new probe similar to HE that reacts with superoxide to yield the specific hydroxylated product OH-TPP-E⁺ [11,15]. We have previously used HE as a probe to monitor the superoxide produced by mitochondria, and have successfully separated HE oxidation products using MEKC in less than 3 min [16], 10-times faster than the reported HPLC approach (~30 min) [11]. Similarly, here we demonstrate that the MEKC separation of TPP-HE oxidation products is completed within 3 min. The MEKC method also results in dramatic improvements in the limits of detection (i.e. attomole, 10⁻¹⁸ mole OH-TPP-E⁺) and injection volumes (i.e. nanoliter) compared to those of the HPLC method (i.e. femtomole, 10⁻¹⁵ mole OH-TPP-E⁺ and microliter, respectively). Both the low LOD and small sample requirements of the MEKC method make it suitable for monitoring superoxide levels in small samples in shorter times.

In numerous reports, superoxide has been detected by measuring the 2-hydroxy-ethidium resulting from the reaction of HE with superoxide [10-12,16,27,28]. The oxidation of TPP-HE by superoxide also forms the specific superoxide product, OH-TPP-E⁺ via the same molecular mechanism. It can be said that the formation of 2-hydroxy-ethidium is superoxide specific since little or no 2-hydroxy-ethidium formation is detected [11,12] when similar concentrations of other ROS, such as hydrogen peroxide, hydroxyl radicals, or reactive nitric species such as peroxynitrite, react with HE. Similarly, the formation of OH-TPP-E⁺ is considered superoxide specific because hydrogen peroxide, hydroxyl radical, peroxynitrite, and hypochlorous acid generate only 0.2%, 4%, 0.9% and 0.2%, respectively, of the OH-TPP-E⁺ formed from the reaction of TPP with equimolar superoxide concentrations [15].

As reported previously, HE cannot serve as the basis for detecting superoxide in the matrix [16] because HE cannot compete with the extremely fast reaction of Mn-SOD with superoxide (HE, $2 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$ vs. Mn-SOD, $1 \times 10^9 \text{ M}^{-1}\text{s}^{-1}$ [15]). As a derivative from the HE probe, TPP-HE has a rate constant for the reaction between superoxide ($4 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$ [15]) that is twice that of HE. In addition to this feature, another major difference between TPP-HE and HE is that TPP-HE has a triphenylphosphonium group, which promotes permeation through the inner mitochondrial membrane and accumulates in the matrix according to the mitochondrial membrane potential [15,20]. Based on Nernstian behavior, for mitochondria with 120 mV of membrane potential, the TPP-HE concentration in the matrix is 100 times higher relative to that outside the mitochondria [20]. Since the measured membrane potentials of the isolated mitochondria used in this study are similar (i.e. 120 to 140 mV, data not shown), the TPP-HE concentration in the matrix would be ~ 100 times higher, resulting in a favorable reaction of this probe with superoxide. In contrast, other superoxide probes such as HE, with distributions not affected by mitochondrial membrane potential, are expected to be found at equal concentrations on both sides of the mitochondrial inner membrane. The membrane-potential enhanced accumulation of TPP-HE in the matrix and its faster reaction rates with superoxide make it possible to observe the oxidation product OH-TPP-E⁺.

The mitochondrial membrane potential plays an important role in superoxide detection using the approach described here. We found that when using high concentrations of inhibitors (e.g. > 10 μM), there was a decrease in OH-TPP-E⁺ accumulation (data not shown). This is consistent with previous reports that found that high concentrations of inhibitors cause a decrease in mitochondrial membrane potential [35-38], thereby affecting the concentration of TPP-HE accumulated in the matrix and the kinetics of the reaction of TPP-HE with superoxide. For instance, the membrane potential of isolated mitochondria from rat liver or hepatocyte cells decreased more than 10% when rotenone concentrations varied from 2.5 to 50 μM [35,36] or when antimycin A concentration were administered at ~ 4 μM [37,38]. According to the Nernst equation, even a 10% drop in membrane potential (e.g. from 120 mV to 108 mV) will cause considerable redistribution of the probe across the mitochondrial inner membrane (e.g. 37% decrease in probe concentration). Therefore, we anticipate that similar changes in membrane potential likely affect the production and accumulation of OH-TPP-E⁺ rendering the present

