



Original Contribution

Real-time monitoring of superoxide accumulation and antioxidant activity in a brain slice model using an electrochemical cytochrome c biosensor

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ABSTRACT

The overproduction of reactive oxygen species and the resulting damage are central to the pathology of many diseases. The study of the temporal and spatial accumulation of reactive oxygen species has been limited because of the lack of specific probes and techniques capable of continuous measurement. We demonstrate the use of a miniaturized electrochemical cytochrome c (Cyt c) biosensor for real-time measurements and quantitative assessment of superoxide production and inactivation by natural and engineered antioxidants in acutely prepared brain slices from mice. Under control conditions, superoxide radicals produced from the hippocampal region of the brain in 400- μ m-thick sections were well within the range of detection of the electrode. Exposure of the slices to ischemic conditions increased the superoxide production twofold and measurements from the slices were stable over a 3- to 4-h period. The stilbene derivative and anion channel inhibitor 4,4'-diisothiocyano-2,2'-disulfonic stilbene markedly reduced the extracellular superoxide signal under control conditions, suggesting that a transmembrane flux of superoxide into the extracellular space may occur as part of normal redox signaling. The specificity of the electrode for superoxide released by cells in the hippocampus was verified by the exogenous addition of superoxide dismutase (SOD), which decreased the superoxide signal in a dose-dependent manner. Similar results were seen with the addition of the SOD mimetic cerium oxide nanoparticles (nanoceria), in that the superoxide anion radical scavenging activity of nanoceria with an average diameter of 15 nm was equivalent to 527 U of SOD for each 1 μ g/ml of nanoceria added. This study demonstrates the potential of electrochemical biosensors for studying real-time dynamics of reactive oxygen species in a biological model and the utility of these measurements in defining the relative contribution of superoxide to oxidative injury.

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Introduction

Reactive oxygen and nitrogen species (ROS and RNS), including superoxide, nitric oxide, hydrogen peroxide (H₂O₂), and hydroxyl and peroxynitrite radicals, are potent oxidizing and nitrating agents that are produced under a variety of physiological and pathophysiological conditions. Physiological levels of these species appear to be involved in myriad of physiological processes. For example, endogenously generated oxidants act as second messengers, transcriptional regulators, and modulators of ion channels and enzyme activity [1,2]. The principle source of superoxide production under physiological conditions is thought to be the mitochondria, in which the rate of superoxide production in vitro is estimated to be 0.15–2% of the total cellular oxygen consumption [3,4]. Other potential sources of superoxide

production include xanthine oxidases (XODs) and the nicotinamide adenine dinucleotide phosphate (NADPH) family of oxidases. Of these, the latter seems to be unique in that superoxide production is thought to play an important role in normal cellular redox signaling [5,6]. The activity of both XOD and NADPH oxidases can be markedly increased after tissue injury or disease states [7,8].

Overproduction of ROS and RNS has been associated with development of a wide variety of neurodegenerative diseases as a result of their high chemical reactivity and potential for inducing oxidative damage to proteins, cells, and tissues [9–11]. Methods for monitoring ROS and RNS levels in intact, living tissues is a critical first step in unraveling their physiological roles in both healthy and disease states. Unfortunately, continuous in situ monitoring of these species in biological systems has been very challenging because of their high reactivity, low concentrations, and short half-lives. Moreover, the study of kinetics in cell-free systems is difficult because of the many interrelated coupled redox reactions that change dynamically over time.

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Cerebral ischemia is a leading cause of death and long-term disability. During ischemia, blood flow to the tissue is inadequate to sustain the metabolism of the tissue, resulting in a progressive decline in mitochondrial function and uncoupling of the electron transport chain [12]. Consequently, many of the biological cascades involved in ischemic cell death have been related with overproduction of ROS, including superoxide, which contributes to oxidative damage [13–16]. Moreover, XOD and NADPH oxidases have been implicated as the principle contributors of superoxide generation leading to tissue damage after ischemia–reperfusion injury [17,18]. In support of this hypothesis, inhibition of NADPH oxidase decreases ROS levels and preserves integrity of the blood–brain barrier and neuronal function [17].

Although many biochemical processes contribute to the oxidative load generated by superoxide under normal and pathological conditions, the damaging effects of superoxide are thought to be restricted to the cells generating this free radical. The transcellular movement of superoxide across biological membranes is not thought to contribute to any appreciable extent except in erythrocytes [19]. Unlike H_2O_2 , which freely diffuses across membranes, superoxide is relatively impermeable owing to its low water solubility in the charged state. Although the neutral, protonated form of superoxide (pK_a 4.9) could traverse biological membranes, its low intracellular concentration provides little driving force for diffusion into adjacent cellular compartments. Moreover, the identification and localization of the superoxide dismutase (SOD) family of isozymes in the mitochondria (SOD 2), the cytosol (SOD 1), and more recently the extracellular matrix (SOD 3) suggest that vectorially produced superoxide resides and reacts within defined cellular compartments. This notion has been challenged by the findings that superoxide may cross mitochondrial and plasma membrane through voltage-dependent anion channels (VDACs). Biochemical evidence for VDAC distribution in the plasma membrane arose from the finding that VDAC1 is present in caveolae, a specialized domain of the plasma membrane involved in endocytosis [20]. Multiple functions have been ascribed to VDACs, including purine nucleotide transport (ATP and ADP), anion-channel-like activity, and transmembrane redox regulation arising from VDACs' NADH reductase activity [21–24].

Evidence that superoxide may selectively cross through anion channels was originally proposed by Lynch and Fridovich in XOD-loaded lipid vesicles [19] and later by Mao and Poznansky [25] in erythrocyte ghost membranes and in human amniotic cells [26]. Han et al. [27] showed that ~55% of the mitochondrial-generated superoxide exited across the outer mitochondrial membrane through 4,4'-diisothiocyano-2,2'-disulfonic stilbene (DIDS)-sensitive anion channels in endosomes isolated from isolated mitochondria from the heart. More recently, a DIDS-sensitive superoxide flux was reported across the plasma membranes of both epithelial-derived endosomes [28] and endothelial cells. Despite the mounting evidence for the existence of superoxide-permeable channels, little is known regarding the mobility of this free radical and the extent to which this superoxide contributes to the extracellular oxidant load. Understanding this relationship can potentially reshape our views on how the transcellular movement of free radicals can influence oxidative damage in adjacent cells and tissues.

In general, measurement of ROS in living organisms has been a significant analytical challenge. Most ROS are highly reactive and short lived and therefore difficult to detect in complex biological matrices. Additionally, ROS often are produced and/or neutralized in subcellular compartments, which requires detection methods directed to specific subcellular localization. There are few methods that measure superoxide anion directly (i.e., electron paramagnetic resonance) and most techniques utilize indirect

absorbance or fluorescence measurements [29] or oxidation products, most of which are relatively nonspecific and have limited temporal or spatial resolution. Thus the goals of this study were threefold. First, using an electrochemical cytochrome *c* (Cyt *c*) biosensor we wished to quantitatively monitor, in real time, superoxide levels in living brain tissue. Second, using an in vitro ischemic brain slice model, we wished to demonstrate proof of concept of electrode specificity using several superoxide scavengers, including superoxide dismutase and cerium dioxide nanoparticles. Last, we wished to evaluate the role of VDACs in the transmembrane flux of superoxide in brain slices during control and ischemic conditions.

Materials and methods

Reagents and stock solutions

XOD from bovine milk (EC 1.17.3.2), Cyt *c* from horse heart, SOD, hypoxanthine (HX), 11-mercapto-1-undecanol (MU), 3-mercapto-1-propionic acid (MPA), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), *N*-hydroxysuccinimide (NHS), and nanoceria (cerium oxide (CeO_2) nanoparticles with 15-nm average diameter determined by field emission scanning electron microscopy and dynamic light scattering) were purchased from Sigma (St Louis, MO, USA) and used as received. Sodium phosphate (monobasic), sodium hydroxide, sodium phosphate (dibasic, anhydrous), potassium chloride, EDTA, and ethyl alcohol were purchased from Fisher Scientific (Springfield, NJ, USA). Sulfuric acid (95.4%) was purchased from J.T. Baker (Phillipsburg, NJ, USA). DIDS was purchased from Sigma–Aldrich, dissolved in dimethyl sulfoxide and used at a final concentration of 500 μ M. All reagents were of analytical grade and were used without further purification. All solutions were prepared using distilled, deionized water (Millipore, Billerica, MA, USA; Direct-Q system) with a resistivity of 18.2 M Ω cm.

Instrumentation

Cyclic voltammetry (CV) and amperometric experiments were carried out with a CHI electrochemical analyzer (CH Instruments, Austin, TX, USA). All experiments were carried out using a three-electrode system with a conventional cell equipped with a Ag/AgCl electrode (Ag/AgCl/3 M NaCl) as reference electrode, a platinum wire (BAS; MW-1032) as counterelectrode, and a Cyt *c* functionalized gold wire microelectrode with a protruding tip 1.5 mm long and with a diameter of 0.25 mm.

