Superoxide dismutase and hydrogen peroxide cause rapid nitric oxide breakdown, peroxynitrite production and subsequent cell death

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Abstract

Isolated copper/zinc superoxide dismutase (Cu/Zn-SOD) or manganese superoxide dismutase (Mn-SOD) together with hydrogen peroxide (H₂O₂) caused rapid breakdown of nitric oxide (NO) and production of peroxynitrite (ONOO⁻) indicated by the oxidation of dihydrorhodamine-1,2,3 (DHR) to rhodamine-1,2,3. The breakdown of NO by this reaction was inhibited by cyanide (CN⁻) or by diethyldithiocarbamate (DETC), both Cu/Zn-SOD inhibitors, and the conversion of DHR to rhodamine-1,2,3 was inhibited by incubating Cu/Zn-SOD with either CN⁻ or with high levels of H₂O₂ or by including urate, a potent scavenger of ONOO⁻. In the presence of phenol, the reaction of SOD, H₂O₂ and NO caused nitration of phenol, which is known to be a footprint of ONOO⁻ formation. H₂O₂ addition to macrophages (cell line J774) expressing the inducible form of NO synthase (i-NOS) caused rapid breakdown of the NO they produced and this was also inhibited by CN⁻ and by DETC. Subsequent ONOO⁻ production by the macrophages, via this reaction, was inhibited by CN⁻, high levels of H₂O₂ or by urate. H₂O₂ addition to i-NOS macrophages also caused cell death which was, in part, prevented by DETC or urate. We also found inhibition of mitochondrial respiration with malate and pyruvate as substrates, when isolated liver mitochondria were incubated with Cu/Zn-SOD, H₂O₂ and NO. Inhibition of mitochondrial respiration was partly prevented by urate. The production of ONOO⁻ by SOD may be of significant importance pathologically under conditions of elevated H₂O₂ and NO levels, and might contribute to cell death in inflammatory and neurodegenerative diseases, as well as in macrophage-mediated host defence. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Superoxide dismutase; Nitric oxide; Hydrogen peroxide; Peroxynitrite; Cell death; Macrophage

1. Introduction

Nitric oxide (NO) reacts with molecular oxygen (O₂), superoxide (O₂⁻) and transition metals to yield nitrogen dioxide (NO₂), peroxynitrite (ONOO⁻), and metal–nitrosyl adducts, respectively, and each of these products has characteristic physiological and/or pathological roles [1]. In fact, the known toxicity of NO in, for example, the neurotoxicity of stroke and ischaemia, Alzheimer’s disease, Parkinson’s disease, multiple sclerosis, motor neuron disease and inflammatory diseases, is probably mediated by these secondary products, particularly ONOO⁻ (reviewed in [2]).

Abbreviations: CN⁻, cyanide; Cu/Zn-SOD, copper/zinc superoxide dismutase; DETC, diethyldithiocarbamate; DHR, dihydrorhodamine-1,2,3; H₂O₂, hydrogen peroxide; i-NOS, inducible nitric oxide synthase; Mn-SOD, manganese superoxide dismutase; NO, nitric oxide; O₂⁻, superoxide; OH⁻, hydroxyl; ONOO⁻, peroxynitrite

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unpaired electron which can rapidly combine with the unpaired electron of NO to form ONOO$^-$. Levels of O$_2^-$ are kept low in the cell by the enzyme superoxide dismutase (SOD), which dismutates O$_2^-$ to hydrogen peroxide (H$_2$O$_2$) and O$_2$. Two major forms of SOD exist in mammals: (a) copper/zinc (Cu/Zn) SOD, found mainly in the cell cytoplasm, but also extracellularly, and (b) manganese (Mn) SOD, which is localised to the mitochondrial matrix. Both forms of SOD catalyse the dismutation reaction of O$_2^-$; however, in the presence of H$_2$O$_2$, SOD can produce potentially damaging oxidants such as the hydroxyl (OH$^-$) radical, and high levels of SOD can be damaging to cells [3].

