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Original Contribution

Removal of H_2O_2 and generation of superoxide radical: Role of cytochrome *c* and NADH

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ABSTRACT

In cells, mitochondria, endoplasmic reticulum, and peroxisomes are the major sources of reactive oxygen species (ROS) under physiological and pathophysiological conditions. Cytochrome *c* (cyt *c*) is known to participate in mitochondrial electron transport and has antioxidant and peroxidase activities. Under oxidative or nitrative stress, the peroxidase activity of Fe^{3+}cyt *c* is increased. The level of NADH is also increased under pathophysiological conditions such as ischemia and diabetes and a concurrent increase in hydrogen peroxide (H_2O_2) production occurs. Studies were performed to understand the related mechanisms of radical generation and NADH oxidation by Fe^{3+}cyt *c* in the presence of H_2O_2. Electron paramagnetic resonance (EPR) spin trapping studies using 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) were performed with NADH, Fe^{3+}cyt *c*, and H_2O_2 in the presence of methyl-β-cyclodextrin. An EPR spectrum corresponding to the superoxide radical adduct of DMPO encapsulated in methyl-β-cyclodextrin was obtained. This EPR signal was quenched by the addition of the superoxide scavenging enzyme Cu,Zn-superoxide dismutase (SOD1). The amount of superoxide radical adduct formed from the oxidation of NADH by the peroxidase activity of Fe^{3+}cyt *c* increased with NADH and H_2O_2 concentration. From these results, we propose a mechanism in which the peroxidase activity of Fe^{3+}cyt *c* oxidizes NADH to $NAD^{\bullet}$, which in turn donates an electron to O_2, resulting in superoxide radical formation. A UV–visible spectroscopic study shows that Fe^{3+}cyt *c* is reduced in the presence of both NADH and H_2O_2. Our results suggest that Fe^{3+}cyt *c* could have a novel role in the deleterious effects of ischemia/reperfusion and diabetes due to increased production of superoxide radical. In addition, Fe^{3+}cyt *c* may play a key role in the mitochondrial "ROS-induced ROS-release" signaling and in mitochondrial and cellular injury/death. The increased oxidation of NADH and generation of superoxide radical by this mechanism may have implications for the regulation of apoptotic cell death, endothelial dysfunction, and neurological diseases. We also propose an alternative electron transfer pathway, which may protect mitochondria and mitochondrial proteins from oxidative damage.

In mammalian cells, mitochondria, endoplasmic reticulum, and peroxisomes are the major sources of reactive oxygen species (ROS) under physiological and pathophysiological conditions [1–3]. Under normal physiological conditions ~1–2% of the oxygen consumed by the heart is converted into ROS [4]. In heart, ~30% of the total volume is occupied by mitochondria [5]. Electron leakage from the electron transport chain in mitochondria occurs with partial reduction of oxygen with generation of ROS [6]. In addition, sulfhydryl oxidases generate disulfide bonds with the reduction of oxygen to H_2O_2 in the mitochondrial intermembrane space (IMS) [7]. Cardiac ischemia leads to a decline in mitochondrial respiratory function that can be exacerbated upon reperfusion [8]. During ischemia and reperfusion in the heart, the production of ROS increases [8,9]. ROS generation also increases under pathophysiological conditions such as diabetes, atherosclerosis, vascular and neurodegenerative diseases, and cancer [10]. The molecular mechanisms involved in the generation of ROS are not yet fully understood; therefore, it is necessary to unravel the source(s) and molecular mechanisms involved in the formation of ROS under pathophysiological conditions.

Under physiological conditions, NADH is a substrate for complex I (NADH:ubiquinone oxidoreductase) in the electron transport chain (ETC) in mitochondria [11]. In the heart, the level of NADH increases during both ischemia and reperfusion as a consequence of anaerobic glycolysis [12–15]. Moreover, the level of NADH also increases under pathophysiological conditions such as diabetes and cancer [16,17]. Recently, it was found that the increase in NADH oxidation induces a concurrent increase in superoxide radical production during ischemia [9]. Therefore, it is very crucial to explore the role of NADH in the generation of ROS during both ischemia and reperfusion in heart and under pathophysiological conditions such as diabetes.