Fabrication of the Cyt *c* biosensor

Gold wires with a diameter of 0.5 mm were cleaned electrochemically in 0.1 M H_2SO_4 by cycling the potential between 0 and +1.4 V at a scan rate of 0.1 V s^{-1} until the characteristic cyclic voltammogram for gold was obtained. The cleaned Au wire electrodes were then rinsed with water and ethanol. An electrodeposited layer of gold nanoparticles was formed by applying a potential of -0.2 V for 60 s to the electrode immersed in a $HAuCl_4$ solution at a concentration of 0.01 M. Immediately after the gold deposition, the electrodes were thoroughly washed with water and ethanol and incubated for 96 h at +4 °C in an ethanolic solution containing a mixture of carboxyl- and hydroxyl-terminated thiols (1.25 mM MPA and 3.75 mM MU). The thiol-modified electrodes were rinsed with ethanol and water to remove any unattached thiol molecules. To facilitate Cyt *c* immobilization, the carboxyl groups of the surface thiols were first activated with EDC and NHS. This was performed by incubating the electrodes in an aqueous solution containing

200 mM EDC and 50 mM NHS for 30 min. Cyt *c* was covalently immobilized by incubating the thiol-modified gold wire electrode in a Cyt *c* solution at a concentration of 5×10^{-6} M for 2 h. Finally the Cyt *c*-modified electrodes were thoroughly rinsed with 0.1 M sodium phosphate buffer (PBS) solution to remove any unattached or weakly adsorbed Cyt *c*. The electrodes were stored at $+4^\circ\text{C}$ in a 0.1 M PBS solution containing 100 μM EDTA at pH 7.5 until use.

Electrochemical monitoring of the superoxide radical in mouse brain slices using the biosensor

CD-1 mice of either sex were used for these experiments. All animals used in this study were housed in the St. Lawrence University's vivarium, fed ad libitum, and kept in a normal light/dark cycle. All procedures were approved by the St. Lawrence University Animal Care and Use Committee and performed in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. Adult (2–4 months of age) mice, which have a more mature, fully developed central nervous system [30] and retain greater regional connectivity and physiological interactions [31], were sacrificed via rapid decapitation; their brains were quickly removed and placed in a chilled, choline-based slicing solution containing (in mM), 24 choline bicarbonate, 135 choline chloride, 1 kynurenic acid, 0.5 CaCl_2 , 1.4 Na_2PO_4 , 5 glucose, 1 KCl, 20 MgCl_2 , 5 mM ascorbic acid (315 mOsm) [32]. Transverse hippocampal slices 400 μm thick were cut along a rostral-to-caudal axis (-1.2 to -2.8 mm bregma) using a Leica VT1200 Vibratome (Leica Microsystems, Wetzlar, Germany) and allowed to recover for 1 h in control artificial cerebral spinal fluid containing (in mM) 124 NaCl, 3 KCl, 2.4 CaCl_2 , 1.3 MgSO_4 , 1.24 KH_2PO_4 , 26 NaHCO_3 , 5 glucose and bubbled with 5% CO_2 , 95% O_2 gas (pH 7.4, 300 mOsm). The culture medium contained 50% minimum essential medium (Hyclone Scientific, Logan, UT, USA), 25% horse serum, 25% Hanks' balanced salt solution (supplemented with 28 mM glucose, 20 mM Hepes, and 4 mM NaHCO_3), 50 U/ml penicillin, and 50 $\mu\text{g}/\text{ml}$ streptomycin, pH 7.2 [30]. Solution osmolarity was measured using a vapor pressure osmometer and corrected to 295 mOsm (Wescor, Logan, UT, USA). Hippocampal slices were placed in a culture dish and maintained in a humidified incubator (Galaxy 14S; Eppendorf New Brunswick, Cambridge, UK) at 37°C with 5% CO_2 during the experiments.

Amperometric measurements of enzymatically generated superoxide were performed in a conventional electrochemical cell, under constant stirring in air-saturated 0.1 M PBS with 100 μM EDTA at pH 7.5 or in culture medium, at an applied potential of $+0.15$ V. The current–time amperometric curves used to calibrate the Cyt *c* biosensor were generated upon addition of various XOD concentrations (ranging from 2.5 to 80 mU/ml) to the air-saturated PBS or culture medium containing 100 μM HX. All electrochemical experiments were performed at 37°C .

Electrochemical measurements for endogenously produced superoxide in hippocampal brain slices were performed by placing the recording electrode midline along the dorsal–ventral axis of the slice. The sections used for the recording were located -2.06 mm from Bregma and included the hippocampus, dentate gyrus, thalamus, and portions of the hypothalamus. All the electrochemical recordings of superoxide in brain slices with the three-electrode system were performed in the incubator. Under control conditions, the O_2 and CO_2 concentrations ($\sim 17\%$ $\text{O}_2/5\%$ CO_2) reached steady state in approximately 30 min after the door was closed. Control recordings under normoxic and normocapnic conditions lasted approximately 180 min, at which time, the response of the electrode to accumulated superoxide had reached a steady state. Electrochemical experiments with brain slices for superoxide monitoring under ischemic conditions were carried out in the same incubator at 37°C by switching gas conditions to 84% N_2 , 15% CO_2 , and 1% O_2 . In experiments that required the addition of nanoceria or the addition of DIDS, these materials were delivered directly to the culture well plate in the incubator remotely with a syringe actuator connected by polyethylene tubing.

Results

Biosensor calibration and characterization

Cyt *c* immobilization and electrochemical characterization

The detection principle of the superoxide anion radical is based on the redox reaction between the Cyt *c*, immobilized onto the surface of a gold wire electrode, and the generated superoxide [11,33]. A new combination of self-assembled monolayer of mixed thiols of MPA and MU was used in this work to covalently immobilize Cyt *c* onto the gold wire electrodes, modified with gold nanoparticles, through thiol chemistry. The immobilized Cyt

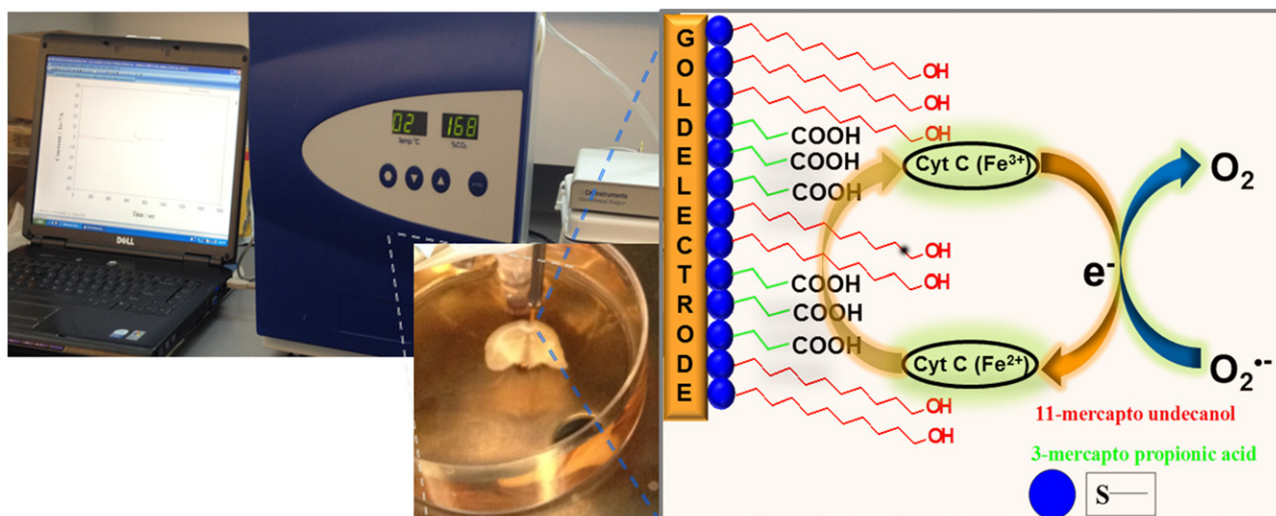


Fig. 1. Experimental setup for real-time monitoring of superoxide in brain slices. The right side shows a schematic representation of the Cyt *c* immobilized onto the gold wire electrode through the self-assembly of mixed-thiol monolayers and the redox process of Cyt *c* in the presence of superoxide.

c is reduced by superoxide and the reduced Cyt c is regenerated electrochemically at the electrode surface. The concentration of superoxide was quantified by constant potential amperometry with the electrode poised at the potential of 0.15 V vs Ag/AgCl, which corresponds to the oxidation potential of Cyt c. The electrogenerated current is proportional to the concentration of the superoxide at the electrode surface. A schematic diagram of the reaction process at the gold electrode is shown in Fig. 1. The immobilization of the Cyt c onto the gold electrode surface was confirmed by measuring the oxidation and reduction peaks of Cyt c using CV. The peak current (I_p) for both the cathodic and the anodic currents were linearly proportional to scan rate, suggesting that the electrode reaction was typical of a surface-controlled process (Supplementary Material S1). Surface coverage (Γ) of the electrode with Cyt c, calculated using the Laviron equation [34,35] by integrating the reduction peak, shows a surface concentration of $3.17 \times 10^{-11} \text{ mol cm}^{-2}$ redox active Cyt c. The low surface coverage indicates the electrochemical behavior of a thin layer in which the redox process at the electrode surface is surface confined and diffusionless.