superoxide measurement qualitative even when the determination of OH-TPP-E⁺ is quantitative. In future studies, simultaneous monitoring of OH-TPP-E⁺ accumulation and mitochondrial membrane potential using membrane potential probes, such as tetramethylrhodamine methyl ester or rhodamine 123 [39], may prove to be an effective measure to develop a quantitative method.

In cellular systems, superoxide outside the mitochondria can also be produced by enzymes such as NAD(P)H oxidase, xanthine oxidase, or cytochrome P450 [40-42], and can interfere with the in vivo determinations of superoxide released into the cytosol by mitochondria. Since isolated mitochondria were used in our study, these other sources of superoxide do not bias the measurements of superoxide released outside the mitochondrial inner membrane. One can foresee that superoxide production by other subcellular environments outside the mitochondria could be investigated using procedures similar to those described here. For instance, monitoring superoxide production by components of the cytosolic fraction could be carried out by isolating this fraction using ultracentrifugation, then incubating it with TPP-HE (or HE), and lastly, analyzing the resulting oxidation products by MEKC-LIF.

Mitochondrial ETC is the source of superoxide in mitochondria. ETC is composed of four enzyme complexes, which transfer electrons, thus creating a proton gradient across the inner membrane. Complexes I and III are the two main sites of superoxide generation in the ETC [2]. Several components within complex I have been proposed as the site of the superoxide generation, including flavine mononucleotide (FMN)-containing flavoprotein, iron-sulfur clusters and semiquinone-binding sites [43]. Because all of these possible superoxide generation sites in Complex I are located on the matrix side, superoxide production by Complex I occurs only in the mitochondrial matrix [2,43], which is consistent with the findings of this study (Figure 6). In contrast, when menadione forms superoxide at Complex I, OH-TPP-E⁺ is detected in both the matrix and outside mitochondria (Figure 6). Complex I may be accepting an electron from menadione at a specific site of Complex I, and forming a semiquinone that would then relocate near the inner or outer leaflet of the mitochondrial inner membrane. The semiquinone would then transfer its electron to molecular oxygen to produce superoxide [32, 44,45].

Superoxide production by Complex III has been reported to occur at both sides of the inner membrane and likely occurs at the “o” center of this complex [5]. Complex III inhibitor, antimycin A, stimulates superoxide production by binding to center “i” and blocking the electron transfer from center “o” [5,43]. Using this inhibitor, our study reports that antimycin A also induces Complex III to release superoxide into both the matrix and outside the inner membrane.

For a given mitochondria source and subcellular region (e.g. either the matrix, or the region outside the mitochondrial inner membrane), the method presented here is useful for qualitatively comparing the effects of rotenone, antimycin A and menadione treatments. For the matrix, treating the mitochondria from 143B cells with 1 to 10 μM rotenone induced OH-TPP-E⁺ accumulation that was from 2- to 12-fold greater compared to the control (0 μM), while the OH-TPP-E⁺ levels are close to zero outside the inner membrane at various rotenone concentrations. Treatments with 1 to 10 μM antimycin A caused 2- to 10-fold and 2- to 4-fold increases in OH-TPP-E⁺ accumulation into the matrix and outside inner membrane of 143B cell mitochondria, respectively. Treatments with 1 to 10 μM menadione increased OH-TPP-E⁺ accumulation 4- to 20-fold and 2- to 4-fold in the matrix and outside inner membrane of 143B cell mitochondria, respectively. Measurements of mitochondria from liver and muscle tissue showed results similar to those for mitochondria from 143B cells, indicating that the OH-TPP-E⁺ accumulation and superoxide production induced by rotenone, antimycinA and menadione remains consistent for different types of mitochondria.

