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Mitochondrial Electron Transport Chain-Derived Superoxide Exits Macrophages: Implications for Mononuclear Cell-Mediated Pathophysiological Processes

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Abstract

The involvement of mitochondrial electron transport chain (METC)-derived superoxide anion radical in cell protooncogene activation, mitogenic responses, and cancerous growth has recently received much attention. In order for METC-derived superoxide to participate in any of the above processes, its exit from mitochondria would be a critical step. Detection of intracellular superoxide showed that mitochondrial respiration is the major source of cellular superoxide in unstimulated or resting monocytes/macrophages. However, direct evidence for the exit of superoxide from mitochondria is presently lacking. Here we show that METC-derived superoxide does exit from mitochondria in unstimulated monocytes/macrophages. Release of superoxide was first found to occur with substrate-supported mitochondria isolated from these cells. We also observed the presence of extracellular superoxide with the intact unstimulated/resting cells. Extracellular superoxide was markedly diminished (>90%) by the mitochondrial inhibitor, rotenone, or the uncoupler, carbonyl cyanide *p*-(trifluoromethyl) phenylhydrazone. Furthermore, cells with a deficient METC exhibited significant reduction (>90%) in extracellular superoxide, demonstrating that with intact cells METC-derived superoxide not only exits from mitochondria, but can be released extracellularly. Superoxide anion radical released from mitochondria could react with exogenous nitric oxide, forming peroxynitrite. Mitochondria-derived extracellular superoxide could also oxidize low-density lipoprotein (LDL). These results thus resolve any uncertainty on the ability of superoxide to exit from mitochondria. This study for the first time also identifies mitochondria as the major source of extracellular superoxide in unstimulated resting monocytes/macrophages, which has implications for the involvement of these mononuclear cells in various pathophysiological situations.

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1. INTRODUCTION

Polymorphonuclear leukocytes (PMNs), monocytes, and macrophages are important cells in the innate immune response to microorganisms [1]. All of these three cell types are derived from the same progenitor cell population in the bone marrow. Blood monocytes are an intermediate stage, which then further differentiates in tissues to various macrophage populations. Mononuclear phagocytes at different levels of maturation have different functional and metabolic characteristics [1]. One of the distinguishing biochemical characteristics of these cells is their ability to synthesize and release reactive oxygen species (ROS). In fact, these were some of the first cells characterized for their ability to synthesize and release ROS in significant amounts [2, 3]. The generation of ROS by these cells was initially linked to their microbiocidal activities [4]. More recently, the generation of ROS by these cell types has been linked to their involvement in various pathophysiological processes, such as atherosclerosis [5], neurodegenerative disorders [6], and cancer [7].

One of the primary cellular sources of ROS in phagocytic cells is the plasma membrane NAD(P)H oxidase, which generates superoxide [8]. During phagocytosis, this superoxide generation is directed toward the engulfed material within the phagolysosome [9]. Alternatively, depending upon the agent used to activate NAD(P)H oxidase, superoxide production can also be directed toward the extracellular milieu [9]. The dismutation of superoxide results in the production of hydrogen peroxide, which can be used as a substrate by myeloperoxidase (MPO) to form much more biologically reactive oxidants, such as hypochlorous acid (HOCl) and singlet oxygen [10]. MPO is found in myeloid progenitor cells, in PMNs, and monocytes, but typically not in mature macrophages [11].

Another characteristic which distinguishes macrophages from PMNs and monocytes is their utilization of mitochondrial respiration for the generation of cellular energy [12]. In contrast, PMNs and monocytes rely primarily on glycolysis or the hexose monophosphate shunt for energy [13]. This is not due to that PMNs and monocytes do not have mitochondria. In fact, mitochondria are an important cellular site for heme biosynthesis in myeloid precursors, including the heme moieties for enzymes like MPO and components of NAD(P)H oxidase. It is important to note that the differentiation of peripheral blood-derived monocytes to macrophages is accompanied by the maturation of the mitochondrial electron transport chain (METC) [14]. In most cell types, mitochondria are considered the primary source of cellular superoxide and hydrogen peroxide [15, 16]. We have suggested previously that this is also true of unstimulated or resting macrophages [14, 17]. In this regard, we show that more extracellular superoxide can be measured from resting rat alveolar macrophages than from either peritoneal macrophages or peripheral PMNs [17].

It has long been known that both complexes I and III of the METC are involved in the reduction of O₂ to form superoxide [18, 19]. Because superoxide is charged, the prevalent view has been that METC-derived superoxide does not cross the mitochondrial inner membrane. Recently, mitochondrial respiratory chain-dependent superoxide has been shown to be released into the intermembrane space of mitochondria [19, 20]. Although it is charged, the existence of anion channels in the mitochondrial inner membrane may allow superoxide to pass through [21, 22]. Indeed, anion channels exist in the plasma membrane of erythrocytes, which allow superoxide to exit these cells [23]. In addition, the interaction between the negative charge of superoxide and the high negative mitochondrial transmembrane potential may also favor the exit of superoxide from mitochondria. We thus hypothesized that METC-derived superoxide might be able to exit mitochondria. In this study, with unstimulated human ML-1 cell-derived monocytes/macrophages, we have provided direct evidence that not only does superoxide exit the mitochondria, but exits the cells also under physiological conditions. Accordingly, this source of ROS could contribute to pathophysiological reactions mediated by macrophages which accumulate in tissues.