Biosensor calibration

Biosensor calibration and optimization were carried out in standard solutions of superoxide generated by the HX/XOD system. XOD catalyzes the oxidation of HX in the presence of molecular oxygen, to uric acid and H_2O_2 , with the production of superoxide as an intermediate in this reaction [36]. The calibration curve obtained with the enzymatically generated superoxide was used to quantify the superoxide levels produced in brain slices. Because superoxide can undergo spontaneous dismutation to H_2O_2 , the steady-state sensor signal was quantified by taking into account both generation and dismutation of the enzymatically generated superoxide [37–41]. Biosensor calibration was performed using an optimum HX concentration of 100 μM . The amperometric responses of the Cyt c biosensor generated upon addition of variable amounts of XOD ranging from 2.5 to 80 mU/ml to the reaction cell containing HX were measured and correlated with the theoretically estimated superoxide concentration. The calibration curve of the biosensor in culture medium showed in Fig. 2 demonstrates the dependence of the sensor signal as a function of XOD activity as well as the theoretically estimated steady-state superoxide concentration. The sensor signal was proportional to the square root of the XOD concentration for enzymatic activities up to 80 mU/ml (Fig. 3). There was little difference between the biosensor responses to superoxide generated in PBS and in the culture medium. The linear ranges for the detection of superoxide from the calibration curves were 0–1.23 and 0–1.32 μM , with sensitivities of 11.78×10^2 and $14.45 \times 10^2 \text{ A M}^{-1} \text{ m}^{-2}$, in PBS and culture medium, respectively. The sensitivity of the Cyt c electrode is superior to that reported in literature with other sensor configurations, including a mixed long-chain thiol-based Cyt c electrode ($2.76 \times 10^2 \text{ A M}^{-1} \text{ m}^{-2}$) [41] and a mixed short-chain thiol Cyt c electrode ($0.56 \times 10^2 \text{ A M}^{-1} \text{ m}^{-2}$) [37].

The sensor response to superoxide was observed in less than 1 s after XOD injection. A steady-state limiting current value was reached in 4–5 s. The detection limit of the biosensor, calculated according to the $3\sigma_s/R'$ criteria (R' is the slope of the linear calibration curve and σ_s is the standard deviation of the amperometric signal of the blank solution), were 4.1 and 2.3 nM in PBS and culture medium, respectively. The biosensor demonstrated good functionality and high sensitivity in the culture medium. The enhanced signal may be due to a higher stability of the superoxide radical in the culture medium, whereas in PBS superoxide tends to decompose spontaneously at faster rates into H_2O_2

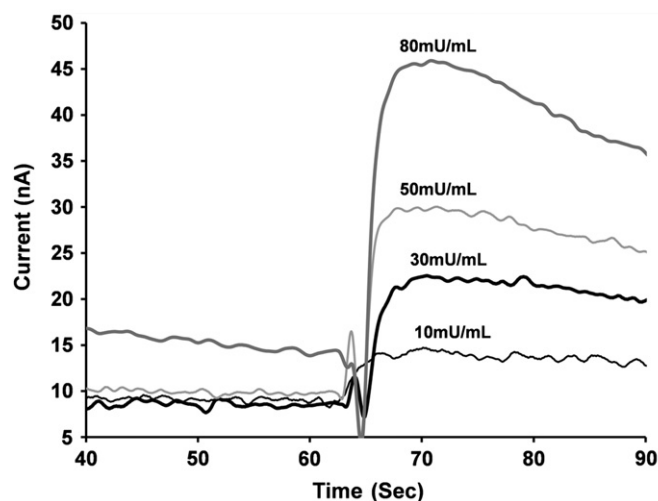


Fig. 2. Typical amperometric current–time response of the Cyt c biosensor upon addition of various concentrations of XOD in the presence of 100 μM HX. Measurements were performed in culture medium at an applied potential of 0.15 V. The current difference between the baseline and the maximum value reached at the plateau at 70 s was used to build the calibration curve of the biosensor. The decrease in the current after reaching the plateau could be due to the disproportionation of the enzymatically produced superoxide radicals into H_2O_2 .

[42]. The biosensors were stable up to 7 days with no change in response when stored in PBS at $+4^\circ\text{C}$. Cyclic voltammograms upon repeated scans showed stable redox peaks for 200 consecutive cycles, which demonstrates the stability of the biosensor (Supplementary Material S2).

Biosensor selectivity

Electrochemically active uric acid is formed as a by-product of the HX/XOD superoxide generation system used to calibrate the biosensor. Because of the short half-life of the superoxide radical, we studied the response selectivity against uric acid and the specificity of the sensor toward the superoxide radical. In the optimized configuration of mixed thiols, the biosensor showed no response when concentrations of uric acid of up to 50 μM were added into the medium (Supplementary Material S3). In addition, the sensor showed no response to H_2O_2 , which can be explained by the low applied potential of 0.15 V. To further confirm that the signal is solely associated with the produced superoxide radicals, SOD was added to inactivate the superoxide radicals. When a concentration of 10 U/ml SOD was added to a solution containing 1.04 μM superoxide (theoretical concentration), the electrochemical response was suppressed entirely until it reached the baseline signal, indicating that the superoxide radicals generated were completely inactivated by SOD (Fig. 4). The results of these studies demonstrate that the signal is specific to superoxide radicals, with no interference from the electrochemically active reaction by-products. The observed selectivity indicates that the modification of the electrode surface with the layer of densely packed thiols is effective at blocking diffusion of interfering species to the electrochemically active surface [41]. The thiol layer also reduces protein adsorption, ensuring good functionality of the sensor in the culture medium.

Real-time measurement of superoxide in hippocampal brain slice using the Cyt c biosensor

The biosensor allows continuous monitoring of superoxide radicals in the extracellular matrix with high temporal resolution, high sensitivity, and high selectivity. This was achieved by placing