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Very recently, a study has shown that respiration-dependent detoxification of H_2O_2 protects rat brain mitochondria [18]. In addition, oxidized cytochrome *c* (Fe^{3+}cyt c) protects complex IV against H_2O_2-induced oxidative damage [19]. However, the role of mitochondrial proteins and the molecular mechanisms involved in the removal of H_2O_2 are not fully understood.

Cytochrome *c* (cyt *c*) is a small, globular heme protein that exists in high concentrations (0.5–5 mM) in the inner membrane of mitochondria [20]. At least 15% of cyt *c* is tightly bound to the inner membrane and the remainder is loosely attached to the inner membrane and can be readily mobilized [21]. Physiologically, cyt *c* mediates electron shuttling between cytochrome *c* reductase (complex III) and cytochrome *c* oxidase (complex IV) during mitochondrial respiration [21]. The loosely associated cyt *c* participates in electron transport, mediates superoxide removal, and prevents oxidative stress [21–23], whereas the tightly bound cyt *c* accounts for the peroxidase activity [24]. Upon interaction with cardiolipin, a mitochondria-specific phospholipid, cyt *c* has been shown to alter its tertiary structure and gain peroxidase activity [25]. Under conditions of oxidative and nitrosative stress, the peroxidase activity of cyt *c* also increases [26,27]. Release of cyt *c* from the inner mitochondrial membrane into the cytosol is a proapoptotic factor [28,29]. In the early event of apoptosis, the redox function of cyt *c* in the respiratory chain switches to a peroxidase function [25,30]. The increased peroxidase activity of cyt *c* is implicated in various neurodegenerative diseases, such as Parkinson disease, Alzheimer disease, and amyotrophic lateral sclerosis (ALS) [31]. To gain a better understanding of the role of Fe^{3+}cyt *c* in oxidative damage, pathological conditions, and removal of H_2O_2, we have employed the powerful, sensitive, and specific technique of electron paramagnetic resonance (EPR) spin trapping along with UV–visible absorption spectroscopy to investigate the oxidation of NADH and generation of free radicals by the peroxidase activity of Fe^{3+}cyt *c* and redox change of cyt *c*.

Materials and methods

Materials

Fe^{3+}cyt *c* (from horse heart), hydrogen peroxide, NADH, and sodium hypochlorite (4% chlorine available in solution) were purchased from Sigma. Diethylenetriaminepentaacetic acid (DTPA), 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO), and methyl-β-cyclodextrin (Me-β-CD) were obtained from Aldrich. Purified 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) was purchased from Dojindo Laboratories (Kumamoto, Japan). Microcrystalline particulate of lithium phthalocyanine (LiPc) was synthesized in our laboratory and used as an oximetry probe [32].

Preparation of the hypochlorite (HOCl)-oxidized Fe^{3+}cyt c (M-Fe^{3+}cyt c)

M-Fe^{3+}cyt *c* was prepared as described previously [26]. Fe^{3+}cyt *c* (1 mM) was incubated with HOCl (4 mM) at room temperature for 15 min. The reaction mixture was diluted 10-fold and concentrated with an Amicon Ultra-4 5000 MWCO concentrator. This process was repeated five times to remove excess HOCl.

EPR measurements

EPR spectra were recorded using quartz flat cells at room temperature with a Bruker ESP 300 or 300E spectrometer operating at X-band with 100-kHz modulation frequency and a TM_{110} cavity. Room temperature EPR spectra were recorded using the following parameters: microwave frequency 9.779 GHz, modulation frequency 100 kHz, modulation amplitude 1 G, microwave power 20 mW, number of scans 10, scan time 30 s, and time constant 82 ms. EPR spectral recording began 2 min after the addition of H_2O_2. All the experiments were carried out in phosphate buffer (50 mM and pH 7.4) containing 0.1 mM DTPA. Reactions were initiated by the addition of H_2O_2. Experiments were performed under anaerobic conditions by preparing the reaction mixture followed by transfer to quartz flat cell that was then sealed inside a nitrogen-filled glove box.