2. MATERIALS AND METHODS

2.1. Materials

Lucigenin, rotenone, antimycin A, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP), succinic acid (sodium salt), pyruvic acid (sodium salt), maleic acid (disodium salt), adenosine diphosphate (ADP), ascorbic acid (sodium salt), ethidium bromide, chloramphenicol, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), potassium cyanide (KCN), tetramethyl-1,4-phenylene-diamine (TMPD), copper-zinc superoxide dismutase (Cu,ZnSOD), manganese SOD (MnSOD), low-density lipoprotein (LDL), and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO). 5-(Dimethoxyphosphoryl)-5-methyl-1-pyrroline-*N*-oxide (DEPMPO) and Mn(III) tetrakis (1-methyl-4-pyridyl)porphyrin (MnTMPyP) were products from Cayman Chemical (Ann Arbor, MI). RPMI-1640, Dulbecco's phosphate-buffered saline (PBS), penicillin, and streptomycin were obtained from Life Technologies (Grand Island, NY). Fetal bovine serum (FBS) was purchased from Biowhittaker (Walkersville, MD). FITC-conjugated murine monoclonal antibodies to CD11b, CD14, and CD16 were products of Harlan (Indianapolis, IN). Rabbit polyclonal anti-p47^{phox} antibody and mouse monoclonal anti-p67^{phox} antibody were purchased from Santa Cruz Biotech (Santa Cruz, CA) and Transduction Laboratories (Lexington, KY), respectively. The nitric oxide donor, 5-nitrosoglutathione was obtained from Calbiochem (San Diego, CA). Tissue culture flasks were purchased from Corning (Cambridge, MA).

2.2. Culture and Differentiation of ML-1 Cells to Monocytes/Macrophages

Human monoblastic ML-1 cells were obtained from Dr. Ruth W. Craig. The cells were cultured at 37°C in an atmosphere of 5% CO₂ in RPMI-1640 medium supplemented with penicillin (50 units/ml), streptomycin (50 µg/ml), and 7.5% FBS in 150 cm² tissue culture flasks. The cell differentiation to monocytes/macrophages was initiated by incubation of ML-1 cells (3 × 10⁵/ml) with TPA (0.3 ng/ml) for 3 days, and then the medium was removed. The cells were fed with fresh media without further addition of TPA. The cells

were cultured for another 3 days. Cells at this time were characteristic of monocytes/macrophages [24, 25] and were harvested for further experiments.

2.3. Isolation of Mitochondria from Monocytes/Macrophages

Mitochondria were isolated from the freshly harvested monocytes/macrophages according to the method of Rickwood et al. [26] with minor modifications. Briefly, the cells ($4-6 \times 10^6$ cells per sample) were washed once with PBS. The cell pellet was resuspended in 5 ml sucrose buffer (0.25 M sucrose, 10 mM Hepes, 1 mM EGTA, and 0.5% BSA, pH 7.4), homogenized in a Dounce tissue grinder on ice. The homogenate was centrifuged at $1,500 \times g$ for 10 min at 4°C . The supernatant was collected and centrifuged at $10,000 \times g$ for 10 min at 4°C . The resulting mitochondrial pellet was washed twice with 5 ml sucrose buffer and then resuspended in 1 ml sucrose buffer. The mitochondrial protein was measured with the Bio-Rad protein assay dye based on the method of Bradford [27] with BSA as the standard.

2.4. Detection of Superoxide Production by Lucigenin-Derived chemiluminescence (CL)

Lucigenin-derived CL was used to detect intracellular mitochondria-derived superoxide production with intact cells [28, 29] and intramitochondrial superoxide production with isolated mitochondria [30, 31]. In brief, for detection of intracellular superoxide production, monocytes/macrophages (1×10^6 cells) were suspended in 2 ml complete PBS (PBS containing 0.5 mM MgCl_2 , 0.7 mM CaCl_2 , and 0.1% glucose). For detecting intramitochondrial superoxide production with isolated mitochondria, mitochondria (0.5 mg protein) were incubated with 6 mM succinate in 1 ml respiratory buffer (70 mM sucrose, 220 mM mannitol, 2 mM Hepes, 2.5 mM KH_2PO_4 , 2.5 mM MgCl_2 , 0.5 mM EDTA, and 0.1% BSA, pH 7.4). After adding lucigenin (1 or 5 μM), the CL was measured continuously with a Berthold LB9505 six-channel luminometer at 37°C for 30 min (for intact cells) or 60 min (for isolated mitochondria). The concentrations of lucigenin used in this study have been characterized to be non-redox cycling concentrations which can be used to specifically detect biological superoxide production [29–32].