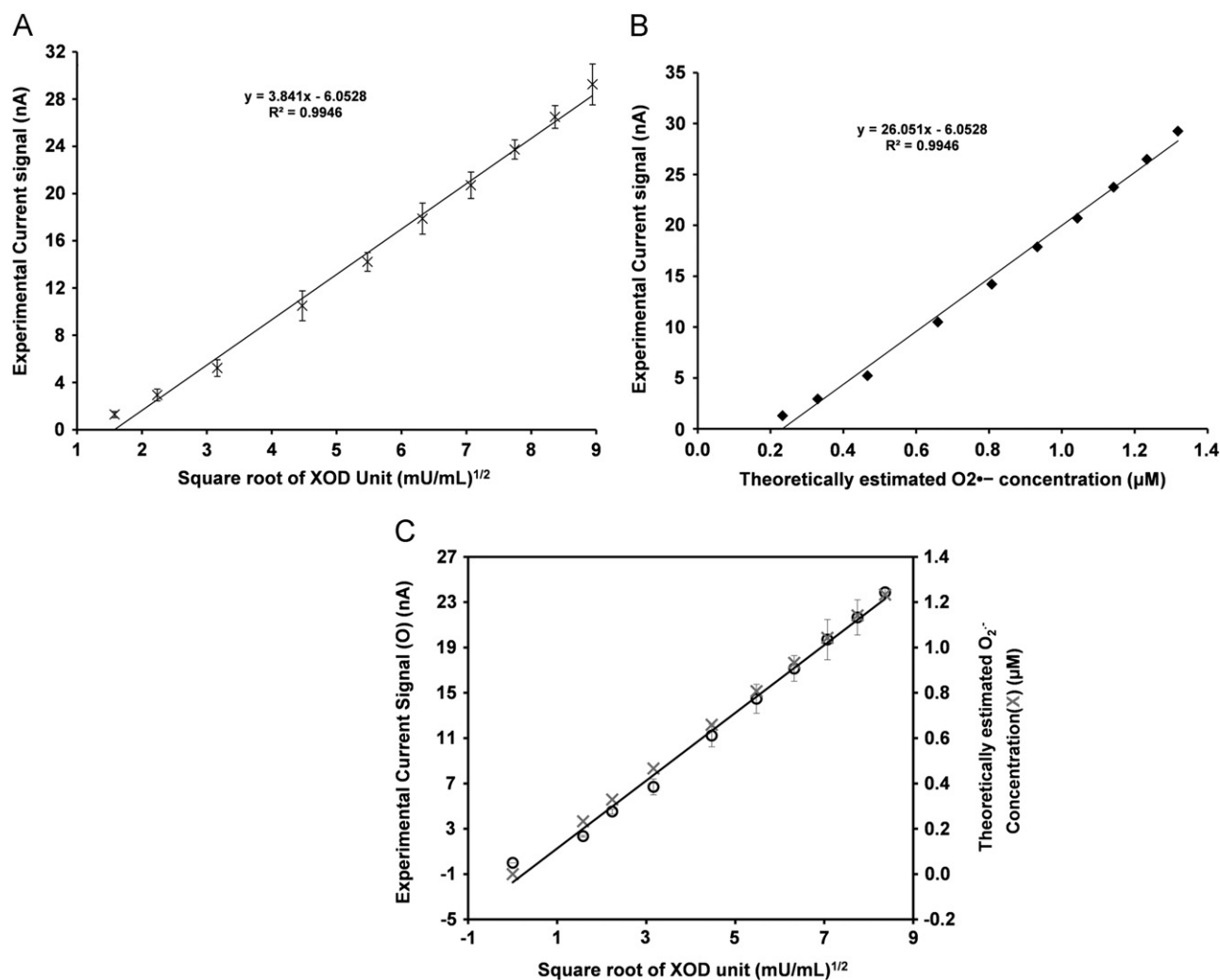


Fig. 3. Biosensor calibration using in situ enzymatically generated superoxide by the HX and XOD system. (A) Calibration and linear range of the biosensor in culture medium. (B) Dependence of the experimental sensor response on the theoretically estimated superoxide concentration in culture medium. (C) Dependence of the experimental sensor signal and the theoretically estimated steady-state superoxide concentration on XOD activity in 0.1 M PBS containing 100 μM EDTA at pH 7.5. The HX concentration was 100 μM and XOD concentrations were 2.5, 5, 10, 20, 30, 40, 50, 60, 70, and 80 mU/ml.

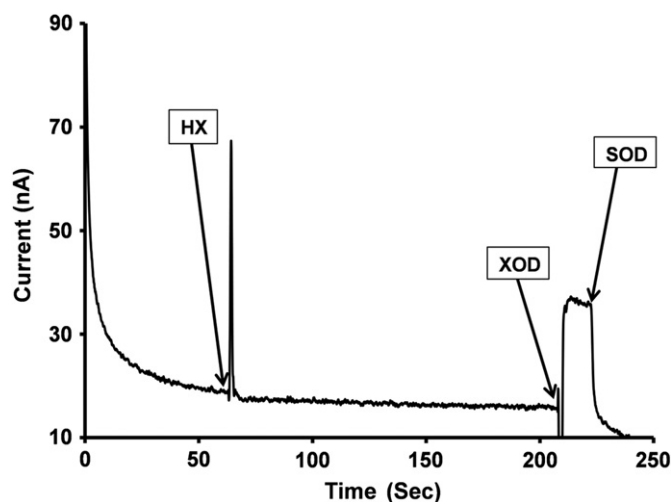


Fig. 4. In situ generation of superoxide anion radicals by addition of 100 μM HX and 50 mU/ml XOD to the reaction cell (arrows indicates the injection time) and then corresponding amperometric response upon addition of XOD. Also shown is the current drop upon addition of 10 U/ml SOD, which suppresses the enzymatically generated superoxide anion radicals.

the electrode on the rostral surface of the brain slice without penetrating the tissue. The biosensor was fabricated with an L-shape configuration to provide a horizontal surface on which to position the slice for the duration of the experiment. Fig. 5 shows the amperometric response of the Cyt c biosensor in contact with the brain slice. After the sensor reached a baseline response, the brain slice was placed onto the surface of the electrode and an immediate increase in the current was recorded. The current response stabilized and reached a maximum value in ~20–30 min after placing the slice onto the sensor surface. The sensor response corresponding to the superoxide produced by the brain slice was calculated by measuring the current difference between the maximum response reached after placing the slice onto the electrode and the background signal. The background signal was measured in the same dish under identical experimental conditions after removing the slice from the electrode surface. For quantitative analysis, the superoxide concentration in brain slices was calculated using the calibration curve obtained with the HX/XOD system in the culture medium. Under normal physiological conditions, the superoxide concentration in the extracellular matrix quantified by the biosensor was 1.95 μM. To demonstrate that the measured superoxide is originating from the brain tissue,

a control experiment was conducted under the same experimental conditions but by placing the brain slice in the recording dish ~ 1 cm away from the electrochemical active surface. When the electrode was not in direct contact with the brain tissue, the sensor did not show any amperometric response (Fig. 5; inset). This indicates that only superoxide levels that are generated when the biological tissue is in direct contact with the electrode are measured.

To further demonstrate the specificity of the signal toward the superoxide radicals originating from the extracellular space of the brain slice, we used the exogenous addition of SOD to facilitate the conversion of superoxide to hydrogen peroxide (Fig. 6). Each sequential addition of 500 U/ml SOD in the reaction cell decreased the amperometric current by approximately 14%, which suggests that the superoxide radicals were inactivated by the added SOD. Three sequential additions of 500 U/ml SOD reduced the electrode signal near to baseline levels, suggesting that the exogenously

added SOD resulted in the dismutation of the superoxide produced. The residual current in Fig. 6 can be attributed to the capacitive current associated with the brain slice. After the slice was removed from the electrode surface, the current reached a background value that is slightly higher than the initial baseline current recorded at the beginning of the experiment. This finding is probably the result of several factors, including nonspecific adsorption of proteins, the presence of residual tissue detritus on the electrode surface, or mechanical damage of the Cyt c layer produced when placing and removing the brain slice to/from the electrode surface. To account for these effects, the superoxide concentration was estimated using the residual current measured after removing the slice from the electrode surface as baseline value.

Evaluation of transmembrane flux of superoxide in brain slices through VDACS

To examine the potential role of the transmembrane flux of superoxide into the extracellular space, DIDS (0.5 mM final concentration for each addition; 2 mM total) was added to the culture medium during the recordings. Previous studies have shown that superoxide can penetrate a variety of biological membranes through the VDACS, which are inhibited with DIDS [27,28]. The mechanism by which DIDS inhibits anion channels is not known, although the inhibition seems to occur in the extracellular space and is reversible in some systems. Under control conditions, the addition of DIDS to the culture medium resulted in a decrease in the superoxide signal (Fig. 7) with each subsequent addition. Because it was not possible to stir the solution during the experiment while recording, it is likely the DIDS concentration was not uniform throughout the solution. In separate experiments, the addition of DIDS alone had no effect on sensor output.

Real-time continuous monitoring of superoxide dynamics during ischemia

Immediately after the transition to hypoxic/hypercapnic gas mixtures in the incubator, superoxide levels began to increase and reached a steady state after ~ 180 min (Fig. 8). Compared to the normoxic superoxide levels, peak superoxide levels increased approximately twofold during ischemia. Based on our calculations this resulted in an average increase in superoxide from 1.95 to 4.1 μM . In contrast to our findings during normoxia, the

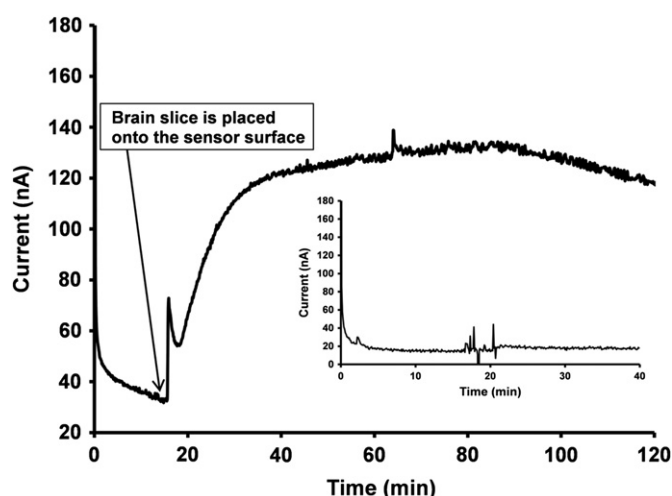


Fig. 5. Real-time amperometric response of the Cyt c biosensor in contact with the hippocampal brain slice. The increase in the current indicates the release of extracellular superoxide radicals from the brain slice under normal physiological conditions. The inset shows a control experiment with the brain slice placed in the measurement cell but far away from the electrode surface.

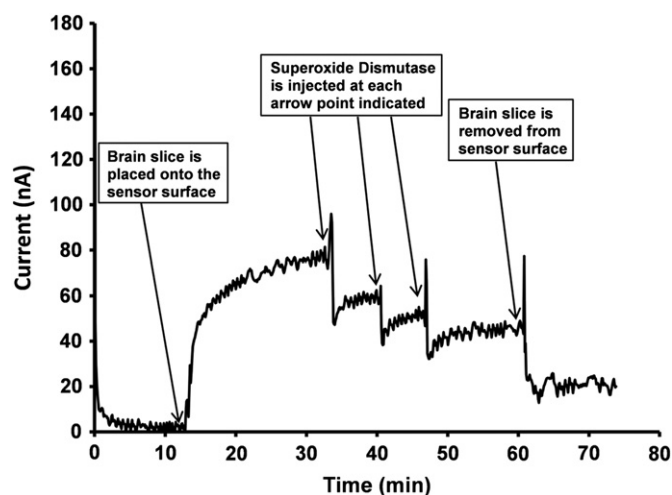


Fig. 6. Specificity of the biosensor response toward superoxide released by the hippocampal brain slice under normal physiological conditions. With each addition of 500 U/ml SOD there is a decrease in the amperometric response, indicating specific inactivation of the produced superoxide radicals. The arrows indicate the times of SOD addition and the exposure of the brain to/from the electrode surface.

