Published in final edited form as:

Anal Bioanal Chem. 2013 July ; 405(18): 6053–6060. doi:10.1007/s00216-013-7022-y.

Capillary electrophoretic analysis of hydroxyl radicals produced by respiring mitochondria

Margaret A. Donoghue^{a,b,c}, Xin Xu^{a,d}, David A. Bernlohr^b, and Edgar Arriaga^{*,a,b}

^aDepartment of Chemistry and Biochemistry, University of Minnesota, Minneapolis, MN 55455, USA

^b Department of Molecular Biology and Biophysics, University of Minnesota, Minneapolis, MN 55455, USA

Abstract

Here we report the use of a capillary electrophoretic method with laser induced fluorescence detection to evaluate hydroxyl radicals produced by respiring mitochondria. The probe, hydroxyphenylfluorescein (HPF), is separated from the product, fluorescein, in under 5 minutes with zeptomol and attomol limits of detection for fluorescein and HPF, respectively. Purification of the probe with a C-18 SPE column is necessary to reduce the fluorescein impurity in the probe stock solution from 0.4% to less than 0.001%. HPF was responsive to hydroxyl radicals produced by isolated mitochondria from L6 cells, and this signal was blunted when DMSO was added to scavenge hydroxyl radicals and when carbonyl cyanide m-chlorophenylhydrazone was added to depolarize the mitochondria. The method was used to compare hydroxyl radical levels in mitochondria isolated from brown adipose tissue of lean and obese mice. Mitochondria from obese mice produced significantly more hydroxyl radicals than those from lean mice.

Keywords

Hydroxyl radicals; Mitochondria; Obesity; Micellar electrokinetic chromatography; Capillary electrophoresis

Introduction

There has long been an association between oxidative stress and a number of disease states, such as obesity, diabetes, insulin resistance, and the aging process [1-2]. Reactive oxygen species (ROS) are the primary source of oxidative stress. Most of cellular ROS are produced by the mitochondria and levels fluctuate due to signaling cascades or functional changes in these organelles. Dysfunction of the mitochondria itself or various antioxidants leads to increased levels of ROS. Methods for the detection of ROS in biological systems are important to pinpoint the mechanisms by which oxidative damage occurs.

In mitochondria, the cascade of ROS begins with superoxide production at complexes I and III in the electron transport chain (ETC). Superoxide dismutase converts superoxide to hydrogen peroxide, which if not cleared enzymatically is transformed into hydroxyl radicals via Fenton chemistry. While normally present at low levels, high concentrations of ROS can lead to mitochondrial DNA damage, protein carbonylation, and other forms of cellular

* Corresponding author: 207 Pleasant St. SE, Minneapolis, MN 55455, USA arriaga@umn.edu.

^cCurrent addresses: Department of Biochemistry, Wake Forest School of Medicine, Winston-Salem, NC 27157, USA

^dCurrent addresses: Department of Mechanical and Aerospace Engineering, University of Florida, Gainesville, FL 32611, USA

damage. Detection of individual ROS in diseased cells is needed for a better understanding of the underlying cellular defects. ROS have several features that make them difficult to analyze in biological systems. Many ROS have very short lifetimes, such as 1×10^{-9} s for hydroxyl radicals. Additionally, these species are present at low concentrations in mitochondria: 1×10^{-10} M for superoxide and 5×10^{-9} M hydrogen peroxide [1,3-4]. Due to its short lifetime, steady state concentrations of hydroxyl radicals are not typically measured. However, the rate of formation in submitochondrial particles has been reported to be 0.6-0.7% the rate of hydrogen peroxide formation ($0.16 \text{ nmol min}^{-1} \text{ mg protein}^{-1}$) [5].

Hydroxyl radicals are of particular interest because of their high reactivity and propensity to cause damage at the site of formation. Hydroxyl radicals have been implicated in the formation of lipid peroxidation products, which lead to highly reactive α,β -unsaturated aldehydes. These reactive compounds can modify proteins via carbonylation and reduce function [6]. Reduced mitochondrial and metabolic function have been observed in obese, glucose-intolerant mice [7]. Many of the mechanisms behind this dysfunction are not completely known. Previous reports describe increased total ROS and superoxide levels in visceral adipose tissue under conditions of obesity [2,8]. In agreement, increases in protein carbonylation and the lipid aldehyde 4-hydroxynonenal (4-HNE), tied to hydroxyl radicals, have also been observed in adipose tissue under these conditions [2,7,9]. Thus sensitive methods to monitor selectively hydroxyl radicals are needed to determine the link between oxidative stress and cellular dysfunction.