2.5. Electron Paramagnetic Resonance (EPR) Detection of Superoxide

DEPMPO-spin trapping was used to detect superoxide production by intact cells and isolated mitochondria. For detection of superoxide production by intact cells, monocytes/macrophages (1×10^6 cells) were incubated with 10 mM DEPMPO at 37°C in complete PBS. For detection of superoxide production by isolated mitochondria, mitochondria (0.5 mg protein) were incubated with 6 mM succinate and 10 mM DEPMPO at 37°C in respiration buffer. After 15 min of incubation, the sample was immediately transferred to an EPR flat cell. The EPR spectra were recorded at room temperature with a spectrometer (model ER 300, IBM-Bruker) operating at X-band as described before [29, 33].

2.6. Measurement of Superoxide Production by Cytochrome c Reduction Assay

SOD-inhibitable ferricytochrome c reduction was used to measure extracellular superoxide. Briefly, the monocytes/macrophages (1×10^6 cells) were incubated with ferricytochrome c (120 μM) in 2 ml complete PBS in the presence or absence of Cu,ZnSOD (300 units/ml) at

37°C for 30 min. After incubation, the cells were pelleted, and the reduced cytochrome c in the supernatant was measured at 550 nm [29].

2.7. SOD Gel Assay

Non-denaturing polyacrylamide gel electrophoresis assay was used to detect cellular SOD. In brief, monocytes/macrophages (0.5×10^6 cells/ml) were incubated with either Cu,ZnSOD (500 units/ml) or MnSOD (500 units/ml) in complete PBS at 37°C for 30 min. After this incubation, the cells were pelleted and washed extensively. The cells were then lysed, and the cellular CuZnSOD and MnSOD were separated and detected in the native gel according to the method of Beauchamp and Fridovich [34].

2.8. Production of Monocytes/Macrophages with Deficient METC

For producing METC-deficient cells, 40 ng/ml of ethidium bromide (EB) or 20 μ M chloramphenicol (CAP) was added to ML-1 cells 30 min before initiation of the differentiation to monocytes/macrophages as described above, except that the medium was supplemented with pyruvate (100 μ g/ml) and uridine (50 μ g/ml). These concentrations of either EB or chloramphenicol were not cytotoxic to the cells.

2.9. Measurement of Mitochondrial O₂ Consumption

To measure the substrate-supported mitochondrial O₂ consumption in intact cells, 4×10^6 control monocytes/macrophages or EB- or CAP-treated cells were suspended in 1 ml of buffer A (250 mM sucrose, 20 mM Hepes, and 10 mM MgCl₂, pH 7.1). Then, 1 ml of buffer A containing 0.1 mg/ml of digitonin was added to the cell suspension to make the cell membrane permeable to substrates, followed by gentle mixing at room temperature for 1 min. After addition of 8 ml ice-cold buffer A, the cells were pelleted by centrifugation at 4°C. The cell pellet was washed once with buffer A and kept on ice. Immediately prior to measurement of O₂ consumption, the cell pellet was resuspended in 2.5 ml pre-warmed (37°C) respiration medium (buffer A supplemented with 1 mM ADP and 2 mM K₂HPO₄/K₂HPO₄, pH 7.1), and the substrate-supported mitochondrial O₂ consumption was measured polarographically with a Clark-type oxygen electrode at 37°C. The mitochondrial substrates used were pyruvate/malate (6 mM/6 mM), succinate (6 mM), and ascorbate/tetramethyl-1,4-phenylenediamine (1 mM/0.2 mM).

2.10. Measurement of Monocyte/Macrophage Surface Marker Expression

The expression of the cell surface markers (CD11b, CD14, and CD16) was analyzed by a Coulter Profile flow cytometer using FITC-conjugated mouse monoclonal antibodies to human CD11b, CD14, and CD16, respectively. The FITC-conjugated mouse IgG was used as control. Prior to incubation with the antibodies, control monocytes/macrophages or EB-or CAP-treated cells were resuspended in a buffer containing 10 mM Hepes, 150 mM NaCl, 0.1% NaN₃, and 1% BSA. The cells (5×10^5 cells) were incubated in the above buffer with 10 μ l of appropriate antibody in a final volume of 100 μ l at 4°C for 30 min. After incubation, the cells were washed twice with the above ice-cold buffer and resuspended in 0.5 ml of the same buffer. For all measurements, approximately 1×10^5 cells were analyzed, and the results are presented as the mean channel number of the fluorescent cells.

2.11. Measurement of NAD(P)H Oxidase Activity

The activity of plasma membrane NAD(P)H oxidase was determined by measuring the superoxide production following stimulation with TPA (30 ng/ml). Extracellular superoxide was measured by the cytochrome c reduction assay as described above (see Section 2.6).