As mentioned above, superoxide dismutases (e.g. Mn-SOD and Cu/Zn-SOD) potentially compete with TPP-HE for superoxide. Although TPP-HE is able to compete with the dismutase of Mn-SOD for superoxide, the relative fraction of superoxide reacting with Mn-SOD remains unknown [12,15]. This topic can be further investigated by using either inhibitors for Mn-SOD (e.g. diethyldithiocarbamate [46]), or by analyzing tissues and cell lines derived from Mn-SOD knockout mice (i.e. SOD deficient [47]). On the other hand, cytosolic Cu/Zn-SOD has been reported inactive in intact isolated mitochondria [16,48] and it is not expected to affect the measurements of superoxide released outside the inner membrane.

In summary, the superoxide detection method presented here uses MEKC-LIF to separate the oxidation products of TPP-HE and then detect superoxide specific OH-TPP-E⁺. This method can detect the release of superoxide inside and outside isolated mitochondria under basal conditions and upon treatment with respiration inhibitors (e.g. rotenone, menadione and antimycin A). Low limits of detection (i.e. attomole OH-TPP-E⁺), short analysis time (i.e. < 3 min), and small sample capability (nanoliter volumes) make this qualitative method suitable for monitoring superoxide levels in small samples such as single muscle fibers under different contractile regimes [49,50], or in tissue biopsies [51] associated with aging or oxidative stress [52,53].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work was supported by grant from the National Institutes of Health (R01-AG-20866) and Edgar A. Arriaga was supported by an NIH Career Award (1K02-AG21453). The authors thank Joe Sikora for the liver tissue and Dr. LaDora Thompson for the skeletal muscle tissue.

References

1. Beckman KB, Ames BN. The free radical theory of aging matures. *Physiol Rev* 1998;78:547–581. [PubMed: 9562038]
2. Orrenius S, Gogvadze V, Zhivotovsky B. Mitochondrial oxidative stress: implications for cell death. *Annu Rev Pharmacol Toxicol* 2007;47:143–183. [PubMed: 17029566]
3. Ashok BT, Ali R. The aging paradox: free radical theory of aging. *Exp Gerontol* 1999;34:293–303. [PubMed: 10433385]
4. St-Pierre J, Buckingham JA, Roebuck SJ, Brand MD. Topology of superoxide production from different sites in the mitochondrial electron transport chain. *J Biol Chem* 2002;277:44784–44790. [PubMed: 12237311]
5. Muller FL, Liu Y, Van Remmen H. Complex III releases superoxide to both sides of the inner mitochondrial membrane. *J Biol Chem* 2004;279:49064–49073. [PubMed: 15317809]
6. Han D, Antunes F, Canali R, Rettori D, Cadenas E. Voltage-dependent anion channels control the release of the superoxide anion from mitochondria to cytosol. *J Biol Chem* 2003;278:5557–5563. [PubMed: 12482755]
7. Barbacanne MA, Souchard JP, Darblade B, Iliou JP, Nepveu F, Pipy B, Bayard F, Arnal JF. Detection of superoxide anion released extracellularly by endothelial cells using cytochrome c reduction, ESR, fluorescence and lucigenin-enhanced chemiluminescence techniques. *Free Radic Biol Med* 2000;29:388–396. [PubMed: 11020659]
8. Du G, Mouithys-Mickalad A, Sluse FE. Generation of superoxide anion by mitochondria and impairment of their functions during anoxia and reoxygenation in vitro. *Free Radic Biol Med* 1998;25:1066–1074. [PubMed: 9870560]
9. Kruglov AG, Yurkov IS, Teplova VV, Evtodienko YV. Lucigenin-derived chemiluminescence in intact isolated mitochondria. *Biochemistry (Mosc)* 2002;67:1262–1270. [PubMed: 12495424]

