Absence of Electron Transport (Rho\(^0\) State) Restores Growth of a Manganese-Superoxide Dismutase-deficient Saccharomyces cerevisiae in Hyperoxia

EVIDENCE FOR ELECTRON TRANSPORT AS A MAJOR SOURCE OF SUPEROXIDE GENERATION IN VIVO\(^*\)

(Received for publication, May 19, 1993, and in revised form, August 19, 1993)

David M. Guidot, Joe M. McCord, Richard M. Wright, and John E. Repine
From the Webb-Waring Institute of Biomedical Research, Denver, Colorado 80262

To address the possibility that electron transport is a biologically significant source of superoxide anion (O\(^2-\)) during exposure to hyperoxia in vivo, we constructed Saccharomyces cerevisiae strains with selective disruptions in the gene encoding the mitochondrial manganese-containing superoxide dismutase (Mn-SOD) and/or genes encoding proteins critical for complexes in electron transport. We hypothesized that complete absence of electron transport would restore growth in hyperoxia to a Mn-SOD-deficient yeast. We found that yeast deficient in Mn-SOD activity failed to grow normally in hyperoxia (95% O\(_2\), 5% CO\(_2\)). In contrast, Mn-SOD-deficient yeast with complete absence of electron transport (the Rho\(^0\) state) grew normally in hyperoxia. By comparison, Mn-SOD-deficient yeast which were deficient only in cytochrome-c-oxidase, the terminal step in electron transport, had only partially restored growth in hyperoxia. Our results indicate that electron transport is a major source of O\(^2-\) in vivo, and that the principal site of this O\(^2-\) production is proximal to the cytochrome-c-oxidase complex.

Aerobic respiration, the fundamental source of energy production for most eukaryotic organisms, presents a biological dilemma. Oxygen consumption is an absolute requirement for nonglycolytic ATP generation and is nontoxic under normoxic conditions. However, high concentrations of oxygen damage cells via excess formation of toxic oxygen intermediates, including superoxide anion (O\(^2-\)), hydrogen peroxide (H\(_2\)O\(_2\)), and the hydroxyl radical ('OH) (1-5). O\(^2-\) produced when molecular oxygen undergoes a one-electron reduction, is actually a weak oxidant and its discrete molecular targets are largely unknown in vivo. O\(^2-\) can release iron from ferritin and initiate the Fenton reaction by reducing ferric iron (Fe\(^{3+}\)) to ferrous iron (Fe\(^{2+}\)) (6, 7). Ferrous iron is particularly damaging to the cell because it can initiate lipid peroxidation in membranes and other targets (8). Recently, evidence has accumulated that O\(^2-\) can react with nitric oxide to form peroxynitrite, a strong oxidant which could not only cause oxidative injury directly but also may give rise to the most powerful biologic oxidant, the hydroxyl radical (9). Whether or not these or other reactions involving O\(^2-\) account for any of the putative biological injury of O\(^2-\) remains speculative and an area of intense interest. However, because reactions involving O\(^2-\) may be key sources of the more powerful oxidants, H\(_2\)O\(_2\), and 'OH, intracellular control of O\(^2-\) concentration is undoubtedly vital to cell integrity.

Multiple antioxidant defense systems have evolved to reduce the toxicity of oxygen radicals. For example, aerobic cells possess a group of enzymes, the superoxide dismutases (SOD),\(^1\) whose sole function appears to be to reduce cellular O\(^2-\) by dismutation of O\(^2-\) to H\(_2\)O\(_2\) and O\(_2\) (10, 11). Other antioxidant systems, such as peroxidases, ascorbate, and vitamin E, also contribute to cellular oxidant-antioxidant balance (12). However, this balance can be upset by increased production of oxygen radicals. Indeed, O\(^2-\) generation during hyperoxia appears to overwhelm cellular defenses which are adequate under normoxic conditions.

Identification of the source(s) of O\(^2-\) in cells exposed to hyperoxia, as well as the specific targets for O\(^2-\) within the cell, is of considerable interest. Studies have demonstrated O\(^2-\) production by the mitochondrial electron transport chain, cytochrome P-450 system, oxidation of hemoglobin and catecholamines, xanthine oxidase, prostaglandin metabolism, and numerous other biological reactions in vitro (13). However, the degree to which each of these reactions contributes to hyperoxic cell injury is largely unknown in vivo. Evidence that O\(^2-\) contributes to hyperoxic cell injury is largely inferential and is mostly based only on experiments using chemical inhibitors of electron transport.