2.12. Immunoblotting Analysis of the Expression of p47^{phox} and p67^{phox}

Control monocytes/macrophages or EB- or CAP-treated cells were lysed with lysis buffer containing 25 mM Tris-HCl, pH 7.4, 0.5 mM EDTA, 0.5 mM EGTA, 0.5% Triton X-100, 10 mM mercaptoethanol, 1 µg/ml of leupeptin, 1 µg/ml of aprotinin, 0.5 mM phenylmethylsulfonyl fluoride, 25 mM sodium fluoride, and 1 mM sodium orthovanadate. After sonication, the cell lysates were centrifuged at $14,000 \times g$ for 5 min at 4°C. The supernatants were collected, and protein concentrations were measured as described above (Section 2.3). Prior to electrophoresis, the samples were diluted with 2 times concentrated loading buffer (125 mM Tris-Cl, pH 6.8, 20% glycerol, 4% SDS, 5% 2-mercaptoethanol, and 25 µg/ml of bromophenol blue), and heated at 95°C for 5 min. Equal amounts of protein from each sample were run on 10% SDS-PAGE gels, and transferred electrophoretically to nitrocellulose membranes (Amersham Pharmacia Biotech, Piscataway, NJ). The membranes were blocked with 5% non-fat dried milk in TTBS buffer (50 mM Tris-HCl, pH 7.4, 0.9% NaCl, and 0.1% Tween-20) at 4°C overnight. Then the membranes were incubated with specific antibodies to p47^{phox} and p67^{phox} in the same buffer at room temperature for 2 hr, followed by washing with TTBS (10 min for three times). The membranes were then incubated with a secondary antibody conjugated with horseradish peroxidase (Santa Cruz Biotech) in TTBS containing 5% dried milk at room temperature for 1 hr, followed by washing in TTBS (10 min for 3 times). Finally, the membranes were developed using an enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech) and exposed to x-ray film.

2.13. Detection of Peroxynitrite Production

The formation of peroxynitrite was detected by luminol-derived CL following incubation of the monocytes/macrophages with S-nitrosoglutathione in the presence or absence of bicarbonate [35]. The buffer and cell concentration were identical to those used for the determination of lucigenin-derived CL with intact cells (Section 2.4). For this assay, horseradish peroxidase is not included in the incubation as it was when luminol-derived CL was used to detect hydrogen peroxide production.

2.14. Measurement of LDL Oxidation

Monocytes/macrophages (1×10^6 cells) were incubated with LDL (0.2 mg protein) in 1 ml of phenol red-free Ham's F-12 medium at 37°C for 6 hr. After this incubation, the oxidation of LDL was determined directly in the medium by the thiobarbituric acid-reactive substance (TBARS) assay as described by Buege and Aust [36]. The results are expressed as nanomoles of malondialdehyde (MDA) equivalents per milligram of LDL protein.

2.15. Statistical analyses

All data are expressed as mean $\pm$ S.E. from at least three separate experiments unless otherwise indicated. Differences between the mean values of multiple groups were analyzed by one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test. Statistical significance between the values was considered at $p < 0.05$.

3. RESULTS

3.1. Mitochondria as the Major Source of Intracellular Superoxide

In this study, we used human ML-1 cell-derived monocytes/macrophages as a model system to examine if the METC-derived superoxide can exit from the mitochondria. Although mitochondria are generally considered the major source of cellular superoxide, direct experimental evidence in support of this notion is still lacking. This is due, at least in part, to the limitation of methods for directly detecting intracellular superoxide. Recently, a lucigenin-based CL assay has been characterized which enables a direct assessment of intracellular mitochondrial superoxide production [28–32]. We therefore used this assay to assess the production of intracellular superoxide in the unstimulated monocytes/macrophages. As shown in Figure 1a, a marked lucigenin-derived CL response was elicited following incubation of the unstimulated cells with 1 μ M lucigenin, indicating the constant production of intracellular superoxide by the unstimulated cells.

To determine the contribution of mitochondrial respiration to this intracellular superoxide production, we examined the effects of several agents known to affect mitochondrial respiration. Rotenone (ROT) is a specific inhibitor of mitochondrial complex I, and FCCP is a mitochondrial uncoupler. Both chemicals are able to inhibit mitochondrial ROS production [31]. KCN is a mitochondrial complex IV inhibitor that causes electrons to build up, leading to enhanced production of superoxide. The nearly complete inhibition by ROT or FCCP and marked increase by KCN of the lucigenin-derived CL (Figure 1b) demonstrate that mitochondria are the major source of intracellular superoxide production in these unstimulated intact cells.

3.2. Release of Superoxide from Isolated Mitochondria

To further examine METC-derived superoxide generation and its possible exit from mitochondria, studies with succinate-supported isolated mitochondria were undertaken using lucigenin-derived CL and DEPMPO-spin trapping. DEPMPO detects extramitochondrial/extracellular superoxide. As shown in Figure 2a, the strong intramitochondrial lucigenin-derived CL was greatly inhibited by the membrane permeable SOD-mimetic, MnTMPyP [37], but not by the membrane impermeable Cu,ZnSOD. In contrast to lucigenin-derived CL, formation of the DEPMPO-hydroxyl (DEPMPO-OH) adduct derived with succinate-supported mitochondria was completely inhibited by Cu,ZnSOD (Figure 2b). Catalase was not inhibitory (not shown), indicating the primary involvement of superoxide in the formation of the DEPMPO-OH adduct. Production of an SOD-inhibitable DEPMPO-OH adduct was also observed with pyruvate/malate-supported mitochondria in the presence of antimycin A (data not shown).