To date, there have been a number of methods to detect hydroxyl radicals. Electron spin trapping has been used to monitor hydroxyl radicals in a number of systems, yet suffers from high detection limits, the need for special instrumentation, and instability of the hydroxyl radical-spin trap adduct [10]. Chemiluminescent probes for hydroxyl radicals have been used, but tend to be nonspecific for hydroxyl radicals [10-11] or require highly reproducible reaction conditions which are not obtainable in biological systems [12]. HPLC and capillary electrophoresis (CE) separation methods have been paired with absorbance and electrochemical detection methods for analysis of hydroxyl radical probes such as salicylic acid, phenylalanine, and terephthalic acid [10,13-17]. Unfortunately, these probes are often biologically active, which may alter the biological system of interest. Several commercial fluorescent probes for ROS, such as dichlorodihydrofluorescein, respond to multiple ROS [18]. Additionally, auto-oxidation of the probes may lead to increased background levels and false positives [19].

One selective probe of interest is 2-[6-(4'-hydroxy)phenoxy-3*H*-xanthen-3-on-9-yl]benzoic acid, or hydroxyphenylfluorescein (HPF) [19]. HPF reacts with hydroxyl radicals, to form fluorescein, which is fluorescent (**Figure 1a**). This probe is highly selective for hydroxyl radicals, although some reaction occurs with peroxynitrite. Furthermore, there is no significant autooxidation of HPF under experimental conditions [19]. This probe has been used previously [20-24], however these assays have used fluorescence detection on a plate reader or microscopy. Although HPF has a lower molar absorptivity ($\epsilon_{454} = 28,000$) and quantum efficiency (0.006) than fluorescein [19], fluorescence from un-reacted probe can be observed in sensitive analysis methods. To improve the analysis of hydroxyl radicals, we investigated here the use of a CE type, micellar electrokinetic chromatography with laser induced fluorescence detection (MEKC-LIF).

CE methods have been used for the detection of other ROS, in which ROS-specific and non-specific products must be separated to avoid bias. MEKC separated the specific and non-specific products of MitoSox used in detection of superoxide [25-26]. A microfluidic-based separation to detect ROS and reactive nitrogen species (RNS) with the fluorescent probes 2-chloro-1,3-dibenzothiazolinedicyclohexene (DBZTC) and 4-amino,5-aminomethyl-2,7-

difluorescein (DAF-FM) was recently developed [27]. CE-LIF has also been used to detect generic ROS, hydrogen peroxide, and glutathione [28-30]. These reports highlight other benefits of separations over microscopy. Namely, only a few nanoliters of sample are required to run an analysis due to the sensitive nature of this technique.

Here we report the use of MEKC-LIF to measure the conversion of HPF to fluorescein upon exposure to hydroxyl radicals. The method has low limits of detection and rapid analysis times. The use of the probe was verified in vitro and applied to mitochondria isolated from cultured L6 rat myoblasts. Additionally, levels of hydroxyl radicals were compared in brown adipose tissue from lean and obese mice. Mitochondria from mice on a high fat diet produced higher levels of hydroxyl radicals, which correlates with a recent report on hydrogen peroxide production in both coupled and uncoupled mitochondria [31]. Such results support the idea that oxidative damage is an important factor in obesity.

Materials and methods

Reagents

2-[6-(4'-hydroxy)phenoxy-3*H*-xanthen-3-on-9-yl]benzoic acid (HPF) and fluorescein were purchased from Invitrogen (Eugene, OR). Sodium tetraborate and ethylene glycol tetraacetic acid (EGTA) were from Fluka (Buchs, Switzerland). Sodium dodecyl sulfate (SDS) was purchased from Bio-Rad (Hercules, CA), mannitol from Riedel-de Haën (Seelze, Germany), and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) from Acros (Geel, Belgium). The potassium phosphate monobasic, citric acid, and NaOH were from Mallinckrodt (Phillipsburg, NJ). Dulbecco's modified Eagle medium (DMEM), bovine serum, and 10× trypsin were from Gibco (Invitrogen, Carlsbad, CA). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Buffers

