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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-μm-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 μg/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.

Introduction

Reactive oxygen and nitrogen species (ROS and RNS), including superoxide, nitric oxide, hydrogen peroxide (H_2O_2), 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 impermeative 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 μM. 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 MΩ 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