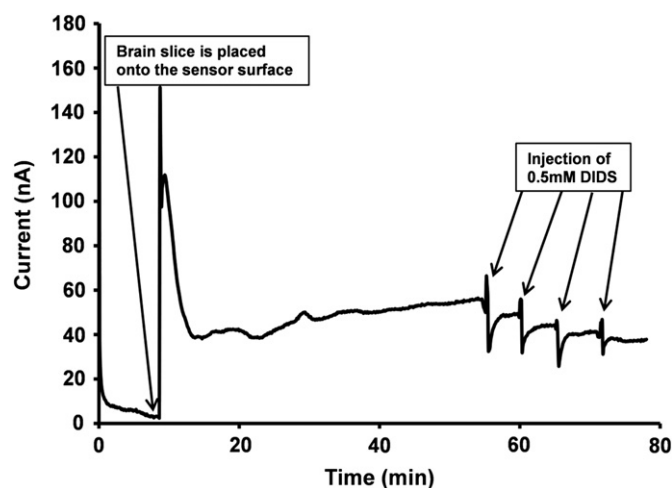


Fig. 7. Effects of four consecutive additions of DIDS (0.5 mM final concentration in the reaction cell) on the amperometric response of the Cyt c biosensor. The arrows indicate the times of DIDS addition.

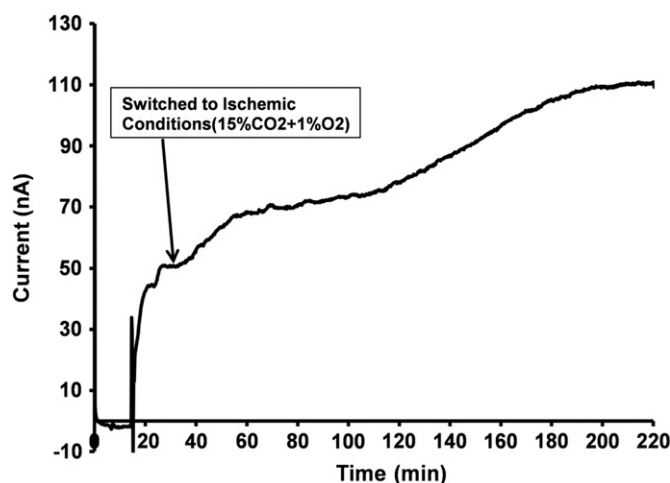


Fig. 8. Typical current–time response of Cyt *c* biosensor showing continuous monitoring of superoxide in mouse hippocampal brain slice exposed to simulated ischemic conditions. The arrow indicates the time when the brain slice was switched to ischemic insult by exposing the slice to hypoglycemic, acidic, and hypoxic conditions (glucose was lowered to 2 mM and the cell was bubbled with 15% CO₂ and 1% oxygen).

sequential addition of DIDS had no effect on the extracellular levels of superoxide.

Assessing antioxidant activity of cerium oxide nanoparticles in ischemic brain slices

In this study, we used the Cyt *c* biosensor to quantitatively assess the time-course inactivation of extracellular superoxide by nanoceria. Previous work from our lab showed that nanoceria is capable of scavenging a variety of free radicals, including nitric oxide, superoxide, and peroxynitrite, in brain slices [32] and the catalytic activity of nanoceria reduces superoxide to H₂O₂. The addition of nanoceria with an average particle size of 15 nm induced an immediate decrease in the superoxide signal under control conditions, confirming the neutralizing activity of these nanoparticles against superoxide (Fig. 9). Consecutive addition of 1 µg/ml nanoceria decreased the superoxide levels by $14.5 \pm 4.5\%$ ($n=5$) per addition. After two consecutive nanoparticle additions, the superoxide levels remained stable for the remainder of the experiment. In contrast, the addition of the same amount of nanoceria during ischemia decreased the superoxide signal by $8.1 \pm 2.2\%$ ($n=3$) per addition. Moreover, after two sequential nanoparticle additions, the superoxide signal decreased progressively toward baseline for the remainder of the experiment. Supplementary Material S4 shows the decrease in the extracellular superoxide concentration for both SOD and nanoceria. Based on these findings, a 1 µg/ml nanoceria concentration induced a decrease in the superoxide level under normoxic conditions equivalent to that of 527 SOD activity units, as calculated from the amperometric responses of the biosensor in brain slices.

Discussion

This study demonstrates the potential of electrochemical microbiosensors for studying the release, accumulation, and mobility of the superoxide radical, and potentially of other oxidative stress markers, in brain tissue with high spatial and temporal resolution and can facilitate our fundamental understanding of the neurophysiology of oxidative-stress-related diseases.

Current methods for monitoring the superoxide anion radical in hippocampal brain slices include colorimetric and fluorescence assays that measure intracellular superoxide [43]. A drawback of these methods is the difficulty of obtaining a continuous, quantitative, real-time measurement of superoxide production in biological tissue. Most often researchers interested in assessing oxidative damage rely on downstream markers such as lipid and protein oxidation [44–46]. Because of their small size (micrometer to submicrometer diameter) electrochemical sensors can provide real-time measurements of ROS and RNS species [47]. The microbiosensor used in this work consists of a gold wire electrode modified with mixed layers of thiols and immobilized Cyt *c* as a biorecognition element [33]. Electrochemical sensors for the detection of superoxide have been reported in the literature [48] but these have seldom been used to measure real-time production of superoxide in intact biological tissues. Most studies have relied on standard solutions of enzymatically produced superoxide, with few examples using cell cultures. Rarely have the studies demonstrated the applicability of similar devices to measure extracellular superoxide in biological tissues [49–51]. There are no reports on the use of electrochemical microbiosensors for studying superoxide mobility across membranes and their application for studying oxidant and antioxidant mechanisms of natural or synthetic antioxidants in the brain. Our ability to measure superoxide in such a small volume of tissue in this

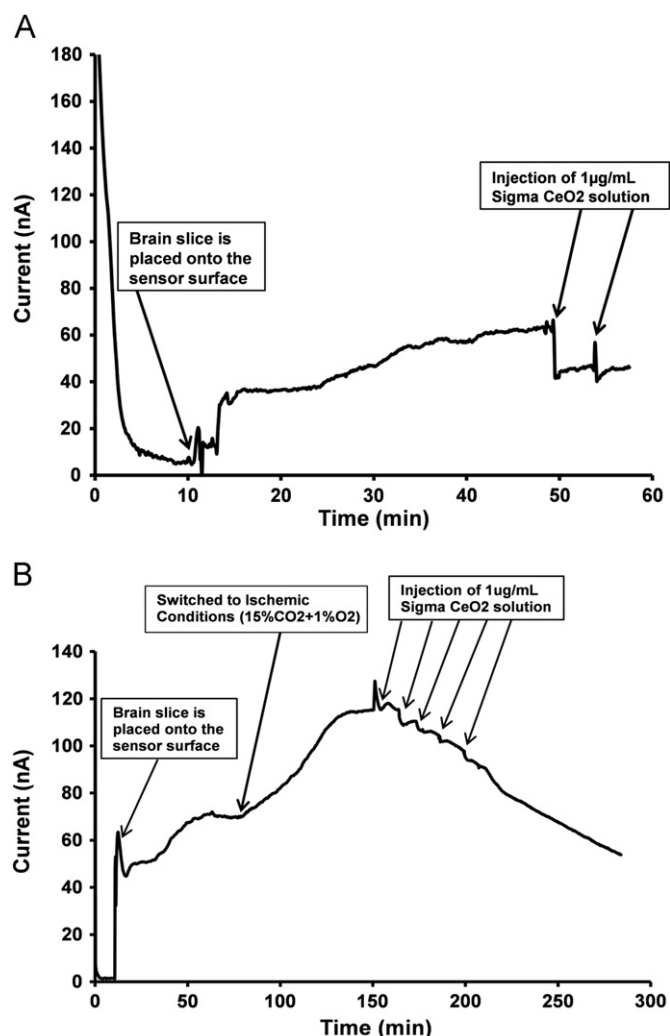


Fig. 9. Effects of nanoceria (successive additions of 1 mg/ml to the reaction cell) on the superoxide level quantified by the Cyt *c* biosensor under (A) physiological and (B) simulated ischemic conditions.

study suggests that these electrodes should be equally sensitive in vivo.