The extraction buffer to purify HPF contained 10 mM citric acid and 20 mM sodium phosphate (pH = 2.6). The MEKC buffer was 10 mM borate and 15 mM sodium dodecyl sulfate (SDS) (pH 9.3). The sample buffer contained 10 mM phosphate and 15 mM SDS (pH 7.4). The Fenton reactions were run in 100 mM phosphate (pH 7.4) with the exception of the xanthine/xanthine oxidase control reaction, which was in 50 mM phosphate buffer with 0.1 mM EDTA added. The buffer for isolation of mitochondria from L6 cells was comprised of 220 mM mannitol, 70 mM sucrose, 2 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and 0.35 mM EGTA (pH 7.4). The isolation buffer for use with adipose tissue was 220 mM mannitol, 70 mM sucrose, 20 mM Tris-HCl, 1 mM EDTA, 0.1 mM EGTA (pH 7.4) with 0.1% bovine serum albumin (BSA). The final wash buffer was the same, only without the addition of BSA. Mitochondria isolated from L6 cells were incubated in respiration buffer comprised of 10 mM HEPES, 125 mM KCl, 5 mM MgCl₂, and 2 mM K₂HPO₄ (pH 7.4). The respiration buffer for adipose mitochondria was the same but also contained 20 μM EGTA. All buffers were prepared in deionized water and filtered through a 0.22-μm filter prior to use.

Cell culture conditions

The rat myoblast L6 cells were obtained from American Tissue Culture Collection (Manassas, VA). Cells were cultured in DMEM containing 10% (v/v) bovine serum and 10 μg/mL gentamicin. Cultures were maintained at 5% CO₂ and 37 °C and split every 3 -4 days.

Animals

C57Bl/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME) and maintained on normal low fat rodent chow or maintained for 11 weeks on a high fat diet (Bioserve F3282) to induce obesity. The mice were sacrificed at 12 weeks of age and the interscapular brown fat depot was harvested for immediate use. All animal procedures were approved by the University of Minnesota Institutional Animal Care and Use Committee. The mice fed with high-fat and normal chow diets had average body weights of 45 ± 6 g and 29 ± 2 g, respectively.

Mitochondrial Preparations

Mitochondria were isolated from L6 cells with previously reported procedures [25,32]. Briefly, cells were released from culture dishes with $0.5\times$ trypsin and incubated for 20 min on ice with 1% protease inhibitor in isolation buffer. Cells were lysed with 90 strokes of a Dounce homogenizer. The homogenate was centrifuged at $600g$ to remove nuclei, unbroken cells, and lipids. Fractions enriched for mitochondria were prepared by centrifugation of the supernatants at $10,000g$. Mitochondria were isolated from adipose tissue by first mincing the tissue in ice-cold isolation buffer. The isolation buffer volume was then increased to 1:7 wt/vol and cells were lysed with 15 strokes on a glass-teflon homogenizer at 1000 rpm. The adipocyte homogenates were centrifuged $700g$. Mitochondria were re-suspended in respiration buffer and protein was quantified with a BCA protein assay (Thermo Scientific, Rockford, IL).

The integrity of the mitochondria following isolation can be evaluated by measurement of the membrane potential with a triphenylphosphonium (TPP^+)-selective electrode (World Precision Instruments, Sarasota, FL). After correction for TPP^+ binding to the mitochondrial membranes [33], mitochondria isolated from L6 cells had a membrane potential of ~ 135 mV, similar to those reported in literature [34].

Purification of HPF

Upon receipt, there is a significant fluorescein impurity present in the HPF. To reduce the fluorescein background, the probe was cleaned up via a solid-phase extraction procedure. A $10\ \mu\text{L}$ aliquot of the HPF stock solution from the manufacturer was diluted to 1 mL in the extraction buffer and loaded onto a Discover DSC-18 SPE column (Supelco, Bellefonte, PA). Columns were prepared by flushing sequentially with 1 mL water, 1 mL 50:50 water:methanol, 1 mL methanol, and 1 mL water. To elute fluorescein, the column was washed four times with 50:50 water:methanol. Two washes with 20:80 water:methanol eluted the retained HPF. The two fractions containing HPF were evaporated and reconstituted in dimethylformamide to approximately 2.5 mM. The actual concentration of the probe stock solution was determined by comparison of the HPF peak area of the purified sample to the original solution of HPF. This procedure reduced the fluorescein impurity to less than 0.001%.

Sample preparation for $\cdot\text{OH}$ oxidation product

Mitochondria isolated from L6 cells were incubated at a concentration of 0.3 mg/mL mitochondrial protein with $10\ \mu\text{M}$ HPF and 10 mM succinate, a substrate for respiration, in $50\ \mu\text{L}$ respiration buffer for 30 min at 37°C . For the various controls, mitochondria were incubated at 37°C for 5 min in the presence of CCCP or DMSO prior to addition of the HPF. The brown adipose tissue derived mitochondria (3-5 mg/mL protein) were incubated similarly with $10\ \mu\text{M}$ HPF and 10 mM glutamate/5 mM malate in $100\ \mu\text{L}$ respiration buffer. After incubation, samples were diluted in sample buffer and sonicated for four min to aid dissolution of the mitochondrial membrane prior to analysis by MEKC-LIF.