ONOO$^-$ has been implicated in a wide range of pathological processes [2]. Nitrification of protein tyrosine residues is a convenient marker of ONOO$^-$ production in vivo [4], and nitrification of neurofilaments by ONOO$^-$ is enhanced by SOD [5]. This may be significant in neurodegenerative disorders such as amyotrophic lateral sclerosis (ALS), where missense mutations in SOD have been shown to cause degeneration of motor neurons in a proportion of familial ALS patients [6]. However, the only known significant source of ONOO$^-$ in vivo is the reaction of NO with O$_2^-$, and the rate of O$_2^-$ production is normally very low in cells. We report here for the first time an alternative source of ONOO$^-$: the reaction of NO, H$_2$O$_2$ and SOD, which may have pathological implications, especially under conditions with elevated NO and H$_2$O$_2$ levels.

2. Materials and methods

2.1. Measurement of NO levels

Measurement of NO levels were performed using a Clark-type NO electrode (World Precision Instruments) (based on [7]) inserted through the top of a sealed, thermostatted vessel. The NO electrode was connected to a chart recorder and NO decay estimated by calculating the gradient of decay at specific NO concentrations. Experiments were performed by injecting an aliquot of NO saturated water (see below) into the vessel, which contained 1 ml of sodium phosphate buffer (0.1 M), pH 7.4 at 37°C, stirred continuously at a constant speed. Most NO measurements were performed in air-saturated buffer, however, where anoxic conditions were required, this was provided by including 1.25 mM ascorbate and 2.5 µg/ml ascorbate oxidase, which removed all O$_2$ from the vessel, without producing any H$_2$O$_2$.

2.2. Preparation of NO

NO saturated water was prepared by purging a gas-tight glass vial filled with distilled de-ionised water with nitrogen for approximately 20 min and then bubbling pure NO gas (MG Gas Products) through the deoxygenated vial for a further 15 min. The concentration of NO in NO saturated water was taken as 2.0 mM at 20°C [8].

2.3. Preparation of washed Cu/Zn-SOD

Washed Cu/Zn-SOD was prepared by centrifuging a sample of enzyme in a centrifplus$^{®}$ concentrator (molecular mass cut-off of 10 kDa) at 3000×g for 135 min at 4°C and resuspending the sample at the desired concentration.

2.4. Fluorescence measurements

The formation of ONOO$^-$ was determined from the oxidation of non-fluorescent dihydrorhodamine-1,2,3 (DHR) to fluorescent rhodamine-1,2,3. Rhodamine-1,2,3 fluorescence was measured using a Perkin-Elmer MPF-44E fluorescence spectrophotometer connected to a chart recorder (excitation wavelength of 500 nm, emission wavelength of 536 nm; slit widths 2.5 and 3.0 nm, respectively). Experiments were performed in sodium phosphate buffer (0.1 M), pH 7.4 at 37°C in 3-ml fluorescence cuvettes to which was added 50 µM DHR (Molecular Probes). The fluorimeter was calibrated by adding known quantities of rhodamine-1,2,3 in sodium phosphate buffer. Also, known amounts of authentic ONOO$^-$ (see below) were added to calibrate the relationship between ONOO$^-$ formation and DHR conversion to rhodamine-1,2,3.

2.5. Preparation of ONOO$^-$

ONOO$^-$ was synthesised by reacting acidified
H₂O₂ with sodium nitrite (NaNO₂) at 4°C [9,10]. 5 ml NaNO₂ (0.6 M) were mixed with 5 ml acidified H₂O₂ (0.6 M HCl, 0.7 M H₂O₂) in a simple flow reactor (constructed by joining two 10 ml pipettes at their bases with a plastic Y-junction (3 mm internal diameter)). The solutions in their respective pipettes were allowed to flow together under gravity and mixed together in a plastic tube attached to the bottom of the Y-junction. The reaction mixture was then quenched into 5 ml NaOH (1.5 M) whilst stirring and residual H₂O₂ degraded by manganese dioxide (MnO₂) (2 g) incubation for 30 min. MnO₂ was removed by filtering the solution through an 0.45 µM nitrocellulose filter. Freeze fractionation of this solution produced an upper concentrated yellow band of ONOO⁻₃. ONOO⁻₃ concentrations were estimated spectrophotometrically (ε₃₀₂ = 1670 M⁻¹ cm⁻¹ [11]).