Quantitation of the observed free radical signals was performed by computer simulation of the spectra with comparison of the double integral of the observed signal to that of a TEMPO standard (1 μM) measured under identical conditions [33].

EPR oximetry experiments were carried out as described previously [34].

UV–visible spectrophotometry

Optical spectra were measured on a Cary 50 Bio UV–visible spectrophotometer. The concentration of HOCl was measured by using the molar extinction coefficient of hypochlorite ($\varepsilon_{292\ nm} = 350\ M^{-1}\ cm^{-1}$) in 5 mM sodium hydroxide solution at pH 11–12 [35].

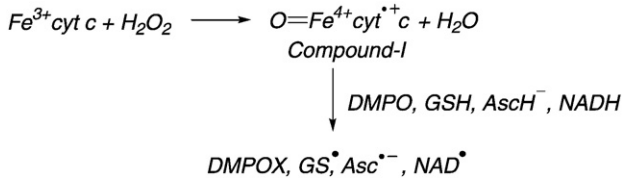
The reduction of Fe^{3+}cyt *c* was carried out in phosphate buffer (50 mM and pH 7.4) containing 0.1 mM DTPA. Reactions were initiated by the addition of H_2O_2. UV–visible spectra were recorded 5 min after the addition of H_2O_2.

Results

EPR spin trapping studies of the generation of superoxide radical by Fe^{3+}cyt c in the presence of NADH and H_2O_2

It has been demonstrated that Fe^{3+}cyt *c* acts as a peroxidase and is involved in the detoxification of H_2O_2 [24]. During peroxidase activity, Fe^{3+}cyt *c* reacts with H_2O_2 to form the peroxidase compound I-type intermediate, as shown in Scheme 1. The peroxidase activity of Fe^{3+}cyt *c* oxidizes various endogenous antioxidants such as ascorbate, GSH, and NADH in the presence of H_2O_2, as shown in Scheme 1 [36].

The levels of NADH in the ischemic and postischemic reperfused hearts are higher than in the normal heart [14]. The level of NADH decreases during postischemic reperfusion in the heart [14]. To gain insight into the molecular mechanisms involved in the formation of ROS during ischemia/reperfusion, we wanted to study the oxidation of NADH and free radical generation by the Fe^{3+}cyt *c* and H_2O_2 system. EPR spin trapping is a powerful technique to study ROS formation. EPR spin trapping studies using the spin trap DMPO were carried out to investigate the oxidation of NADH by Fe^{3+}cyt *c* and H_2O_2. EPR spectra were recorded from Fe^{3+}cyt *c* (0.1 mM) in the presence of H_2O_2 (0.5 mM), NADH (1 mM), DMPO (50 mM), and Me-β-CD (0.1 M) along with DTPA (0.1 mM). A prominent EPR signal was seen, corresponding to the superoxide radical adduct of DMPO (DMPO–OOH), as shown in Fig. 1, spectrum A. From the EPR spectrum the calculated isotropic hyperfine values are $a_N = 13.49$ G, $a_{H1} = 10.78$ G, and $a_{H2} = 1.39$ G, which are in agreement with reported values [37]. In the absence of Fe^{3+}cyt *c*, a trace level of DMPO–OOH signal was obtained, as shown in Fig. 1, spectrum B. A very weak quartet EPR signal of intensity ratio 1:2:2:1 was obtained in the absence of Me-β-CD, as shown in Fig. 1, spectrum C. From the EPR spectrum, the calculated isotropic hyperfine coupling constant is 14.9 G



Scheme 1. Mechanism of Fe^{3+}cyt c activation to a peroxidase compound I-type intermediate by H_2O_2 and its oxidation of substrates.