MEKC-LIF analysis

The home-built CE instrument has been previously described [25]. It consists of an Argon ion laser (Melles Griot, Irvine, CA) for excitation at 488 nm. A bandpass filter (Omega Optical, Brattleboro, VT) centered at 535 ± 17.5 nm permitted the fluorescent signal of interest to reach a photomultiplier tube (PMT) (R1477; Hamamatsu, Bridgewater, NJ) biased at 1000 V for detection. Signal measured by the PMT was collected by LabView at 10 Hz with a NiDaq I/O board (PCI-MIO-16XE-50, National Instruments, Austin, TX). Detection was post-column in a sheath-flow cuvette. Alignment of the optical components was done with 10^{-10} M fluorescein. Limits of detection (signal/noise = 3) were calculated from the peak intensities of analytes injected at standard concentrations. The limit of detection for fluorescein was approximately 14 zeptomole.

Mitochondrial samples were analyzed in triplicate by MEKC-LIF. Prior to use, the fused silica capillaries (PolymicroTechnologies, Phoenix, AZ) were conditioned by flushing with 0.5 M NaOH followed by a water flush. The capillary was then conditioned with the run buffer for 45 min. Hydrodynamic injections at 1.4 psi for 1 s introduced 3.7 nL of sample into the fused silica capillary (50 μ m i.d., 150 μ m o.d.) with a total and effective length of 36 cm. Separations were conducted at 400 V/cm. The data collected by LabView were converted to binary files and analyzed with IgorPro (Wavemetrics, Lake Oswego, OR) software. Electrophoretic mobilities of the analytes were calculated by dividing the length of the capillary by the migration time and electric field strength. Results are reported as means $\pm$ standard deviation. Significance was determined with a Student *t* test.

Results and discussion

Separation and detection of HPF and fluorescein

Upon receipt, the commercial HPF contained approximately 0.4% mole fluorescein. Although this is a small percentage, when 500 nM HPF was analyzed with MEKC-LIF the fluorescein peak was very intense ($t_M = 254$ s) relative to that of HPF ($t_M = 274$ s) (**Figure 1b, top**), which is unacceptable for detection of low fluorescein levels resulting from HPF reaction with hydroxyl radicals. To reduce the amount of fluorescein present in the probe stock solution, the probe was purified twice with solid-phase extraction. This procedure reduced the fluorescein impurity to less than 0.001% mole (**Figure 1b, bottom**). After purification the amount of fluorescein observed in the HPF solution was stable over the course of several weeks.

As seen above, the MEKC conditions used here were adequate to separate fluorescein and HPF with high resolution ($R = 4.9$) in 5 min (**Figure 1b**). The net electrophoretic mobilities of fluorescein and HPF were $(3.55 \pm 0.02) \times 10^{-4} \text{ cm}^2\text{V}^{-1}\text{s}^{-1}$ and $(3.28 \pm 0.02) \times 10^{-4} \text{ cm}^2\text{V}^{-1}\text{s}^{-1}$, respectively. Limits of detection were ~ 11 attomole for HPF and ~ 14 zeptomole for fluorescein. The HPF signal was linear as described by $y = (3.3 \pm 0.6) \times 10^7 x - (0.2 \pm 0.3)$, ($R^2 = 0.999$), where *y* is the peak area and *x* is the concentration of analyte. The fluorescein signal was also linear ($R^2 = 0.995$) as described by $y = (1.79 \pm 0.05) \times 10^{10} x + (0.0 \pm 0.1)$. Linearity was evaluated over 1.5 orders of magnitude for both analytes.

The Fenton reaction is a common method to generate hydroxyl radicals in solution and was used here to produce hydroxyl radicals to test HPF response to this ROS. HPF (10 μ M) was incubated with 40 μ M FeSO₄ and an excess (1 mM) of H₂O₂ in phosphate buffer. The reaction mixture was then analyzed by MEKC-LIF at different incubation times (**Figure 2**). After 15 min of incubation time, the fluorescein peak at $t_M = 240$ s has increased significantly in area, as most of the iron has reacted to produce hydroxyl radicals (**Figure 2a**). A small increase was further observed from 15 to 35 min, after which the signal intensity remained constant for 75 min, indicating that the probe was stable in the reaction

mixture. The bar graphs in **Figure 2b** show this trend more clearly, where the ratio of fluorescein peak area to HPF peak area has been plotted. A Fenton reaction control lacking hydrogen peroxide confirmed that the increase in fluorescein area was due to hydroxyl radicals. In the absence of hydrogen peroxide and the presence of 50 μM FeSO_4 to catalyze the Fenton reaction, no increase in peak area was observed. This data was also used to assess migration time and peak area reproducibilities of the method. Migration time reproducibilities were 1.4% and 1.1% (relative standard deviation, RSD; $n = 32$) for fluorescein and HPF, respectively. Peak area reproducibilities were 4.3% and 6.8% (RSD; $n = 9$) respectively.