2.6. Nitration of phenol

Phenol nitration was measured using a scanning spectrophotometer (Hitachi U-2000) and scans taken after each reaction addition. Experiments were performed using 5 mM phenol in air-saturated sodium phosphate (0.1 M), pH 7.4 at 37°C.

2.7. Induction of i-NOS in macrophages

Macrophages (J774 murine macrophage cell line ATCC TIB 67) were maintained in suspension culture in Dulbecco’s Modified Eagle Medium (DMEM) with 4500 mg/l glucose and pyridoxine HCl, 10% foetal calf serum, 100 units/ml penicillin and 100 µg/ml streptomycin. Only cells with >95% viability (measured by trypan blue exclusion) were used for experiments. Inducible NO synthase (i-NOS) was induced in the macrophages (approximately 2×10⁵ cells/ml) by adding Salmonella typhimurium lipopolysaccharide (10 µg/ml) and interferon-γ (mouse recombinant, 50 units/ml) and incubating the cells at 37°C for 18 h in stirrer culture (5% CO₂). Activated cells were then resuspended (approximately 5×10⁶ cells/ml) in a Krebs–Hepes medium, pH 7.4. Measurements of NO decay and rhodamine-1,2,3 fluorescence caused by H₂O₂ addition to i-NOS macrophages were performed as described above in the presence of 1 mM l-arginine.

2.8. Assay of cell death

Estimates of i-NOS macrophage cell death caused by H₂O₂ addition were counted as cell membrane rupture (measured by trypan blue exclusion) after incubating the cells on plastic well plates at 37°C in DMEM containing 10% foetal calf serum. H₂O₂ additions (100 µM) were made every 15 min to the cells for 60 min and cell death counted at 30 and 60 min. Assays including the Cu/Zn-SOD inhibitor diethyldithiocarbamate (DETC) (10 mM) and the ONOO⁻ scavenger urate (100 µg/ml) were also included with appropriate controls. Washed Cu/Zn-SOD was prepared by centrifuging a sample of enzyme in a Centriplus concentrator (molecular mass cut-off of 10 kDa) at 3000×g for 135 min at 4°C and resuspending the sample at the desired concentration.

2.9. Preparation of liver mitochondria

Isolation medium was as follows: 250 mM sucrose, 5 mM Tris, 5 mM EGTA (pH 7.4) at 4°C. Liver was removed from a male rat (300 g) and placed in isolation medium on ice. The liver was chopped and rinsed and then homogenised with a Dounce homogeniser. The homogenate was then centrifuged at 3500 rpm for 5 min and then the supernatant centrifuged at 8500 rpm for 10 min. The resulting pellet was resuspended in medium and again centrifuged at 8500 rpm for 10 min. The final pellet was resuspended in approximately 250 µl medium and protein concentration assayed by Biuret test.