10. Zhao H, Kalivendi S, Zhang H, Joseph J, Nithipatikom K, Vasquez-Vivar J, Kalyanaraman B. Superoxide reacts with hydroethidine but forms a fluorescent product that is distinctly different from ethidium: potential implications in intracellular fluorescence detection of superoxide. *Free Radic Biol Med* 2003;34:1359–1368. [PubMed: 12757846]
11. Zhao H, Joseph J, Fales HM, Sokoloski EA, Levine RL, Vasquez-Vivar J, Kalyanaraman B. Detection and characterization of the product of hydroethidine and intracellular superoxide by HPLC and limitations of fluorescence. *Proc Natl Acad Sci U S A* 2005;102:5727–5732. [PubMed: 15824309]
12. Fink B, Laude K, McCann L, Doughan A, Harrison DG, Dikalov S. Detection of intracellular superoxide formation in endothelial cells and intact tissues using dihydroethidium and an HPLC-based assay. *Am J Physiol Cell Physiol* 2004;287:C895–902. [PubMed: 15306539]
13. Tarpey MM, Wink DA, Grisham MB. Methods for detection of reactive metabolites of oxygen and nitrogen: in vitro and in vivo considerations. *Am J Physiol Regul Integr Comp Physiol* 2004;286:R431–444. [PubMed: 14761864]
14. Liochev SI, Fridovich I. Lucigenin (bis-N-methylacridinium) as a mediator of superoxide anion production. *Arch Biochem Biophys* 1997;337:115–120. [PubMed: 8990275]
15. Robinson KM, Janes MS, Pehar M, Monette JS, Ross MF, Hagen TM, Murphy MP, Beckman JS. Selective fluorescent imaging of superoxide in vivo using ethidium-based probes. *Proc Natl Acad Sci U S A* 2006;103:15038–15043. [PubMed: 17015830]
16. Meany DL, Thompson L, Arriaga EA. Simultaneously monitoring the superoxide in the mitochondrial matrix and extramitochondrial space by micellar electrokinetic chromatography with laser-induced fluorescence. *Anal Chem* 2007;79:4588–4594. [PubMed: 17492834]
17. Mukhopadhyay P, Rajesh M, Hasko G, Hawkins BJ, Madesh M, Pacher P. Simultaneous detection of apoptosis and mitochondrial superoxide production in live cells by flow cytometry and confocal microscopy. *Nat Protoc* 2007;2:2295–2301. [PubMed: 17853886]
18. Mukhopadhyay P, Rajesh M, Yoshihiro K, Hasko G, Pacher P. Simple quantitative detection of mitochondrial superoxide production in live cells. *Biochem Biophys Res Commun* 2007;358:203–208. [PubMed: 17475217]
19. Johnson-Cadwell LI, Jekabsons MB, Wang A, Polster BM, Nicholls DG. ‘Mild Uncoupling’ does not decrease mitochondrial superoxide levels in cultured cerebellar granule neurons but decreases spare respiratory capacity and increases toxicity to glutamate and oxidative stress. *J Neurochem* 2007;101:1619–1631. [PubMed: 17437552]
20. Ross MF, Kelso GF, Blaikie FH, James AM, Cocheme HM, Filipovska A, Da Ros T, Hurd TR, Smith RA, Murphy MP. Lipophilic triphenylphosphonium cations as tools in mitochondrial bioenergetics and free radical biology. *Biochemistry (Mosc)* 2005;70:222–230. [PubMed: 15807662]
21. Meany DL, Poe BG, Navratil M, Moraes CT, Arriaga EA. Superoxide released into the mitochondrial matrix. *Free Radic Biol Med* 2006;41:950–959. [PubMed: 16934678]
22. Whiting CE, Arriaga EA. CE-LIF analysis of mitochondria using uncoated and dynamically coated capillaries. *Electrophoresis* 2006;27:4523–4531. [PubMed: 17117462]
23. Labajova A, Vojtiskova A, Krivakova P, Kofranek J, Drahota Z, Houstek J. Evaluation of mitochondrial membrane potential using a computerized device with a tetraphenylphosphonium-selective electrode. *Analytical Biochemistry* 2006;353:37–42. [PubMed: 16643832]
24. Murphy MP, Smith RAJ. Drug delivery to mitochondria: the key to mitochondrial medicine. *Advanced Drug Delivery Reviews* 2000;41:235–250. [PubMed: 10699318]
25. Alonso A, Almendral MJ, Curto Y, Criado JJ, Rodriguez E, Manzano JL. Determination of the DNA-binding characteristics of ethidium bromide, proflavine, and cisplatin by flow injection analysis: usefulness in studies on antitumor drugs. *Anal Biochem* 2006;355:157–164. [PubMed: 16842734]
26. Duffy CF, Fuller KM, Malvey MW, O’Kennedy R, Arriaga EA. Determination of electrophoretic mobility distributions through the analysis of individual mitochondrial events by capillary electrophoresis with laser-induced fluorescence detection. *Anal Chem* 2002;74:171–176. [PubMed: 11795787]
27. Benov L, Szejnberg L, Fridovich I. Critical evaluation of the use of hydroethidine as a measure of superoxide anion radical. *Free Radic Biol Med* 1998;25:826–831. [PubMed: 9823548]