The selectivity of HPF for hydroxyl radical was also evaluated. Incubation of HPF with 50 μM hydrogen peroxide alone produced no change in the fluorescein to HPF ratio (**Figure 2b**). This concentration of hydrogen peroxide was chosen because it is on the high end of values commonly reported in biological systems [3-4]. Samples containing 50 μM hydrogen peroxide only generated 0.3% the response of hydroxyl radicals, so the data agrees well with literature [19]. HPF was also incubated with 50 μM xanthine and an excess of xanthine oxidase to test its reactivity with superoxide. A higher response was observed compared to literature [19], 6% instead of 1%, however was still much lower than the response to hydroxyl radicals. This discrepancy may arise from the use of enzymatic production of superoxide instead of a chemical addition (KO_2), as well as the presence of EDTA in the xanthine/xanthine oxidase reaction mixture. Although EDTA was included to chelate free iron, there is evidence this may enhance production of hydroxyl radicals [35-36]. Because the expected steady state superoxide concentration within biological cells is low, (e.g. the superoxide level in the mitochondrial matrix is $\sim 10^{-10}$ M [26]) relative to those generated by the xanthine/xanthine oxidase reaction, the interference of superoxide in the analysis of hydroxyl radical levels in biological systems is likely negligible. Superoxide and hydrogen peroxide are the two primary ROS other than hydroxyl radicals. Thus, the low reactivity of HPF to these ROS, shows it can be used reliably in biological systems to monitor hydroxyl radicals.

Detection of fluorescein in mitochondria enriched fractions

Under basal conditions, mitochondria are the main source of ROS within a cell, where ROS are produced by the ETC. ROS production increases when mitochondria cease to function properly [2], such as when the ETC has dysfunctional proteins or is blocked by chemical inhibitors. To get a clearer picture of how dysfunction is related to ROS and disease, methods capable of sensitive and specific detection of hydroxyl radicals produced by mitochondria are needed. We explored the use of the MEKC-LIF method described here to analyze hydroxyl radicals in mitochondrial preparations.

Subcellular fractions enriched for mitochondria were obtained by differential centrifugation and incubated with HPF for 30 min, with and without substrate (succinate). Results from these experiments are shown in **Figure 3**. In actively respiring mitochondria an increase in hydroxyl radicals was seen over mitochondria with no substrates (**Figure 3b**). Although the increase is small, it is a significant difference. The electropherograms in **Figure 3a** also show that the increase in fluorescein peak area can be stimulated with the addition of Fe^{2+} to the mitochondrial fraction. DMSO, a scavenger of hydroxyl radicals [37], decreases the response of the probe. These data illustrate that we can monitor hydroxyl radical production in isolated cellular fractions effectively.

When compared to the HPF standard, mitochondrial preparations containing no exogenous ETC substrates did show an increase in the fluorescein to HPF ratio. This increase in signal can have several possible contributions. Upon isolation, mitochondria contain a small amount of the substrates needed for respiration. Therefore, even though no additional

succinate was added to fuel the ETC a small level of respiratory activity may still be possible. To evaluate this theory, the ratio of fluorescein to HPF was measured in mitochondria in which respiration had been decoupled with CCCP. CCCP dissipates the mitochondrial membrane potential by passing protons across the mitochondrial inner membrane. This uncoupling decreases the levels of superoxide [38] and hydrogen peroxide [39] produced by mitochondria utilizing succinate as a respiration substrate. A reduction in these ROS should result in a decrease in hydroxyl radicals as well. These results are compared to mitochondria respiring normally with succinate and to DMSO treated mitochondria in **Figure 4**. Upon treatment with CCCP, the ratio of fluorescein to HPF decreases as expected. The amount of fluorescein produced is not significantly different compared to mitochondria that were incubated with DMSO.

The lack of a significant difference between the CCCP and DMSO treated mitochondria suggests that the hydroxyl radicals being detected are indeed produced by the mitochondria. However, this may result from the limited statistical power of our measurements. It would have been anticipated that other sources of hydroxyl radicals may be present in the sample [40]. In the future, it will be relevant to establish whether other contaminating organelles in the mitochondrial-enriched sample, such as lysosomes and autophagosomes, contribute to the levels of hydroxyl radical detected. Lysosomes in particular contain a source of iron, which may lead to an increase in hydroxyl radicals independent of the mitochondrial ETC. A small part of the signal may also be due to peroxynitrite in the system [41].