3.3. Effects of METC Inhibitors on Extracellular Superoxide

The results with DEPMPO-spin trapping and effects of SOD (Figure 2b) demonstrate that with substrate-supported isolated mitochondria, METC-derived superoxide can exit from the mitochondria. As such, we next examined the possible exit of mitochondria-derived superoxide in intact unstimulated/resting cells. It has been known that activation of a plasma membrane NAD(P)H oxidase in phagocytic cells leads to the extracellular appearance of superoxide as detected by SOD-inhibitable cytochrome c reduction [3]. On the other hand, measurable amounts of SOD-inhibitable cytochrome c reduction can also be detected with unstimulated alveolar macrophages [17]. The assumption has been that this basal superoxide in the extracellular milieu originates from a basal activity of NAD(P)H oxidase. We also observed a significant amount of SOD-inhibitable cytochrome c reduction following incubation of the unstimulated/resting ML-1-derived monocytes/macrophages with ferricytochrome c (Figure 3a), indicating the presence of superoxide in the extracellular milieu. This unstimulated level of SOD-inhibitable cytochrome c reduction was reduced by >90% in the presence of either rotenone or FCCP (Figure 3b). This indicates that mitochondrial respiration rather than the basal activity of the plasma membrane NAD(P)H oxidase appears to be the major source of this extracellular superoxide.

DEPMPO-spin trapping was used to further demonstrate the extracellular release of superoxide originating from mitochondrial respiration in intact cells. Incubation of the unstimulated cells with DEPMPO resulted in the production of a mixture of both DEPMPO-superoxide (DEPMPO-OOH) and DEPMPO-OH adducts (Figure 3c). Formation of these spin adducts was completely inhibited by either Cu,ZnSOD or MnSOD, but not by catalase (Figure 3c), indicating the primary involvement of superoxide. Incubation of cells with either Cu,ZnSOD or MnSOD did not result in any detectable increase in the intracellular accumulation of these SODs over the time period examined (Figure 3d). Thus, the DEPMPO-spin trapping experiment (Figure 3c) again demonstrates the presence of mitochondria-derived extracellular superoxide. Consistent with the cytochrome c reduction, formation of the superoxide-derived spin adducts in the extracellular space was reduced (>90%) by ROT or FCCP, and was increased (~50%) by KCN (Figure 3e). KCN blocks complex IV of the METC, thereby causing a backup of electrons and a buildup of METC-derived superoxide. Therefore, the above observations with two independent methods for detecting superoxide in conjunction with mitochondrial inhibitors strongly indicate that not only can superoxide exit from mitochondria, but can exit from the intact cell as well.

3.4. Mitochondria-Derived Extracellular Superoxide in Cells with a Deficient METC

The human mitochondrial genome encodes 13 polypeptides that are essential components of mitochondrial complexes I, III, IV, and V [38]. Development of mitochondrial respiration occurs following the differentiation of both human peripheral blood monocytes to macrophages [14], and human ML-1 cells to monocytes/macrophages [24, 29].

To conclusively attribute the presence of extracellular superoxide to the activity of the METC, ML-1 cells were induced to differentiate to monocytes/macrophages in the presence of EB or CAP. EB and CAP are chemicals that, when used at appropriate concentrations, selectively inhibit mitochondrial DNA synthesis/transcription and translation, respectively

[39, 40]. Treatments with EB or CAP did not affect the differentiation of ML-1 cells to monocytes/macrophages as assessed both morphologically and by determining the expression of monocytic surface antigens (Figure 4b), the plasma membrane NAD(P)H oxidase activity (Figure 4c), and the expression of p47^{phox} and p67^{phox} (Figure 4d), two critical cytosolic factors involved in the assembly of an activated plasma membrane NAD(P)H oxidase complex [41]. However, these treatments did result in the production of mitochondrial respiration-deficient cells as evidenced by significantly reduced substrate-supported mitochondrial O₂ consumption (Figure 4a) and translation of mitochondrial DNA-encoded subunits of the METC (not shown).

As expected, the above treatments also resulted in a marked decrease (>90%) in intracellular production of superoxide as detected by lucigenin-derived CL (Figure 5a). As compared with the control cells, both SOD-inhibitable cytochrome c reduction and the formation of superoxide-derived DEPMPO-spin adducts extracellularly were reduced by >90% with cells differentiated in the presence of either EB or CAP (Figure 5b and 5c). These observations further demonstrate that the METC is the major source of both intracellular and extracellular superoxide in the unstimulated monocytes/macrophages.