28. Zielonka J, Vasquez-Vivar J, Kalyanaraman B. The confounding effects of light, sonication, and Mn (III)TBAP on quantitation of superoxide using hydroethidine. *Free Radic Biol Med* 2006;41:1050–1057. [PubMed: 16962930]
29. Cocheme HM, Murphy MP. Complex I is the major site of mitochondrial superoxide production by paraquat. *Journal of Biological Chemistry* 2008;283:1786–1798. [PubMed: 18039652]
30. Singh MP, Wang FJ, Hoppel CL, Sayre LM. Inhibition of mitochondrial respiration by neutral, monocationic, and dicationic bis-pyridines related to the dopaminergic neurotoxin 1-methyl-4-phenylpyridinium cation (MPP+). *Arch Biochem Biophys* 1991;286:138–146. [PubMed: 1897943]
31. Barrientos A, Moraes CT. Titrating the effects of mitochondrial complex I impairment in the cell physiology. *J Biol Chem* 1999;274:16188–16197. [PubMed: 10347173]
32. Criddle DN, Gillies S, Baumgartner-Wilson HK, Jaffar M, Chinje EC, Passmore S, Chvanov M, Barrow S, Gerasimenko OV, Tepikin AV, Sutton R, Petersen OH. Menadione-induced reactive oxygen species generation via redox cycling promotes apoptosis of murine pancreatic acinar cells. *J Biol Chem* 2006;281:40485–40492. [PubMed: 17088248]
33. Dussmann H, Kogel D, Rehm M, Prehn JH. Mitochondrial membrane permeabilization and superoxide production during apoptosis. A single-cell analysis. *J Biol Chem* 2003;278:12645–12649. [PubMed: 12560329]
34. Gerasimenko JV, Gerasimenko OV, Palejwala A, Tepikin AV, Petersen OH, Watson AJ. Menadione-induced apoptosis: roles of cytosolic Ca(2+) elevations and the mitochondrial permeability transition pore. *J Cell Sci* 2002;115:485–497. [PubMed: 11861756]
35. Petit PX, O'Connor JE, Grunwald D, Brown SC. Analysis of the membrane potential of rat- and mouse-liver mitochondria by flow cytometry and possible applications. *Eur J Biochem* 1990;194:389–397. [PubMed: 2269275]
36. Kataoka M, Fukura Y, Shinohara Y, Baba Y. Analysis of mitochondrial membrane potential in the cells by microchip flow cytometry. *Electrophoresis* 2005;26:3025–3031. [PubMed: 16078196]
37. Tzung SP, Kim KM, Basanez G, Giedt CD, Simon J, Zimmerberg J, Zhang KY, Hockenbery DM. Antimycin A mimics a cell-death-inducing Bcl-2 homology domain 3. *Nat Cell Biol* 2001;3:183–191. [PubMed: 11175751]
38. de Bari L, Atlante A, Guaragnella N, Principato G, Passarella S. D-Lactate transport and metabolism in rat liver mitochondria. *Biochem J* 2002;365:391–403. [PubMed: 11955284]
39. Scaduto RC, Grotyohann LW. Measurement of mitochondrial membrane potential using fluorescent rhodamine derivatives. *Biophysical Journal* 1999;76:469–477. [PubMed: 9876159]
40. Griendling KK, FitzGerald GA. Oxidative stress and cardiovascular injury: Part I: basic mechanisms and in vivo monitoring of ROS. *Circulation* 2003;108:1912–1916. [PubMed: 14568884]
41. Rajmakers MT, Peters WH, Steegers EA, Poston L. NAD(P)H oxidase associated superoxide production in human placenta from normotensive and pre-eclamptic women. *Placenta* 2004;25(Suppl A):S85–89. [PubMed: 15033313]
42. Stokes KY, Clanton EC, Russell JM, Ross CR, Granger DN. NAD(P)H oxidase-derived superoxide mediates hypercholesterolemia-induced leukocyte-endothelial cell adhesion. *Circ Res* 2001;88:499–505. [PubMed: 11249873]
43. Brand MD, Affourtit C, Esteves TC, Green K, Lambert AJ, Miwa S, Pakay JL, Parker N. Mitochondrial superoxide: production, biological effects, and activation of uncoupling proteins. *Free Radic Biol Med* 2004;37:755–767. [PubMed: 15304252]
44. Thor H, Smith MT, Hartzell P, Bellomo G, Jewell SA, Orrenius S. The metabolism of menadione (2-methyl-1,4-naphthoquinone) by isolated hepatocytes. A study of the implications of oxidative stress in intact cells. *J Biol Chem* 1982;257:12419–12425. [PubMed: 6181068]
45. Tamarit J, Cabisco E, Ros J. Identification of the major oxidatively damaged proteins in *Escherichia coli* cells exposed to oxidative stress. *J Biol Chem* 1998;273:3027–3032. [PubMed: 9446617]
46. Sharma P, Mongan PD. Ascorbate reduces superoxide production and improves mitochondrial respiratory chain function in human fibroblasts with electron transport chain deficiencies. *Mitochondrion* 2001;1:191–198. [PubMed: 16120278]
47. Lebovitz RM, Zhang H, Vogel H, Cartwright J Jr, Dionne L, Lu N, Huang S, Matzuk MM. Neurodegeneration, myocardial injury, and perinatal death in mitochondrial superoxide dismutase-deficient mice. *Proc Natl Acad Sci U S A* 1996;93:9782–9787. [PubMed: 8790408]