The hydroxyl radicals that react with HPF are produced both inside and outside the mitochondria. Fluorescein has previously been shown to enter mitochondria [42-43], although it does not accumulate within the mitochondria. With a similar structure and a less negative charge (**Figure 1a**), HPF should act similarly. Analysis of mitochondrial pellets and supernatants after treatment with HPF supported this, where both fractions contained HPF and fluorescein (data not shown). Hydroxyl radicals are short lived, and thus do not typically cross the mitochondrial membrane. However, HPF cannot be used to determine the site of hydroxyl radical production. Hydrogen peroxide can cross the mitochondrial membrane, thus could potentially be produced inside the mitochondrial matrix and converted to hydroxyl radicals outside of the mitochondria. This method is thus valuable to determine the total amount of hydroxyl radicals produced by the mitochondria.

Detection of ·OH in brown adipose tissue from lean and obese mice

Studies have shown that mitochondrial dysfunction is a hallmark of obesity, which is one of the main risk factors for diabetes and insulin resistance. Changes in mitochondrial morphology, impaired fusion and fission, and a decrease in enzymes involved in energy metabolism have been observed in visceral adipose tissue [3]. Increased levels of ROS, lipid peroxides, and protein carbonylation have also been observed in these same depots [2,7,9]. Although ROS and their oxidative products are observed to be elevated in obesity, it is unclear exactly how these relate to the development of insulin resistance and diabetes. More precise understanding of the ROS present in such systems may help answer such questions. In this report, we evaluated the relative levels of hydroxyl radicals produced by mitochondria obtained from the adipose tissue of lean and obese mice.

Mitochondrial enriched fractions isolated from adipose tissue were incubated with glutamate and malate to stimulate respiration and HPF to detect the relative production of hydroxyl radicals. For mice fed a high-fat diet, the ratio of fluorescein to HPF in mitochondria was 1.0 ± 0.3 , compared to 0.3 ± 0.1 in the mitochondria from mice fed with regular chow. This significant difference ($p < 0.05$) indicates that more hydroxyl radicals are produced by the mitochondria from obese mice than from lean mice. This agrees with other work that shows

increased ROS levels occur with obesity, which is in agreement with increased protein carbonylation, an indicator of oxidative damage in obesity [7].

In summary, this paper presents a method that uses MEKC-LIF to separate and detect the oxidation product of HPF, fluorescein, for the analysis of hydroxyl radicals. This method has the benefits of low limits of detection and small sample volumes. Unlike other published methods that focus on microscopy or microplate assays, the use of MEKC-LIF enables sensitive and selective detection of fluorescence only from the product fluorescein. Many of the recently published methods rely on fluorescent imaging [44-46], however, it would be useful to monitor multiple distinct reactive oxygen species in a single sample. A method based on the separation of multiple probes for different ROS will lead to increased flexibility in experimental design. The improved sensitivity of this MEKC method that uses a commercially available product has wider availability than some probes, such as lanthanide-based probes [47] that currently require synthesis. Thus far, separation methods for ROS detection have not focused on hydroxyl radicals, although reports have been published on the use of electrophoretic separation methods for the detection of other ROS [25-28,30].

With this method, hydroxyl radicals can be qualitatively detected in basal and decoupled mitochondria from L6 cells, as well as mitochondria obtained from tissue samples. In particular, it was shown that mitochondria from the brown adipose tissue of obese mice produce more hydroxyl radicals than lean mice. Such use of this detection method will enable further research into the mechanisms by which oxidative stress is associated with obesity and diabetes.

Acknowledgments

This work was supported in part by NIH P30DK050456 and R01AG020866 grants. M.A.D. was supported by NIH Training Grant T32DK007203. The authors thank Rocio Foncea and Wendy Hahn for assistance in obtaining the adipose tissue.