Disruption of electron transport in mammalian cells is lethal because it eliminates ATP generation. Therefore, biochemical inhibitors of electron transport, such as rotenone and cyanide, can only be used in isolated mitochondrial preparations. Experiments suggest that oxygen radicals are produced by electron transport when electron flow is interrupted with these inhibitors (13). However, these observations do not unequivocally mean that the electron transport chain forms significant amounts of O\(^2-\) in vivo. Resolution of this uncertainty has been further hampered because oxygen radicals are extremely short-lived species whose measurement is difficult in vivo.

Since mitochondrial respiration accounts for more than 95% of all oxygen consumption in aerobic cells, we hypothesized that the electron transport chain is a predominant source of O\(^2-\) production in cells exposed to hyperoxia. We chose to address this basic premise in yeast for several reasons. First, yeast are eukaryotic cells with an intracellular organization and bio-

\(^*\) All work was performed during the tenure of Dr. Guidot's Research Fellowship Award from the American Lung Association and supported by grants from the National Institutes of Health (ROI-45652, P50-HL27253, R11-HL08890), American Heart Association, Council for Tobacco Research Inc., Ronald McDonald Childrens Charities, Johnson & Johnson, Inc., Williams Family, Swan, Hill, and American Express Foundations. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

\(^\dagger\) To whom correspondence and reprint requests should be addressed: Box C922, 4300 East Ninth Ave., Denver, CO 80262.

\(^1\) The abbreviations used are: SOD, superoxide dismutase; COXVI, subunit IV of cytochrome-c-oxidase; kb, kilobase pair.
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chemistry that is similar to higher eukaryotes, including mammals. Second, yeast are facultative aerobes that can grow in the absence of functional electron transport, a feature which permits disruption of the respiratory chain at discrete sites. The impact of these specific disruptions on \( \Delta \psi \) generation can then be determined \textit{in vivo}. Third, yeast can be manipulated genetically to create strains deficient in putative sources of \( \Delta \psi \) generation and/or \( \Delta \psi \) scavenging.

MATERIALS AND METHODS

Yeast Growth Medium

Liquid cultures for growth curve determination and DNA and protein extraction were performed in standard YPD liquid medium, composed of 1% Bacto-yeast Extract (Difco, Detroit, MI), 2% Bacto-Peptone (Difco), and 2% dextrose. Selection of transformed strains was conducted in SD agar, composed of 0.67% yeast nitrogen base without amino acids (Difco), 2% dextrose, and 2% Bacto-agar (Difco). Amino acids were added at a concentration of 40 \( \mu \)g/ml.

Construction of Yeast Mutants

Host Strain—\textit{Saccharomyces cerevisiae} strain JM43 (MATa, leu2-3, 112, ura3-52, trp1-289, his4-550) was generously provided by Dr. Joan McEwen of UCLA (14, 15). JM43Rh0 was constructed by treating JM43 with ethidium bromide to selectively purge mitochondrial DNA.

Construction of Yeast Deficient in Cytochrome-c-oxidase (COXVI)

The plasmid pVIST3 (16) was used for disruption of the nuclear gene encoding the subunit VI of cytochrome-c-oxidase (COXVI). pVIST3 contains 1.6 kb from the COXVI genetic locus and bears a single BglII restriction endonuclease cleavage site that lies between the COXVI and thiocyanate promoters. The recombinant fragment was then used to transform JM43 with ethidium bromide to selectively purge mitochondrial DNA.

Construction of Yeast Deficient in Mn-SOD enzyme activity

—The plasmid pVIS23 (18) was used for disruption of the nuclear gene encoding the subunit VI of cytochrome-c-oxidase (COXVI). pVIS23 contains 1.6 kb from the COXVI genetic locus and bears a single BglII restriction endonuclease cleavage site that lies between the COXVI mRNA initiation sites and the COXVI promoter (16); its parent vector is pUC19 (16). The COXVI locus was disrupted by inserting the LEU2 gene into the BglII site of pVIS23 and transformation of JM43 with the 4.8-kb SalI fragment (17). Confirmation of the disruption was achieved using Southern blot analysis, Northern blot analysis, and Western immunoblot analysis of mitochondrial proteins. JM43GD6 is a nuclear petite mutant that selectively lacks subunit VI of cytochrome-c-oxidase.