To explore the potential of this technology, we examined the nature of the superoxide signal in the extracellular space of the brain slice. There is increasing evidence that free radicals play a central role in redox signaling both in health and in disease [9]. Madesh et al. [52] first suggested that extracellular superoxide produced by plasmalemmal NADPH oxidase or superoxide crossing the plasma membrane through a VDAC-like channel modified cell surface proteins to mediate cell signaling involved in programmed cell death. Recent proteomic studies have confirmed the presence of VDACs in the plasma membrane of human cells [53–55], including cultured septal and hippocampal neuronal cells and in membrane preparations from human brain cortex [56]. Although preliminary, this study is the first report of DIDS eliminating the superoxide signal in intact brain tissue. The DIDS-sensitive accumulation of superoxide under control conditions suggests that the transmembrane flux of superoxide occurred through a VDAC-like channel and was not simply an artifact of compromised cell membranes associated with the sectioning process. The VDAC is a highly conserved protein in terms of both its structural and its functional features across species [57]. It has been hypothesized that VDACs perform a variety of functions, including cellular ATP release, maxi-anion channel, volume control, a component of the GABAA receptor, plasma membrane NADH oxidoreductase, and modulation of programmed cell death. Our observation that extracellular superoxide concentrations were unaffected by DIDS during ischemia suggests that VDACs may either become insensitive to the effects of DIDS or close during the ischemic period. Consistent with the latter notion, previous studies have shown that NADH levels increase during the period of ischemia [58–60] and elevated cytosolic NADH concentrations promote the closure of VDACs and apoptotic signals [53,61,62]. The fact that extracellular superoxide is elevated during the ischemic period even with closure of VDACs suggests that there are alternative pathways for transmembrane flux of superoxide or there may be activation of plasmalemmal NADPH oxidase. Additional experiments will be needed to explore these questions but this study highlights the importance of this technology in the study of the biological effects of free radicals.

Previous work from our lab [63–65] and others [66–75] has demonstrated the ability of ceria to neutralize a variety of biologically relevant free radicals. The 3+/4+ valence of nanoceria allows the particles to participate in reversible redox reactions similar to endogenous redox enzymes in cells. Given its high redox potential (1.55 V), ceria can accept electrons from hydroxyl, superoxide, and peroxyxynitrite in the +4 state and donate electrons to H₂O₂ in the +3 state. Our data acquired with the electrochemical Cyt c micro-sensor show that the catalytic activity for ceria is remarkably high with respect to superoxide dismutase; 1 µg of ceria was equivalent to ~527 U of SOD in neutralizing superoxide. The progressive decrease in superoxide accumulation after repeated administration of ceria during ischemia is probably due to the cumulative uptake and intracellular scavenging of superoxide by the nanoparticles. We have previously shown that particles of nanoceria are endocytosed within 1 h and they localize to mitochondria, neurofilaments, and lipid/myelin membranes [63]. Given its regenerative, catalytic nature, nanoceria has become attractive as a potential pharmacological agent and potent antioxidant [66–74]. This being said it has become increasingly clear that the contradictory biological effects of nanoceria (see Yokel and others) [76–79] reported in the literature stem from differences in the physical (i.e., size, ζ potential) and chemical characteristics (presence of dopants or stabilizers) of the particles. Modest changes in the synthetic identity of the particles can confer very different biological outcomes. We have recently explored the therapeutic potential of nanoceria in a murine model of

multiple sclerosis [80] using both custom-synthesized and commercially available nanoceria. We found that the biological effects and deposition of the particles in the brain were dependent on multiple physical/chemical characteristics. Exploring the factors that contribute to the biological action of nanoceria will be an important area of research, as the therapeutic potential of these compounds will be developed in the future.

Conclusions

This work demonstrates the ability of electrochemical biosensors to study real-time dynamics of reactive oxygen species in a biological model. The electrochemical measurements provided evidence of transmembrane mobility and extracellular superoxide release under normal physiological and simulated ischemic conditions in a brain slice model of ischemia. Pharmacological manipulations of VDACs, which facilitate transport of mitochondrial superoxide to the extracellular space, demonstrate the mobility of superoxide measured electrochemically. The dynamics of superoxide overproduction during ischemic brain injury was quantified over time and the specificity of signal was validated using SOD as a model endogenous antioxidant. The potential of this technology for studying the time course of inactivation and providing quantitative assessment of the superoxide scavenging capacity by antioxidant compounds has been demonstrated with an emerging engineered antioxidant, nanoceria, which holds potential for treatment and therapy of oxidative diseases. In summary, this study demonstrates the potential of electrochemical sensors for providing quantitative assessment of real-time changes in extracellular ROS and studying fundamental mechanisms to understand ROS-mediated processes under normal physiological and pathophysiological conditions involved in disease progression and therapy.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.freeradbiomed.2012.10.540>.

References

- [1] Winterbourn, C. C.; Hampton, M. B. Thiol chemistry and specificity in redox signaling. *Free Radic. Biol. Med.* **45**:549–561; 2008.
- [2] Paulsen, C. E.; Carroll, K. S. Orchestrating redox signaling networks through regulatory cysteine switches. *ACS Chem. Biol.* **5**:47–62; 2010.
- [3] Robinson, B. H. The role of manganese superoxide dismutase in health and disease. *J. Inherit. Metab. Dis.* **21**:598–603; 1998.
- [4] Dickinson, B. C.; Peltier, J.; Stone, D.; Schaffer, D. V.; Chang, C. J. Nox2 redox signaling maintains essential cell populations in the brain. *Nat. Chem. Biol.* **7**:106–112; 2011.
- [5] Daiber, A.; Frein, D.; Namgaladze, D.; Ullrich, V. Oxidation and nitrosation in the nitrogen monoxide/superoxide system. *J. Biol. Chem.* **277**:11882–11888; 2002.
- [6] Daiber, A.; Bachschmid, M. Enzyme inhibition by peroxyxynitrite-mediated tyrosine nitration and thiol oxidation. *Curr. Enzyme Inhibit* **3**:103–117; 2007.
- [7] Kuroda, J.; Ago, T.; Matsushima, S.; Zhai, P. Y.; Schneider, M. D.; Sadoshima, J. NADPH oxidase 4 (Nox4) is a major source of oxidative stress in the failing heart. *Proc. Natl. Acad. Sci. USA* **107**:15565–15570; 2010.
- [8] Forstermann, U. Nitric oxide and oxidative stress in vascular disease. *Pflugers Arch. Eur. J. Physiol* **459**:923–939; 2010.
- [9] Zweier, J. L.; Talukder, M. A. H. The role of oxidants and free radicals in reperfusion injury. *Cardiovasc. Res.* **70**:181–190; 2006.