References

1. Cadenas E, Davies KJA. Free Radical Biol Med. 2000; 29:222–230. [PubMed: 11035250]
2. Curtis JM, Hahn WS, Long EK, Burrill JS, Arriaga EA, Bernlohr DA. Trends Endocrinol Metab. 2012; 23:399–406. [PubMed: 22742812]
3. Espiritu DJ, Mazzone T. Diabetes. 2008; 57:2992–2998. [PubMed: 18678613]
4. Gautam DK, Misro MM, Chaki SP, Sehgal N. Apoptosis. 2006; 11:39–46. [PubMed: 16374549]
5. Giulivi C, Boveris A, Cadenas E. Arch Biochem Biophys. 1995; 316:909–916. [PubMed: 7864650]
6. Grimsrud PA, Xie H, Griffin TJ, Bernlohr DA. J Biol Chem. 2008; 283:21837–21841. [PubMed: 18445586]
7. Grimsrud PA, Picklo MJ Sr, Griffin TJ, Bernlohr DA. Mol Cell Proteomics. 2007; 6:624–637. [PubMed: 17205980]
8. Fernandez-Sanchez A, Madrigal-Santillan E, Bautista M, Esquivel-Soto J, Morales-Gonzalez A, Esquivel-Chirino C, Durante-Montiel I, Sanchez-Rivera G, Valadez-Vega C, Morales-Gonzalez JA. Int J Mol Sci. 2011; 12:3117–3132. [PubMed: 21686173]
9. Curtis JM, Grimsrud PA, Wright WS, Xu X, Foncea RE, Graham DW, Brestoff JR, Wiczor BM, Ilkayeva O, Cianflone K, Muoio DE, Arriaga EA, Bernlohr DA. Diabetes. 2010; 59:1132–1142. [PubMed: 20150287]
10. Cheng F-C, Jen J-F, Tsai T-H. J Chromatogr B Analyt Technol Biomed Life Sci. 2002; 781:481–496.
11. Yamaguchi S, Kishikawa N, Ohyama K, Ohba Y, Kohno M, Masuda T, Takadate A, Nakashima K, Kuroda N. Anal Chim Acta. 2010; 665:74–78. [PubMed: 20381693]
12. Miller CJ, Rose AL, Waite TD. Anal Chem. 2011; 83:261–268. [PubMed: 21142069]

13. Kilinc E. *Talanta*. 2005; 65:876–881. [PubMed: 18969882]
14. Michail K, Siraki AG. *Anal Chem*. 2012; 84:6739–6746. [PubMed: 22724922]
15. Biondi R, Xia Y, Rossi R, Paolocci N, Ambrosio G, Zweier JL. *Anal Biochem*. 2001; 290:138–145. [PubMed: 11180947]
16. Marchiarullo DJ, Lim JY, Vaksman Z, Ferrance JP, Putcha L, Landers JP. *J Chromatogr A*. 2008; 1200:198–203. [PubMed: 18555260]
17. Wang Q, Chen X, Lu J, He P, Fang Y. *Electrophoresis*. 2005; 26:3273–3278. [PubMed: 16143980]
18. Kuznetsov AV, Kehrer I, Kozlov AV, Haller M, Redl H, Hermann M, Grimm M, Troppmair J. *Anal Bioanal Chem*. 2011; 400:2383–2390. (Copyright (C) 2013 American Chemical Society (ACS). All Rights Reserved.). [PubMed: 21336935]
19. Setsukinai K, Urano Y, Kakinuma K, Majima HJ, Nagano T. *J Biol Chem*. 2003; 278(5):3170–3175. [PubMed: 12419811]
20. Naito K, Tachikawa T, Fujitsuka M, Majima T. *J Phys Chem C*. 2008; 112:1048–1059.
21. Soh N. *Anal Bioanal Chem*. 2006; 386:532–543. (Copyright (C) 2013 American Chemical Society (ACS). All Rights Reserved.). [PubMed: 16609844]
22. Sugiyama I, Kojima S, Oku N, Sadzuka Y. *Colloid Polym Sci*. 2010; 288:1293–1300.
23. Choi H, Lee DG. *Res Microbiol*. 2012; 163:479–486. [PubMed: 22705395]
24. Mols M, van KR, van MCCJ, Moezelaar R, Abee T. *Environ Microbiol*. 2010; 12:873–885. [PubMed: 20074238]
25. Xu X, Arriaga EA. *Free Radical Biol Med*. 2009; 46(7):905–913. [PubMed: 19168125]
26. Xu X, Arriaga EA. *Analytical Chemistry*. 2010; 82(16):6745–6750. [PubMed: 20704362]
27. Chen Z, Li Q, Sun Q, Chen H, Wang X, Li N, Yin M, Xie Y, Li H, Tang B. *Anal Chem*. 2012; 84:4687–4694. [PubMed: 22551384]
28. Chen Z, Li Q, Wang X, Wang Z, Zhang R, Yin M, Yin L, Xu K, Tang B. *Anal Chem*. 2010; 82:2006–2012. [PubMed: 20143832]
29. Ling Y-Y, Yin X-F, Fang Z-L. *Electrophoresis*. 2005; 26:4759–4766. [PubMed: 16278919]
30. Xu C-X, Yin X-F. *J Chromatogr. A*. 2011; 1218:726–732. [PubMed: 21185567]
31. Schonfeld P, Wojtczak L. *Biochim Biophys Acta*. 2012; 1817:410–418. [PubMed: 22226918]
32. Meany DL, Poe BG, Navratil M, Moraes CT, Arriaga EA. *Free Radical Biol Med*. 2006; 41:950–959. [PubMed: 16934678]
33. Labajova A, Vojtiskova A, Krivakova P, Kofranek J, Drahota Z, Houstek J. *Anal Biochem*. 2006; 353:37–42. [PubMed: 16643832]
34. Murphy MP, Smith RAJ. *Adv Drug Delivery Rev*. 2000; 41:235–250.
35. Li L, Abe Y, Kanagawa K, Shoji T, Mashino T, Mochizuki M, Tanaka M, Miyata N. *Anal Chim Acta*. 2007; 599:315–319. [PubMed: 17870296]
36. Snrychova I, Pospisil P, Naus J. *Photosynth Res*. 2006; 88:323–329. [PubMed: 16755325]
37. Milne PJ, Zika RG, Saltzman ES. *ACS Symp Ser*. 1989; 393:518–528.
38. Turrens JF, Boveris A. *Biochem J*. 1980; 191:421–427. [PubMed: 6263247]
39. Tahara EB, Navarete FDT, Kowaltowski AJ. *Free Radical Biol Med*. 2009; 46:1283–1297. [PubMed: 19245829]
40. Kurz T, Terman A, Brunk UT. *Arch Biochem Biophys*. 2007; 462:220–230. [PubMed: 17306211]
41. Radi R, Peluffo G, Alvarez MN, Naviliat M, Cayota A. *Free Radical Biol Med*. 2001; 30:463–488. [PubMed: 11182518]
42. Cercek L, Cercek B. *Biophys J*. 1979; 28:403–411. [PubMed: 262558]
43. Wenzel U, Schoberl K, Lohner K, Daniel H. *J Cell Physiol*. 2005; 202:379–390. [PubMed: 15452831]
44. Li P, Xie T, Duan X, Yu F, Wang X, Tang B. *Chem--Eur J*. 2010; 16:1834–1840. [PubMed: 20014077]
45. Yuan L, Lin W, Song J. *Chem Commun (Cambridge, U K)*. 2010; 46:7930–7932.
46. Ganea GM, Kolic PE, El-Zahab B, Warner IM. *Anal Chem*. 2011; 83:2576–2581. [PubMed: 21384843]