This strain retains less than 2% of the wild type level of cyanide-sensitive \( \Delta \psi \) consumption.

Construction of Yeast Deficient in Mn-SOD

—The plasmid pFL41 containing the \textit{S. cerevisiae} Mn-SOD gene was provided by Dr. Jennifer Pinkham (New Haven, CT). pFL41 is a 9.7-kb plasmid derived from pBR322. It contains the 1.1-kb yeast URA3 fragment as well as the 2.1-kb Mn-SOD insertion. The 2.1-kb Mn-SOD fragment contains a unique PooII site at the amino terminus of the coding region which was targeted for disruption. The URA3 fragment was cloned into the PooII site of the Mn-SOD gene. The recombinant fragment was then used to transform JM43, JM43Rh0, and JM43GD6 (17). The resulting three yeast strains were each deficient in Mn-SOD but derived from distinct parent strains, two of which had mutations in electron transport genes (Table I). DGY1 was deficient only in Mn-SOD. DGY2 was deficient in Mn-SOD and cytochrome-c-oxidase. DGY3 was the Mn-SOD-deficient strain of JM43Rh0. All strains were otherwise isochromosomal to strain JM43.

Gel Electrophoresis Analysis of SOD Activity—Yeast were grown to end log phase in YPD medium. Protein was extracted by vortexing cultures with glass beads (425-600 \( \mu \)m, Sigma in 2% Triton X-100 (Sigma). Samples were centrifuged at 12,000 \( \times \) g and the supernatants were removed and placed into a Universal Electrophoresis Film Agarose gel (Fisher). SOD activity was determined using nitro blue tetrazolium formation of \( \text{Cu,Zn-SOD} \) and \( \text{Mn-SOD} \) (Sigma). Samples were centrifuged at 12,000 \( \times \) g and the supernatants were removed and placed into a Universal Electrophoresis Film Agarose gel (Fisher). SOD activity was determined using nitro blue tetrazolium

<table>
<thead>
<tr>
<th>Yeast strain</th>
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<th>Mn-SOD activity</th>
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<tbody>
<tr>
<td>JM43</td>
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<td>As strain JM43 but conf: LEU2</td>
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<td>DGY1</td>
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<td>DGY2</td>
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<td>Absent</td>
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DISCUSSION

Our principal findings are 1) that Mn-SOD-deficient yeast did not grow normally in hyperoxia and 2) that yeast deficient in mitochondrial electron transport, even with a coexisting deletion in Mn-SOD, grew normally in hyperoxia. These observations indicate that hyperoxia stimulates biologically significant formation of \( \Delta \psi \) by the mitochondrial electron transport chain \textit{in vivo}, and that Mn-SOD normally decreases the toxicity of \( \Delta \psi \) generated during exposure to hyperoxia.

TABLE I

Legend for yeast with genetic disruptions in electron transport and/or Mn-SOD

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<tr>
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<tr>
<td>DGY3</td>
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<td>As strain JM43GD6 but sod2: URA3</td>
<td>This study</td>
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DGY3  DGY2  DGY1  JM43

sod1  (Cu,Zn-SOD)
sod2  (Mn-SOD)

FIG. 1. Electrophoretic gels stained for SOD enzyme activity of protein extracts from the yeast strains JM43, DGY1, DGY2, and DGY3. The upper band represents sod1 (Cu,Zn-SOD) enzyme activity and the lower band (in the JM43 lane) represents sod2 (Mn-SOD) enzyme activity.

FIG. 2. Panel a, growth in normoxia of the JM43, JM43GD6, and JM43Rho0 yeast strains in YPD broth. Each value represents the mean ± S.E. of three or more determinations. Panel b, growth in normoxia of the DGY1, DGY2, and DGY3 yeast strains in YPD broth. Each value represents the mean ± S.E. of three or more determinations.

FIG. 3. Growth in hyperoxia (95% O₂, 5% CO₂) of the JM43, JM43GD6, and JM43Rho0 yeast strains in YPD broth. Each value represents the mean ± S.E. of three or more determinations.