2.10. O₂ measurements

Measurements of mitochondrial O₂ consumption was performed in the following medium: 20 mM Hepes, 20 mM KCl, 5 mM KH₂PO₄, 5 mM MgCl₂, 225 mM sucrose, pH 7.4 at 37°C. Mitochondria were incubated in the presence of Cu/Zn-SOD, H₂O₂ and NO and also in the presence or absence of urate for 10 min at 37°C with appropriate controls. H₂O₂ and NO were added as 100 µM and 1 µM additions respectively every 2 min for 10 min. All other additions were made in a single dose at the start of incubation with the following concentrations: Cu/Zn-SOD, 50 mg/ml; urate, 50 µg/ml. The inhibition of mitochondrial O₂ consumption
caused by ONOO\(^{-}\) was also recorded. ONOO\(^{-}\) was added as five aliquots of 70 \(\mu\)M over 10 min, giving a total of 350 \(\mu\)M ONOO\(^{-}\). Five \(\mu\)M oxyhaemoglobin was added to each incubated sample before oxygen consumption was measured to ensure that NO (if added) was removed. Mitochondrial \(O_2\) consumption was measured using two different substrates: 5 mM malate and 5 mM pyruvate or 2.5 mM succinate, and state 3 respiration (in the presence of 1 mM ADP) and uncoupled rate (in the presence of 10 \(\mu\)M DNP) recorded. 10 \(\mu\)M rotenone was included when succinate was used as a substrate. All \(O_2\) consumption rates were measured over the range of 50–150 \(\mu\)M \(O_2\).

2.11. Materials

DMEM, penicillin and streptomycin were from Gibco-BRL. All other chemicals used were purchased from Sigma Chemical Co. Cu/Zn-SOD was from bovine kidney, suspended in 3.8 M (NH\(_4\))\(_2\)SO\(_4\), pH 7.0. Mn-SOD was from Escherichia coli.

3. Results

3.1. NO breakdown by SOD and \(H_2O_2\)

When an aliquot of NO is added to an air-saturated medium, it breaks down slowly due to its reaction with \(O_2\) in the medium (Fig. 1). We found that SOD and \(H_2O_2\) cause an increase in the breakdown rate of NO (Fig. 1 and Table 1) and that this reaction also occurred in the absence of \(O_2\) (Table 1). In the presence of a fixed amount of Cu/Zn-SOD, further additions of \(H_2O_2\) continued to cause rapid breakdown of added NO. The observed NO breakdown rate was lower with the second additions of \(H_2O_2\) and NO, but still significantly higher than the NO breakdown caused by \(O_2\) alone. This NO breakdown rate was maintained for at least twelve additions of 100 \(\mu\)M \(H_2O_2\) (3rd and 6th additions shown in Table 1). An increase in NO breakdown catalysed by SOD was also observed with a tenfold lower concentration of \(H_2O_2\) (Table 1). \(H_2O_2\), in the absence of SOD, caused no detectable increase in NO breakdown (Table 1). In the presence of Cu/Zn-SOD, the rate of NO breakdown was signifi- cantly decreased when either CN\(^{-}\) (10 mM) or DETC (10 mM), which inhibit Cu/Zn-SOD, were added to the enzyme, or when SOD was incubated with \(H_2O_2\) (0.33 mM for 10 min), which inactivates SOD (Table 1). DETC (10 mM) alone, DETC (10 mM) plus copper sulfate (CuSO\(_4\)) (10 \(\mu\)M) or CN\(^{-}\) (10 mM) alone did not cause detectable NO breakdown (Table 1). The presence of mannitol (10 mM) (a scavenger of OH\(^{•}\)) did not prevent the observed increased breakdown of NO in the presence of Cu/Zn-SOD and \(H_2O_2\) (Table 1), suggesting that the increased NO breakdown is not mediated by OH\(^{•}\) radicals. \(H_2O_2\) addition to washed Cu/Zn-SOD (to remove any potential free copper ions) caused rapid NO breakdown comparable to unwashed SOD. CuSO\(_4\) and \(H_2O_2\) did not cause a detectable increase in NO breakdown (Table 1). Recently it was shown that Cu/Zn-SOD acquires peroxidase activity at physiological pH only in the presence of bicarbonate [12]. Therefore we measured NO breakdown by Cu/Zn-SOD and \(H_2O_2\) in the presence of 20 mM sodium bicarbonate but found that the NO breakdown rate was similar to that in the absence of bicarbonate (0.180 ± 0.015 nmol NO/min/ml at 200 nM NO,
Mn-SOD also caused rapid NO breakdown in the presence of H₂O₂ (Table 1).