47. Xiao Y, Ye Z, Wang G, Yuan J. *Inorg Chem.* 2012; 51:2940–2946. [PubMed: 22356217]

Figure 1.

(a) Chemical structures of the hydroxyl radical probe HPF and its product fluorescein. (b) Separation of fluorescein and HPF in a sample of ~ 500 nM HPF before and after purification. The separation was performed in a 10 mM borate/15 mM SDS buffer, pH 9.3. Electric field strength was 400 V/cm applied over a 36 cm capillary.

Figure 2.

Production of hydroxyl radicals by the Fenton reaction. (a) Electropherograms at $t = 0, 15,$ and 75 min, as well as a control with no H_2O_2 present in the system. Separation conditions are the same as in Figure 1. (b) Plot of the ratio of fluorescein to HPF in the Fenton reaction at time $= 0, 15, 35,$ and 75 min. Two controls, $0 \mu\text{M H}_2\text{O}_2$ (w/ Fe^{2+}) and $50 \mu\text{M H}_2\text{O}_2$ (w/o Fe^{2+}) show no response. Data reported as mean $\pm \sigma$ for three instrumental replicates.

Figure 3.

Use of HPF to detect hydroxyl radicals produced in enriched mitochondria fractions from L6 cells. (a) Electropherograms show an increase in fluorescein peak area when mitochondria are stimulated with 100 μM Fe^{2+} . Separation conditions are the same as in Figure 1. (b) Bar graphs show the ratio of fluorescein to HPF in mitochondria: without substrates, basally respiring, stimulated with 100 μM Fe^{2+} , and treated with DMSO. Mitochondria (0.15 mg protein/mL) were incubated for 30 min. Data reported as mean $\pm$ σ for three biological replicates.

Figure 4.

The ratio of fluorescein to HPF as a measure of hydroxyl radical production in respiring mitochondria, mitochondria decoupled with 80 μ M CCCP, and mitochondria treated with DMSO. Mitochondria (0.23 mg protein/mL) were incubated for 30 min. Data reported as mean $\pm$ σ for three biological replicates.