This is important in that mitochondrial respiration would represent a constant rather than a transient source of extracellular superoxide from monocytes/macrophages. In data not shown, we also observed, by using horseradish peroxidase-dependent catalase-inhibitable luminol-derived CL, that the mitochondria-derived extracellular hydrogen peroxide with these unstimulated cells was completely abolished in the presence of ROT or FCCP, or in cells with a deficient METC.

3.5. Formation of Extracellular Peroxynitrite

Superoxide anion radical can directly react with nitric oxide at a diffusion-limited rate to form peroxynitrite, a potent oxidant that is both cytotoxic and genotoxic [42]. To examine whether the superoxide exiting from mitochondria reacts with nitric oxide, peroxynitrite formation was determined by luminol-derived CL following incubation of the mononuclear cells/macrophages with S-nitrosoglutathione, a nitric oxide donor. In this case, horseradish peroxidase was not included in the assay. As shown in Figure 6a, bicarbonate-enhanced luminol-derived CL was elicited after incubation of the monocytes/macrophages with S-nitrosoglutathione, indicating the formation of peroxynitrite. The bicarbonate-enhanced luminol-derived CL was greatly diminished (70%) by SOD (Figure 6b), suggesting that the reaction leading to luminol-derived CL occurred mainly in the extracellular space. Consistent with the effects on extracellular superoxide, the production of peroxynitrite was greatly reduced by either ROT or FCCP, or with cells differentiated in the presence of non-cytotoxic concentrations of either EB or CAP (Figure 6b). These results not only further confirm the presence of METC-derived superoxide in the extracellular milieu, but demonstrate that this METC-derived superoxide is also reactive extracellularly.

3.6. Extracellular Oxidation of LDL

Superoxide anion radical has long been suggested to mediate modifications of LDL by several types of cells, including macrophages, smooth muscle cells, and endothelial cells

[43–45]. Because mitochondria-derived superoxide can be released extracellularly by the unstimulated monocytes/macrophages, we next determined whether this mitochondria-derived superoxide could mediate the oxidation of exogenous LDL. As shown in Figure 7, incubation of LDL with the unstimulated monocytes/macrophages resulted in a marked oxidation of LDL as detected by the TBARS assay. The cell-mediated LDL oxidation was greatly inhibited by exogenously added SOD, but not by catalase, indicating the involvement of superoxide. Consistent with the effects on extracellular superoxide, oxidation of LDL was also greatly reduced by either ROT or FCCP, or with cells differentiated in the presence of non-cytotoxic concentrations of either EB or CAP (Figure 7).

4. DISCUSSION

The critical involvement of ROS in the pathogenesis of a diversity of diseases and disorders has increasingly been recognized over the last two decades [46– 50]. Production of ROS, including superoxide, hydrogen peroxide, singlet oxygen, and hydroxyl radicals, is associated with various cellular metabolic pathways [51, 52]. Among the many cellular sources of ROS, mitochondrial respiration consumes about 90% of the O₂ used by cells and is generally considered the major source of cellular superoxide and hydrogen peroxide [51]. As such, mitochondria-derived ROS have been implicated in various pathophysiological processes, including aging [53], neurodegenerative diseases [49], ischemia/reperfusion injury [47], and cytotoxicity induced by both xenobiotics and endobiotics [54]. Moreover, the involvement of mitochondria-derived superoxide in cellular mitogenic responses and cancerous growth has been suggested by multiple studies [55–58]. For example, it was reported that overexpression of mitochondrial manganese superoxide dismutase (MnSOD) in JB6 cells could prevent the anchorage-independent growth and expression of c-jun and c-fos induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA), a potent tumor promoter [59]. Increased MnSOD expression also resulted in suppression of the malignant phenotypes of human melanoma cells and SV40 transformed lung fibroblasts [57, 58]. St. Clair and coworkers further observed that elevated expression of MnSOD in mitochondria led to the reduction of Jun-associated transcription factors and bcl-xL expression in fibrosarcoma cells [60], and suppression of fibrosarcoma metastasis in syngeneic mice [57]. MnSOD catalyzes the dismutation of mitochondrial superoxide to form hydrogen peroxide. Superoxide has been demonstrated to cause activation of protooncogenes and cellular mitogenic responses as well as play a role in multistage carcinogenesis in both in vitro and in vivo models [61– 63]. Accordingly, the inhibitory effects of increased expression of mitochondrial MnSOD on cell tumorigenesis appear to result from the decreased release of superoxide from the mitochondrial matrix to the cytoplasm, where the superoxide may either directly or indirectly act on cellular targets, such as signaling molecules and/or DNA, contributing to cancerous cell growth.