In our model, selective absence of Mn-SOD effectively serves as a bioassay for O₂⁻ generation. Since the only known function of SOD in all organisms is the dismutation of O₂⁻ (11, 19), we presumed that any biological effect which occurs in yeast with a selective deficiency of SOD must be a consequence of O₂⁻. Moreover, because we selectively disrupted only the mitochondrial form of SOD, the observed effects are most likely due to O₂⁻ which is generated within the mitochondria. While elegant studies performed on isolated mitochondria and submitochondrial particles have demonstrated O₂⁻ production in vitro (3, 4), these preparations may be inherently altered since even careful isolation of mitochondria disrupts membranes and, accordingly, O₂⁻ production may not reflect what is occurring in vivo. Our genetic approach provided the opportunity to obtain direct evidence for a role of electron transport in O₂⁻ production during hyperoxic exposure in vivo, evidence which paralleled and supported the studies using biochemical inhibitors of respiration.

We found that Mn-SOD-deficient yeast did not grow normally in hyperoxia. This finding indicates the importance of Mn-SOD in decreasing hyperoxic stress and is consistent with previously described Mn-SOD gene disruptions in yeast (20) and with observations from Mn-SOD-deficient Escherichia coli (21). Since Mn-SOD is located specifically in the mitochondria and accounts for only 15% of the total cellular SOD activity in yeast (the balance is Cu,Zn-SOD), the decreased growth rate of Mn-SOD-deficient yeast during hyperoxic exposure shows that mitochondria produce biologically significant amounts of O₂⁻ in vivo. Elegant studies in E. coli have shown that respiring vesicles produce 3 O₂⁻/10,000 electrons transferred (22), a rate corresponding to an intracellular production of 5 μM/s. Thus, the respiratory chain in E. coli is responsible for the vast majority of the total cellular O₂⁻ burden. Our results suggest that respiration accounts for a substantial portion of the O₂⁻ produc-
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Most likely increases even when O$_2$ tension is normal or low. Under these conditions, mitochondrial respiration is impaired, ATP production is inhibited (23), and cellular stress can be induced by the subsequent O$_2^-$ production and the accompanying depletion of energy stores. In these scenarios, Mn-SOD may be overwhelmed with subsequent mitochondrial injury from unscavenged O$_2^-$. Our results also localize the site of O$_2^-$ production by the electron transport chain to sites which are proximal to cytochrome-c-oxidase, in accordance with studies performed in vitro (3, 22, 24). Selective disruption of this complex failed to restore normal growth in hyperoxia to the Mn-SOD-deficient yeast. Partial restoration of growth in hyperoxia in this strain may reflect the effect that cytochrome-c-oxidase deletion has on the redox state of proximal components in electron transport, specifically coenzyme Q (CoQ). In the normal state, the majority of CoQ exists as the free radical ubisemiquinone which can donate an electron to O$_2$ to form O$_2^-$. However, when electron flow from cytochrome c$_1$ to cytochrome-c-oxidase is inhibited, CoQ exists primarily in the reduced ubiquinol form which will not readily produce O$_2^-$(25). Thus, deletion of cytochrome-c-oxidase may reduce O$_2^-$ generation from the proximal CoQ site. This interpretation is consistent with studies which show no evidence for O$_2^-$ release from cytochrome-c-oxidase in vitro.

The biological consequences of excess mitochondrial O$_2^-$ production are likely substantial. A variety of disease states, including ischemia/reperfusion injury and hyperoxia, are associated with increased mitochondrial oxidant generation, mitochondrial oxidative injury, and/or ATP depletion (4, 26-28). Our work supports prior assumptions regarding the site of O$_2^-$ production within mitochondrial electron transport and the relevant importance of cellular O$_2^-$ generation. Nonetheless, many questions remain regarding the intracellular balance of O$_2^-$ and other reactive oxygen species. For example, sensitive targets for reactive oxygen species, as well as the other biologically relevant sources for their production, remain largely ill-defined or altogether unknown. Precise genetic models, such as this one, provide basic tools not available in mammalian systems to address these issues.

### Table II

<table>
<thead>
<tr>
<th>Yeast</th>
<th>Doubling time in hyperoxia (h)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>JM43</td>
<td>2 ± 0.2</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>DGY1</td>
<td>10.8 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>JM43GD6</td>
<td>2.9 ± 0.1</td>
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</tr>
<tr>
<td>DGY2</td>
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<tr>
<td>JM43Rho0</td>
<td>3.0 ± 0</td>
<td>0.2</td>
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<td>DGY3</td>
<td>2.75 ± 1</td>
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**REFERENCES**

Mitochondrial Superoxide Generation in Hyperoxia