### 3.2. Conversion of DHR to rhodamine-1,2,3 in the presence of SOD, NO and H₂O₂

A fluorescent assay, involving the conversion of non-fluorescent DHR to fluorescent rhodamine-1,2,3, was used in order to detect ONOO⁻ formation during the reaction of NO with SOD and H₂O₂. Calibration of fluorescence was achieved by adding known amounts of rhodamine-1,2,3 to sodium phosphate buffer (0.1 M, pH 7.4) and also by adding authentic ONOO⁻ to DHR (50 µM) in sodium phosphate buffer (0.1 M, pH 7.4). ONOO⁻ production was detected during the reaction of SOD (Cu/Zn- and Mn-) with NO and H₂O₂ (Fig. 2). ONOO⁻ formation, expressed in terms of rhodamine-1,2,3 fluorescence, was recorded in the presence of SOD (50 µg/ml), NO (1 µM) and H₂O₂ (100 µM) (Table 2). Similar levels of rhodamine-1,2,3 fluorescence were observed when Mn-SOD, instead of Cu/Zn-SOD, was added to NO and H₂O₂ (Table 2). In the presence of Cu/Zn-SOD, rhodamine-1,2,3 fluorescence was significantly lowered when CN⁻ (10 mM), which inhibits Cu/Zn-SOD, was added to the enzyme or when SOD was preincubated with H₂O₂ (0.33 mM for 10 min), which inactivates SOD (Table 2). CN⁻ (10 mM) itself did not affect the fluorescence observed by adding authentic ONOO⁻ to dihydrorhodamine-1,2,3 (result not shown). No fluorescence was detected when Cu/Zn-SOD (50 µg/ml) was incubated with urate (50 µg/ml), which is a potent scavenger of ONOO⁻ [13] (Table 2). The reaction of SOD and H₂O₂, SOD and NO, or H₂O₂ and NO did not produce detectable ONOO⁻ (results not shown). Formate (100 mM), mannitol (50 mM) and arginine (100 mM) which all scavenge OH⁻ radicals, did not prevent ONOO⁻ production (Table 2). The addition of EDTA (10 mM), which binds divalent cations, or deferoxamine (10 mM), which primarily chelates ferric ions, had no effect on the observed fluorescence.