48. Inarrea P, Moini H, Rettori D, Han D, Martinez J, Garcia I, Fernandez-Vizarra E, Iturralde M, Cadenas E. Redox activation of mitochondrial intermembrane space Cu,Zn-superoxide dismutase. *Biochem J* 2005;387:203–209. [PubMed: 15537389]
49. Reid MB, Durham WJ. Generation of reactive oxygen and nitrogen species in contracting skeletal muscle: potential impact on aging. *Ann N Y Acad Sci* 2002;959:108–116. [PubMed: 11976190]
50. Chandwaney R, Leichtweis S, Leeuwenburgh C, Ji LL. Oxidative stress and mitochondrial function in skeletal muscle: Effects of aging and exercise training. *Age* 1998;21:109–117.
51. Oshitani N, Kitano A, Okabe H, Nakamura S, Matsumoto T, Kobayashi K. Location of Superoxide Anion Generation in Human Colonic Mucosa Obtained by Biopsy. *Gut* 1993;34:936–938. [PubMed: 7688334]
52. Barja G. Endogenous oxidative stress: relationship to aging, longevity and caloric restriction. *Ageing Res Rev* 2002;1:397–411. [PubMed: 12067594]
53. Beckman KB, Ames BN. Mitochondrial aging: open questions. *Ann N Y Acad Sci* 1998;854:118–127. [PubMed: 9928425]

Abbreviations

CE	capillary electrophoresis
LIF	laser induced fluorescence
MEKC	micellar electrokinetic capillary chromatography
CTAB	cetyltrimethylammonium bromide
ROS	reactive oxygen species
mtDNA	mitochondrial DNA
SOD	superoxide dismutase
ETC	electron transport chain
HE	hydroethidine
TPP-HE	triphenylphosphonium hydroethidine (a.k.a MitoSOX red)

Figure 1.
Formation of ROS by electron transport chain (ETC) in mitochondria.