Although the mechanism involved remains to be determined, our results demonstrate that under physiological conditions METC-derived superoxide can exit from mitochondria and be released into the extracellular milieu of unstimulated monocytes/macrophages (Figure 8). This study has for the first time demonstrated that the activity of the METC is the major source of both intracellular and extracellular superoxide production in unstimulated monocytes/macrophages. Our results are consistent with previous observations that METC-

derived superoxide not only causes direct damage to the mitochondrial compartment, such as inactivation of the mitochondrial aconitase and electron transport chain components [64], but may also act on the targets outside the mitochondria to cause cytotoxic and genotoxic effects [65–69]. The ability of superoxide to exit mitochondria is also consistent with recent suggestions that METC-derived superoxide may act as a signaling molecule involved in modulation of protein kinase cascades and transcription factors, leading to altered gene expression and mitogenic responses [69–71]. The ability of mitochondria-derived superoxide to exit from this organelle as well as the cell suggests a biochemical basis for why all eukaryotic cells have cytosolic Cu,ZnSOD and some also have an extracellular SOD (ECSOD).

Our observation that the METC is the major source of extracellular superoxide in unstimulated monocytes/macrophages also has important pathophysiological implications (Figure 8). Monocytes/macrophages are widely distributed throughout the body, being found in the lungs, liver, kidneys, bone marrow, blood, central nervous system, bone, skin, gastrointestinal tract, and connective tissues. Alteration in either the distribution or the function of monocytes/macrophages has been implicated in diverse disorders, including immune diseases, neuronal injuries, and atherosclerosis [72]. With regard to atherosclerosis, studies have demonstrated that through oxidizing LDL and stimulating vascular smooth muscle cell growth, macrophages are crucially involved in the pathogenesis of this disease [73]. Since superoxide is able to cause cell proliferation and oxidation of LDL (Figure 7) [74], the constant extracellular presence of mitochondria-derived superoxide by macrophages may play an important role in the development of atherosclerosis. The constant extracellular release of mitochondria-derived superoxide in macrophages may also play a role in the pathogenesis of other ROS-associated diseases, such as neurodegenerative diseases, inflammatory disorders, and cancer. In this regard, the involvement of macrophages via production of ROS in the pathogenesis of Alzheimer's disease has recently been suggested [75]. Furthermore, the extracellular mitochondria-derived superoxide from macrophages might also react with cell-derived nitric oxide, forming the highly reactive peroxynitrite (Figure 8). Production of peroxynitrite has been implicated in neuronal injuries induced by diverse insults [76]. Lastly, the exit of superoxide from mitochondria provides a biochemical basis as to why overexpression of MnSOD exerts significant protective effects in a wide variety of cellular systems [55–58, 77].

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ABBREVIATIONS

BSA	bovine serum albumin
CAP	chloramphenicol
CL	chemiluminescence

DEPMPO	5-(dimethoxyphosphoryl)-5-methyl-1-pyrroline- <i>N</i> -oxide
DEPMPO-OH	DEPMPO-hydroxyl adduct
DEPMPO-OOH	DEPMPO-superoxide adduct
EB	ethidium bromide
ECSOD	extracellular SOD
EPR	electron paramagnetic resonance
FBS	fetal bovine serum
FCCP	carbonylcyanide <i>p</i> -(trifluoromethoxy) phenylhydrazine
KCN	potassium cyanide
LDL	low-density lipoprotein
MDA	malondialdehyde
METC	mitochondrial electron transport chain
MPO	myeloperoxidase
MnTMPyP	Mn(III) tetrakis(1-methyl-4-pyridyl)porphyrin
NO	nitric oxide
ONOO⁻	peroxynitrite
PBS	phosphate-buffered saline
PMNs	polymorphonuclear leukocytes
ROS	reactive oxygen species
ROT	rotenone
SOD	superoxide dismutase
TBARS	thiobarbituric acid-reactive substance
TMPD	tetramethyl-1,4-phenylenediamine
TPA	12- <i>O</i> -tetradecanoylphorbol-13-acetate

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FIGURE 1. Mitochondria as the major source of intracellular superoxide in unstimulated monocytes/macrophages

In panel a, a representative profile of lucigenin-derived CL elicited following incubation of unstimulated monocytes/macrophages with 1 μ M lucigenin is shown. In panel b, intracellular superoxide production was detected by lucigenin-derived CL following incubation of unstimulated monocytes/macrophages with 1 μ M lucigenin in the presence or absence of rotenone (ROT) (10 μ M), FCCP (1 μ M), or KCN (0.2 mM). Values in panel b represent the mean $\pm$ S.E. from 5 experiments.

Figure 2. Release of superoxide from isolated mitochondria

In panel a, intramitochondrial superoxide was detected by lucigenin-derived CL following incubation of succinate-supported mitochondria with 5 μ M lucigenin in the presence or absence of MnTMPyP (5 μ M) or Cu,ZnSOD (500 units/ml). Values represent the mean $\pm$ S.E. from 3 experiments. In panel b, formation of the DEPMPO-OH (o) was measured by EPR spectrometry following incubation of succinate-supported mitochondria with 10 mM DEPMPO in the presence or absence of Cu,ZnSOD (500 units/ml).