### Table 1

<table>
<thead>
<tr>
<th>Reaction</th>
<th>NO Breakdown Rate (nmol NO/min/ml)</th>
<th>n</th>
<th>NO at 200 nM</th>
<th>NO at 600 nM</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO</td>
<td>0.033 ± 0.002</td>
<td>6</td>
<td>0.165 ± 0.021</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>H₂O₂+NO</td>
<td>0.031 ± 0.005</td>
<td>3</td>
<td>0.166 ± 0.012</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Cu/Zn-SOD+H₂O₂+NO</td>
<td>0.175 ± 0.018</td>
<td>3</td>
<td>0.454 ± 0.041</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Cu/Zn-SOD+NO (no O₂)</td>
<td>0.028 ± 0.002</td>
<td>3</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Cu/Zn-SOD+H₂O₂+NO (no O₂)</td>
<td>0.159 ± 0.018</td>
<td>3</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Cu/Zn-SOD+3rd addition of (H₂O₂+NO)</td>
<td>0.089 ± 0.007</td>
<td>3</td>
<td>0.269 ± 0.024</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Cu/Zn-SOD+6th addition of (H₂O₂+NO)</td>
<td>0.090 ± 0.001</td>
<td>3</td>
<td>0.254 ± 0.005</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Cu/Zn-SOD+10 µM H₂O₂+NO</td>
<td>0.067 ± 0.000</td>
<td>3</td>
<td>0.230 ± 0.002</td>
<td>3</td>
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<tr>
<td>Cu/Zn-SOD+H₂O₂+NO+CN⁻</td>
<td>0.097 ± 0.01</td>
<td>5</td>
<td>0.183</td>
<td>2</td>
<td></td>
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<tr>
<td>NO+CN⁻</td>
<td>0.034 ± 0.007</td>
<td>3</td>
<td>0.167 ± 0.011</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Cu/Zn-SOD+H₂O₂+NO+H₂O₂ (pre.)</td>
<td>0.061 ± 0.020</td>
<td>3</td>
<td>0.150 ± 0.009</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Cu/Zn-SOD+H₂O₂+NO+DETCl</td>
<td>0.041 ± 0.004</td>
<td>3</td>
<td>0.197 ± 0.014</td>
<td>3</td>
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<tr>
<td>NO+DETCl</td>
<td>0.034 ± 0.00</td>
<td>3</td>
<td>0.178 ± 0.039</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>NO+DETCl+CuSO₄</td>
<td>0.036 ± 0.003</td>
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<td>0.156 ± 0.021</td>
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<tr>
<td>Cu/Zn-SOD+H₂O₂+NO+mannitol</td>
<td>0.283 ± 0.011</td>
<td>3</td>
<td>0.558 ± 0.015</td>
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<td></td>
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<tr>
<td>washed Cu/Zn-SOD+H₂O₂+NO</td>
<td>0.131 ± 0.009</td>
<td>3</td>
<td>0.435 ± 0.028</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>washed Cu/Zn-SOD+H₂O₂+NO+DETCl</td>
<td>0.041 ± 0.000</td>
<td>3</td>
<td>0.180 ± 0.002</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>CuSO₄+H₂O₂+NO</td>
<td>0.038 ± 0.003</td>
<td>3</td>
<td>0.170 ± 0.017</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Mn-SOD+H₂O₂+NO</td>
<td>0.289 ± 0.051</td>
<td>3</td>
<td>1.690 ± 0.095</td>
<td>3</td>
<td></td>
</tr>
</tbody>
</table>

Although 1 µM NO was added in each experiment, NO breakdown rates were recorded at 200 nM NO and 600 nM NO by drawing a tangent to the NO decay curve at these NO concentrations. Experiments were performed in air-saturated sodium phosphate (0.1 M), pH 7.4, at 37°C. Concentrations were: NO (1 µM), Cu/Zn-SOD (50 µg/ml), H₂O₂ (100 µM unless otherwise stated), CN⁻ (10 mM), DETCl (10 mM), mannitol (10 mM), CuSO₄ (10 µM) and Mn-SOD (50 µg/ml). In one treatment SOD was preincubated (pre.) with 0.33 mM H₂O₂ for 10 min to inactivate the enzyme. Results are expressed as mean ± S.D. of n experiments.
in the presence of Cu/Zn-SOD, H$_2$O$_2$ and NO (Table 2).

### 3.3. SOD, H$_2$O$_2$ and NO cause nitration of phenol

We observed an increase in absorbance over the wavelength range 350-450 nm when authentic ONOO$^-$ was added to phenol in sodium phosphate (0.1 M, pH 7.4) (Fig. 3), indicating nitration of phenol [14]. A very low level of nitration was observed when 5 µM NO alone was added to phenol (probably due to NO$_2$ resulting from the reaction of NO with O$_2$); however, phenol nitration was significantly increased when 5 µM NO was added in the presence of Cu/Zn-SOD and H$_2$O$_2$. The concentrations of phenol and SOD used in experiments did not absorb over this wavelength range and H$_2$O$_2$ addition to SOD in the presence of phenol caused no absorbance increase. Further phenol nitration caused by SOD, H$_2$O$_2$ and NO was evident after 15 min suggesting a time-dependence of ONOO$^-$ formation (result not shown).