Figure 2.

TPP-HE oxidation products formed in the presence of the xanthine/xanthine oxidase reaction system. (A) Electropherograms of oxidation products, OH-TPP-E⁺ and TPP-E⁺, with different incubation times. The reaction mixture consisted of 50 μ M TPP-HE, 375 μ M xanthine, and 5 μ g/mL of xanthine oxidase with or without 300 U/mL SOD. Separations were performed in a 40-cm long capillary at -400 V/cm in borate-CTAB buffer. The 488-nm line of an argon-ion laser was used for excitation, and a 635 ± 27.5 nm band-pass filter was used for detection. Traces have been offset vertically for clarity. (B) Corrected peak area ratios of OH-TPP-E⁺ to TPP-E⁺ with different incubation time/conditions. Data are presented as mean $\pm$ SD, $n=3$.

Figure 3. Effects of rotenone on TPP-HE oxidation in the mitochondrial matrix and outside the mitochondrial inner membrane. Electropherograms of TPP-HE oxidation products, OH-TPP-E⁺ and TPP-E⁺, in the matrix (A) and outside the mitochondrial inner membrane (B) after treatment with different concentrations of rotenone for 30 min. The substrate consists of 5 mM Pyruvate, 5 mM Malate. The separation and detection conditions were similar to those described in Figure 1A. (C) Corrected peak areas of OH-TPP-E⁺ and TPP-E⁺ versus rotenone concentrations. The mitochondrial protein concentration is normalized to 1 mg·mL⁻¹. Data are presented as mean $\pm$ SD, n=3.

Figure 4.

Effect of antimycin A and menadione on OH-TPP-E⁺ and TPP-E⁺ accumulation at both sides of the mitochondrial inner membrane. Corrected peak areas of OH-TPP-E⁺ and TPP-E⁺ versus antimycin A (A) or menadione (B) concentrations. The mitochondria are incubated in 10 mM Succinate and 10 μM Rotentone (A) or 5 mM Pyruvate and 5 mM Malate (B) for 30 min. Other conditions were similar to those described in Figure 3.

Figure 5.

Superoxide-dependent and -independent oxidation of TPP-HE induced by rotenone, antimycin A and menadione. Ratio of corrected peak areas of OH-TPP-E⁺ over TPP-E⁺ in either the matrix (A) or outside the inner membrane (B). The mitochondria are incubated in 5 mM Pyruvate and 5 mM Malate for rotenone and menadione treatments, 10 mM Succinate and 10 μM Rotentone for antimycin A treatments for 30 min. Other conditions were similar to those described in Figure 3. Data are presented as means ± SD, n=3. (A) * P < 0.01 vs. control (0 μM). (B) ** P < 0.05 vs. control (0 μM).

Figure 6. The OH-TPP-E⁺ and TPP-E⁺ levels in mitochondria from 143B cells, liver and muscle. Treatments of 5 μ M rotenone, menadione or antimycin A in either the matrix (A) or outside the inner membrane (B). Other conditions were similar to those described in Figure 5.

Figure 7. Superoxide-specific and -independent oxidation of TPP-E⁺ in mitochondria of 143B cells, liver and muscle. Treatments were rotenone, antimycin A or menadione, 5 μ M each. Ratios of corrected peak areas of OH-TPP-E⁺ to TPP-E⁺ outside of the inner membrane (bars up) or in the matrix (bars down). Other conditions were similar to those described in Figure 5.