- [10] Salvemini, D.; Cuzzocrea, S. Oxidative stress in septic shock and disseminated intravascular coagulation. *Free Radic. Biol. Med.* **33**:1173–1185; 2002.
- [11] Arnold, S.; Feng, Z. Q.; Kakiuchi, T.; Knoll, W.; Niki, K. Investigation of the electrode reaction of cytochrome c through mixed self-assembled monolayers of alkanethiols on gold(111) surfaces. *J. Electroanal. Chem.* **438**:91–97; 1997.
- [12] Siemionow, M.; Arslan, E. Ischemia/reperfusion injury: a review in relation to free tissue transfers. *Microsurgery* **24**:468–475; 2004.
- [13] Chrissobolis, S.; Faraci, F. M. The role of oxidative stress and NADPH oxidase in cerebrovascular disease. *Trends Mol. Med.* **14**:495–502; 2008.
- [14] Matsuda, S.; Umeda, M.; Uchida, H.; Kato, H.; Araki, T. Alterations of oxidative stress markers and apoptosis markers in the striatum after transient focal cerebral ischemia in rats. *J. Neural Transm.* **116**:395–404; 2009.
- [15] Oliver, C. N.; Starke-Reed, P. E.; Stadtman, E. R.; Liu, G. J.; Carney, J. M.; Floyd, R. A. Oxidative damage to brain proteins, loss of glutamine synthetase activity, and production of free radicals during ischemia/reperfusion-induced injury to gerbil brain. *Proc. Natl. Acad. Sci. USA* **87**:5144–5147; 1990.
- [16] Floyd, R. A.; Carney, J. M. Free radical damage to protein and DNA: mechanisms involved and relevant observations on brain undergoing oxidative stress. *Ann. Neurol.* **32**(Suppl):S22–S27; 1992.
- [17] Chen, H.; Song, Y. S.; Chan, P. H. Inhibition of NADPH oxidase is neuroprotective after ischemia–reperfusion. *J. Cereb. Blood Flow Metab.* **29**:1262–1272; 2009.
- [18] Ono, T.; Tsuruta, R.; Fujita, M.; Aki, H. S.; Kutsuna, S.; Kawamura, Y.; Wakatsuki, J.; Aoki, T.; Kobayashi, C.; Kasaoka, S.; Maruyama, I.; Yuasa, M.; Maekawa, T. Xanthine oxidase is one of the major sources of superoxide anion radicals in blood after reperfusion in rats with forebrain ischemia/reperfusion. *Brain Res.* **1305**:158–167; 2009.
- [19] Lynch, R. E.; Fridovich, I. Permeation of the erythrocyte stroma by superoxide radical. *J. Biol. Chem.* **253**:4697–4699; 1978.
- [20] Lisanti, M. P.; Scherer, P. E.; Tang, Z.; Sargiacomo, M. Caveolae, caveolin and caveolin-rich membrane domains: a signalling hypothesis. *Trends Cell Biol.* **4**:231–235; 1994.
- [21] Baker, M. A.; Lane, D. J. R.; Ly, J. D.; De Pinto, V. Lawen, A. VDAC1 is a transplasma membrane NADH-ferricyanide reductase. *J. Biol. Chem.* **279**:4811–4819; 2004.
- [22] Darbandi-Tonkabon, R.; Hastings, W. R.; Zeng, C. M.; Akk, G.; Manion, B. D.; Bracamontes, J. R.; Steinbach, J. H.; Mennerick, S. J.; Covey, D. F.; Evers, A. S. Photoaffinity labeling with a neuroactive steroid analogue—6-AZI-pregnanolone labels voltage-dependent anion channel-1 in rat brain. *J. Biol. Chem.* **278**:13196–13206; 2003.
- [23] Blatz, A. L.; Magleby, K. L. Single voltage-dependent chloride-selective channels of large conductance in cultured rat muscle. *Biophys. J.* **43**:237–241; 1983.
- [24] Bahamonde, M. I.; Fernandez-Fernandez, J. M.; Guix, F. X.; Vazquez, E.; Valverde, M. A. Plasma membrane voltage-dependent anion channel mediates antiestrogen-activated maxi Cl[−] currents in C1300 neuroblastoma cells. *J. Biol. Chem.* **278**:33284–33289; 2003.
- [25] Mao, G. D.; Poznansky, M. J. Electron spin resonance study on the permeability of superoxide radicals in lipid bilayers and biological membranes. *FEBS Lett* **305**:233–236; 1992.
- [26] Ikebuchi, Y.; Masumoto, N.; Tasaka, K.; Koike, K.; Kasahara, K.; Miyake, A.; Tanizawa, O. Superoxide anion increases intracellular pH, intracellular free calcium, and arachidonate release in human amnion cells. *J. Biol. Chem.* **266**:13233–13237; 1991.
- [27] 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.* **278**:5557–5563; 2003.
- [28] Mumbengegwi, D. R.; Li, Q.; Li, C.; Bear, C. E.; Engelhardt, J. F. Evidence for a superoxide permeability pathway in endosomal membranes. *Mol. Cell. Biol.* **28**:3700–3712; 2008.
- [29] Kalyanaraman, B.; Darley-Usmar, V.; Davies, K. J. A.; Dennery, P. A.; Forman, H. J.; Grisham, M. B.; Mann, G. E.; Moore, K.; Roberts, L. J.; Ischiropoulos, H. Measuring reactive oxygen and nitrogen species with fluorescent probes: challenges and limitations. *Free Radic. Biol. Med.* **52**:1–6; 2012.
- [30] Njagi, J.; Erlichman, J. S.; Aston, J. W.; Leiter, J. C.; Andreescu, S. A sensitive electrochemical sensor based on chitosan and electropolymerized Meldola blue for monitoring NO in brain slices. *Sens. Actuators B* **143**:673–680; 2010.
- [31] Sullivan, B. L.; Leu, D.; Taylor, D. M.; Fahlman, C. S.; Bickler, P. E. Isoflurane prevents delayed cell death in an organotypic slice culture model of cerebral ischemia. *Anesthesiology* **96**:189–195; 2002.
- [32] Erlichman, J. S.; Hewitt, A.; Damon, T. L.; Hart, M.; Kurascz, J.; Li, A.; Leiter, J. C. Inhibition of monocarboxylate transporter 2 in the retrotapezoid nucleus in rats: a test of the astrocyte–neuron lactate-shuttle hypothesis. *J. Neurosci.* **28**:4888–4896; 2008.
- [33] Sato, Y.; Mizutani, F. Electrochemical responses of cytochrome c on a gold electrode modified with mixed monolayers of 3-mercaptopropionic acid and n-alkanethiol. *J. Electroanal. Chem.* **438**:99–104; 1997.
- [34] Laviron, E. Adsorption, autoinhibition and autocatalysis in polarography and in linear potential sweep voltammetry. *J. Electroanal. Chem. Interfacial Electrochem* **52**:355–393; 1974.
- [35] Laviron, E. General expression of the linear potential sweep voltammogram in the case of diffusionless electrochemical systems. *J. Electroanal. Chem. Interfacial Electrochem* **101**:19–28; 1979.
- [36] Fujita, M.; Tsuruta, R.; Kasaoka, S.; Fujimoto, K.; Tanaka, R.; Oda, Y.; Nanba, M.; Igarashi, M.; Yuasa, M.; Yoshikawa, T.; Maekawa, T. In vivo real-time measurement of superoxide anion radical with a novel electrochemical sensor. *Free Radic. Biol. Med.* **47**:1039–1048; 2009.
- [37] Tammeveski, K.; Tenno, T. T.; Mashirin, A. A.; Hillhouse, E. W.; Manning, P.; McNeil, C. J. Superoxide electrode based on covalently immobilized cytochrome c: modelling studies. *Free Radic. Biol. Med.* **25**:973–978; 1998.
- [38] McCord, J. M.; Fridovich, I. The reduction of cytochrome c by milk xanthine oxidase. *J. Biol. Chem.* **243**:5753–5760; 1968.
- [39] Behar, D.; Czapski, G.; Rabani, J.; Dorfman, L. M.; Schwarz, H. A. Acid dissociation constant and decay kinetics of the perhydroxyl radical. *J. Phys. Chem.* **74**:3209–3213; 1970.
- [40] Chen, X. J.; West, A. C.; Cropek, D. M.; Banta, S. Detection of the superoxide radical anion using various alkanethiol monolayers and immobilized cytochrome c. *Anal. Chem.* **80**:9622–9629; 2008.
- [41] Ge, B.; Lisdat, F. Superoxide sensor based on cytochrome c immobilized on mixed-thiol SAM with a new calibration method. *Anal. Chim. Acta* **454**:53–64; 2002.
- [42] Gobi, K. V.; Mizutani, F. Efficient mediatorless superoxide sensors using cytochrome c-modified electrodes: surface nano-organization for selectivity and controlled peroxidase activity. *J. Electroanal. Chem.* **484**:172–181; 2000.
- [43] Bindokas, V. P.; Jordan, J.; Lee, C. C.; Miller, R. J. Superoxide production in rat hippocampal neurons: selective imaging with hydroethidine. *J. Neurosci.* **16**:1324–1336; 1996.
- [44] Berlett, B. S.; Stadtman, E. R. Protein oxidation in aging, disease, and oxidative stress. *J. Biol. Chem.* **272**:20313–20316; 1997.
- [45] Schopfer, F. J.; Batthyany, C.; Baker, P. R.; Bonacci, G.; Cole, M. P.; Rudolph, V.; Groeger, A. L.; Rudolph, T. K.; Nadtchiy, S.; Brookes, P. S.; Freeman, B. A. Detection and quantification of protein adduction by electrophilic fatty acids: mitochondrial generation of fatty acid nitroalkene derivatives. *Free Radic. Biol. Med.* **46**:1250–1259; 2009.
- [46] Kochanek, P. M.; Berger, R. P.; Bayir, H.; Wagner, A. K.; Jenkins, L. W.; Clark, R. S. Biomarkers of primary and evolving damage in traumatic and ischemic brain injury: diagnosis, prognosis, probing mechanisms, and therapeutic decision making. *Curr. Opin. Crit. Care* **14**:135–141; 2008.
- [47] Wilson, G. S.; Johnson, M. A. In-vivo electrochemistry: what can we learn about living systems? *Chem. Rev.* **108**:2462–2481; 2008.
- [48] Mesároš, Š.; Vaňková, Ž.; Grunfeld, S.; Mesárošová, A.; Malinski, T. Preparation and optimization of superoxide microbiosensor. *Anal. Chim. Acta* **358**:27–33; 1998.
- [49] Buttemeyer, R.; Philipp, A. W.; Mall, J. W.; Ge, B. X.; Scheller, F. W.; Lisdat, F. In vivo measurement of oxygen-derived free radicals during reperfusion injury. *Microsurgery* **22**:108–113; 2002.
- [50] Fabian, R. H.; Dewitt, D. S.; Kent, T. A. In-vivo detection of superoxide anion production by the brain using a cytochrome-c electrode. *J. Cereb. Blood Flow Metab.* **15**:242–247; 1995.
- [51] Scheller, W.; Jin, W.; Ehrentreich-Forster, E.; Ge, B.; Lisdat, F.; Buttemeyer, R.; Wollenberger, U.; Scheller, F. W. Cytochrome c based superoxide sensor for in vivo application. *Electroanalysis* **11**:703–706; 1999.
- [52] Madesh, M.; Hawkins, B. J.; Milovanova, T.; Bhanumathy, C. D.; Joseph, S. K.; Ramachandrarao, S. P.; Sharma, K.; Kurosaki, T.; Fisher, A. B. Selective role for superoxide in InsP3 receptor-mediated mitochondrial dysfunction and endothelial apoptosis. *J. Cell Biol.* **170**:1079–1090; 2005.
- [53] Lisanti, M. P.; Scherer, P. E.; Vidugiriene, J.; Tang, Z. L.; Hermanowskivosatka, A.; Tu, Y. H.; Cook, R. F.; Sargiacomo, M. Characterization of caveolin-rich membrane domains isolated from an endothelial-rich source—implications for human-disease. *J. Cell Biol.* **126**:111–126; 1994.
- [54] Stockwin, L. H.; Blonder, J.; Bumke, M. A.; Lucas, D. A.; Chan, K. C.; Conrads, T. P.; Issaq, H. J.; Veenstra, T. D.; Newton, D. L.; Rybak, S. M. Proteomic analysis of plasma membrane from hypoxia-adapted malignant melanoma. *J. Proteome Res.* **5**:2996–3007; 2006.
- [55] Schindler, J.; Lewandrowski, U.; Sickmann, A.; Friauf, E.; Nothwang, H. G. Proteomic analysis of brain plasma membranes isolated by affinity two-phase partitioning. *Mol. Cell. Proteomics* **5**:390–400; 2006.
- [56] Ramirez, C. M.; Gonzalez, M.; Diaz, M.; Alonso, R.; Ferrer, I.; Santpere, G.; Puig, B.; Meyer, G.; Marin, R. VDAC and ERalpha interaction in caveolae from human cortex is altered in Alzheimer's disease. *Mol. Cell. Neurosci.* **42**:172–183; 2009.
- [57] Shoshan-Barmatz, V.; Israelson, A.; Brdiczka, D.; Sheu, S. S. The voltage-dependent anion channel (VDAC): function in intracellular signalling, cell life and cell death. *Curr. Pharm. Des.* **12**:2249–2270; 2006.
- [58] Perez-Pinzon, M. A.; Mumford, P. L.; Rosenthal, M.; Sick, T. J. Antioxidants, mitochondrial hyperoxidation and electrical recovery after anoxia in hippocampal slices. *Brain Res.* **754**:163–170; 1997.
- [59] Perez-Pinzon, M. A.; Mumford, P. L.; Carranza, V.; Sick, T. J. Calcium influx from the extracellular space promotes NADH hyperoxidation and electrical dysfunction after anoxia in hippocampal slices. *J. Cereb. Blood Flow Metab.* **18**:215–221; 1998.
- [60] Foster, K. A.; Margraf, R. R.; Turner, D. A. NADH hyperoxidation correlates with enhanced susceptibility of aged rats to hypoxia. *Neurobiol. Aging* **29**:598–613; 2008.
- [61] Rostovtseva, T. K.; Bezrukov, S. M. VDAC regulation: role of cytosolic proteins and mitochondrial lipids. *J. Bioenerg. Biomembr.* **40**:163–170; 2008.
- [62] Zizi, M.; Forte, M.; Blachly-Dyson, E.; Colombini, M. NADH regulates the gating of VDAC, the mitochondrial outer membrane channel. *J. Biol. Chem.* **269**:1614–1616; 1994.
- [63] Estevez, A. Y.; Pritchard, S.; Harper, K.; Aston, J. W.; Lynch, A.; Lucky, J. J.; Ludington, J. S.; Chatani, P.; Mosenthal, W. P.; Leiter, J. C.; Andreescu, S.; Erlichman, J. S. Neuroprotective mechanisms of cerium oxide nanoparticles