FIGURE 3. Presence of extracellular superoxide with unstimulated monocytes/macrophages and its diminution in the presence of mitochondrial inhibitors

In panel a, extracellular superoxide was measured by SOD-inhibitable cytochrome c reduction following incubation of unstimulated monocytes/macrophages for the indicated times. In panel b, extracellular superoxide was measured by SOD-inhibitable cytochrome c reduction following incubation of unstimulated monocytes/macrophages with rotenone (ROT) (10 μ M) or FCCP (1 μ M) for 30 min. In panel c, formation of DEPMPO-OOH (o) and DEPMPO-OH (*) was measured by EPR spectrometry following incubation of unstimulated monocytes/macrophages with 10 mM DEPMPO in the presence or absence of

Cu,ZnSOD (500 units/ml), MnSOD (500 units/ml), or catalase (500 units/ml). In panel d, intracellular SOD activity was detected by SOD gel assay after incubation of unstimulated monocytes/macrophages with 500 units/ml Cu,ZnSOD (lane 2) or 500 units/ml MnSOD (lane 3) for 30 min. Lane 1 represents control cells. In panel e, formation of DEPMPO-spin adducts was detected by EPR spectrometry following incubation of unstimulated monocytes/macrophages with rotenone (ROT) (10 μ M), FCCP (1 μ M), or KCN (0.2 mM). In panels a and b, values represent the mean $\pm$ S.E. from 3 separate experiments.

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FIGURE 4. Normal cell surface marker expression, intact plasma membrane NAD(P)H oxidase, and decreased mitochondrial respiration in monocytes/macrophages with a deficient METC

In panel a, substrate-supported mitochondrial oxygen consumption was measured polarographically in digitonin-permeabilized control cells and EB- or CAP-treated cells. Curves represent the average of three experiments. P/M, pyruvate/malate (6 mM/6 mM); ROT, rotenone (0.1 μ M); Succ, succinate (6 mM); AA, antimycin A (0.1 μ M); Asc./TMPD, ascorbate/tetramethyl-1,4-phenylenediamine (1 mM/0.2 mM). The sequence of addition of the above substrates and inhibitors was identical for control, EB-, and CAP-treated cells. In panel b, the expression of monocyte/macrophage surface markers, CD11b, CD14, and CD16

was measured flow cytometrically in control cells and EB- or CAP-treated cells. Values represent mean $\pm$ S.E. from 3 experiments. In panel c, production of superoxide by plasma membrane NAD(P)H oxidase following activation by TPA was measured by SOD-inhibitable cytochrome c reduction in control cells and EB- or CAP-treated cells. Values represent mean $\pm$ S.E. from five experiments. In panel d, the expression of cytosolic p47^{phox} and p67^{phox} was determined by immunoblotting (representative experiment of three).

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FIGURE 5. Diminished presence of extracellular superoxide in unstimulated monocytes/macrophages with a deficient METC

In panel a, intracellular superoxide production was detected by lucigenin-derived CL in unstimulated control cells and EB-or CAP-treated cells. In panels b and c, presence of extracellular superoxide was measured by the SOD-inhibitable cytochrome c reduction assay and the DEPMPO-spin trapping, respectively, in unstimulated control cells and EB- or CAP-treated cells. In panels a and b, values represent the mean $\pm$ S.E. from at least 3 experiments.

FIGURE 6. Formation of peroxynitrite from reaction of nitric oxide with mitochondria-derived superoxide in unstimulated monocytes/macrophages

In panel a, formation of peroxynitrite was detected by luminol-derived CL following incubation of unstimulated monocytes/macrophages with 10 μM luminol in the presence or absence of the indicated concentrations of S-nitrosoglutathione and bicarbonate. Values represent the mean ± S.E. from at least 5 experiments. In panel b, formation of peroxynitrite was detected by luminol (10 μM)-derived CL following incubation of 100 μM S-nitrosoglutathione and 15 mM bicarbonate with unstimulated monocytes/macrophages in the presence or absence of Cu,ZnSOD (500 units/ml), rotenone (ROT) (10 μM), or FCCP (1

μM), or with EB- or CAP-treated cells. Values in both panels represent the mean $\pm$ S.E. from at least 3 experiments.

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FIGURE 7. Oxidation of LDL by unstimulated monocytes/macrophages

LDL oxidation was detected by the TBARS assay following incubation of LDL with the unstimulated monocytes/macrophages in the presence or absence of Cu,ZnSOD (500 units/ml), catalase (500 units/ml), rotenone (ROT) (10 μ M), or FCCP (1 μ M), or with EB- or CAP-treated cells. Values represent the mean $\pm$ S.E. from at least 3 experiments.

FIGURE 8. Schematic illustration of the potential relevance of the exit of mitochondria-derived superoxide in mononuclear cell-mediated pathophysiological processes
 $O_2^{\bullet -}$, superoxide; $NO^{\bullet}$, nitroxide; $ONOO^-$, peroxynitrite. It should be noted that superoxide that exits from mitochondria may also play a role in normal cellular signaling and physiology.