- in a mouse hippocampal brain slice model of ischemia. *Free Radic. Biol. Med.* **51**:1155–1163; 2011.
- [64] Estevez, A.; Erlichman, J. S. Cerium oxide nanoparticles for the treatment of neurological oxidative stress diseases. In *Oxidative Stress: Diagnostics, Prevention, and Ther.* London: Oxford Univ. Press; 2012:255–288.
- [65] Andreescu, S.; Ornatska, M.; Erlichman, J. S.; Estevez, A.; Leiter, J. C. Biomedical applications of metal oxide nanoparticles. In: Matijavic, E., editor. *Fine Particles in Medicine and Pharmacy*. New York: Springer; 2011.
- [66] Chen, J.; Patil, S.; Seal, S.; McGinnis, J. F. Rare earth nanoparticles prevent retinal degeneration induced by intracellular peroxides. *Nat. Nanotechnol* **1**:142–150; 2006.
- [67] Das, M.; Patil, S.; Bhargava, N.; Kang, J. F.; Riedel, L. M.; Seal, S.; Hickman, J. J. Auto-catalytic ceria nanoparticles offer neuroprotection to adult rat spinal cord neurons. *Biomaterials* **28**:1918–1925; 2007.
- [68] D'Angelo, B.; Santucci, S.; Benedetti, E. Di Loreto, S.; Phani, R. A.; Falone, S.; Amicarelli, F.; Ceru, M. P.; Cimini, A. Cerium oxide nanoparticles trigger neuronal survival in a human Alzheimer disease model by modulating BDNF pathway. *Curr. Nanosci.* **5**:167–176; 2009.
- [69] Hirst, S. M.; Karakoti, A.; Singh, S.; Self, W.; Tyler, R.; Seal, S.; Reilly, C. M. Bio-distribution and in vivo antioxidant effects of cerium oxide nanoparticles in mice. *Environ. Toxicol.* ; 2011. in press.
- [70] Hirst, S. M.; Karakoti, A. S.; Tyler, R. D.; Sriranganathan, N.; Seal, S.; Reilly, C. M. Anti-inflammatory properties of cerium oxide nanoparticles. *Small* **5**:2848–2856; 2009.
- [71] Celardo, I.; Pedersen, J. Z.; Traversa, E.; Ghibelli, L. Pharmacological potential of cerium oxide nanoparticles. *Nanoscale* **3**:1411–1420; 2011.
- [72] Colon, J.; Hsieh, N.; Ferguson, A.; Kupelian, P.; Seal, S.; Jenkins, D. W.; Baker, C. H. Cerium oxide nanoparticles protect gastrointestinal epithelium from radiation-induced damage by reduction of reactive oxygen species and upregulation of superoxide dismutase 2. *Nanomed.-Nanotechnol* **6**:698–705; 2010.
- [73] Niu, J.; Azfer, A.; Rogers, L. M.; Wang, X.; Kolattukudy, P. E. Cardioprotective effects of cerium oxide nanoparticles in a transgenic murine model of cardiomyopathy. *Cardiovasc. Res.* **73**:549–559; 2007.
- [74] Tarnuzzer, R. W.; Colon, J.; Patil, S.; Seal, S. Vacancy engineered ceria nanostructures for protection from radiation-induced cellular damage. *Nano Lett.* **5**:2573–2577; 2005.
- [75] Niu, J. L.; Wang, K. K.; Kolattukudy, P. E. Cerium oxide nanoparticles inhibit oxidative stress and nuclear factor-kappa B activation in H9c2 cardiomyocytes exposed to cigarette smoke extract. *J. Pharmacol. Exp. Ther.* **338**:53–61; 2011.
- [76] Hardas, S. S.; Butterfield, D. A. Sultana, R.; Tseng, M. T.; Dan, M.; Florence, R. L.; Unrine, J. M.; Graham, U. M.; Wu, P.; Grulke, E. A.; Yokel, R. A. Brain distribution and toxicological evaluation of a systemically delivered engineered nanoscale ceria. *Toxicol. Sci.* **116**:562–576; 2010.
- [77] Yokel, R. A. Florence, R. L.; Unrine, J. M.; Tseng, M. T.; Graham, U. M.; Wu, P.; Grulke, E. A.; Sultana, R.; Hardas, S. S.; Butterfield, D. A. Biodistribution and oxidative stress effects of a systemically-introduced commercial ceria engineered nanomaterial. *Nanotoxicology* **3**:234–248; 2009.
- [78] Park, E. J.; Choi, J.; Park, Y. K.; Park, K. Oxidative stress induced by cerium oxide nanoparticles in cultured BEAS-2B cells. *Toxicology* **245**:90–100; 2008.
- [79] Auffan, M.; Rose, J.; Orsiere, T.; De Meo, M.; Thill, A.; Zeyons, O.; Proux, O.; Masion, A.; Chaurand, P.; Spalla, O.; Botta, A.; Wiesner, M. R.; Bottero, J. Y. CeO₂ nanoparticles induce DNA damage towards human dermal fibroblasts in vitro. *Nanotoxicology* **3**:U115–161; 2009.
- [80] DeCoteau, W. E.; Estevez, A. Y.; Leo-Nyquist, S.; Heckman, K.; Reed, K.; Erlichman, J. S. Ceria nanoparticles reduce disease severity in a mouse model of multiple sclerosis. Abstract presented at TechConnect World. *Boston* ; 2011.