### Table 2

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Rhodamine-1,2,3 fluorescence (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$t = 2$ min</td>
</tr>
<tr>
<td>Cu/Zn-SOD+H$_2$O$_2$+NO</td>
<td>0.078 ± 0.011</td>
</tr>
<tr>
<td>Cu/Zn-SOD+H$_2$O$_2$+NO+H$_2$O$_2$ (pre.)</td>
<td>0.019 ± 0.000</td>
</tr>
<tr>
<td>Cu/Zn-SOD+H$_2$O$_2$+NO+CN$^-$</td>
<td>0.023 ± 0.007</td>
</tr>
<tr>
<td>Cu/Zn-SOD+H$_2$O$_2$+NO+urate</td>
<td>0.000 ± 0.000</td>
</tr>
<tr>
<td>Cu/Zn-SOD+H$_2$O$_2$+NO+formate</td>
<td>0.073 ± 0.006</td>
</tr>
<tr>
<td>Cu/Zn-SOD+H$_2$O$_2$+NO+mannitol</td>
<td>0.085 ± 0.004</td>
</tr>
<tr>
<td>Cu/Zn-SOD+H$_2$O$_2$+NO+arginine</td>
<td>0.065 ± 0.014</td>
</tr>
<tr>
<td>Cu/Zn-SOD+H$_2$O$_2$+NO+EDTA</td>
<td>0.089 ± 0.010</td>
</tr>
<tr>
<td>Cu/Zn-SOD+H$_2$O$_2$+NO+desferrioxamine</td>
<td>0.079 ± 0.019</td>
</tr>
<tr>
<td>Mn-SOD (50 µg/ml)+H$_2$O$_2$+NO</td>
<td>0.087 ± 0.021</td>
</tr>
</tbody>
</table>

Rhodamine-1,2,3 fluorescence was recorded after 2 and 5 min after NO additions. Experiments were performed in air-saturated sodium phosphate (0.1 M), pH 7.4, at 37°C in the presence of 50 µM DHR. Concentrations were: Cu/Zn-SOD (50 µg/ml), H$_2$O$_2$ (100 µM), NO (1 µM), CN$^-$ (10 mM), urate (50 µg/ml), formate (100 mM), mannitol (50 mM), arginine (100 mM), EDTA (10 mM), desferrioxamine (10 mM) and Mn-SOD (50 µg/ml). In one treatment SOD was preincubated (pre.) with 0.33 mM H$_2$O$_2$ for 10 min to inactivate the enzyme. Results are expressed as mean ± S.D. of $n$ experiments.

Fig. 2. A representative fluorimetric trace showing ONOO$^-$ formation during the reaction of SOD and NO in the presence of H$_2$O$_2$ (conversion of non-fluorescent DHR to fluorescent rhodamine-1,2,3). Rhodamine-1,2,3 fluorescence was measured using an excitation wavelength of 500 nm and an emission wavelength of 536 nm (slit widths 2.5 and 3.0 nm, respectively). Experiments were performed in air-saturated sodium phosphate (0.1 M), pH 7.4, at 37°C.
3.4. \( \text{H}_2\text{O}_2 \) addition to activated macrophages causes rapid NO breakdown

Macrophage NO production was monitored with the NO electrode 18 h after i-NOS induction. Addition of \( \text{H}_2\text{O}_2 \) (100 \( \mu \text{M} \)) to the macrophages caused rapid NO breakdown (approximately 0.33 \( \mu \text{M} \) NO/min/10\(^6\) cells) (Fig. 4a). Addition of isolated Cu/Zn-SOD also caused NO decay in the presence of \( \text{H}_2\text{O}_2 \) (Fig. 4a). The addition of oxyhaemoglobin (1 \( \mu \text{M} \)) removed all the NO allowing the zero NO concentration to be determined. However, in the presence of 10 mM CN\(^-\), or when the cells were incubated with 10 mM DETC for 30 min, \( \text{H}_2\text{O}_2 \) did not cause detectable breakdown of NO produced from activated macrophages (Fig. 4b), suggesting that the NO breakdown was mediated by active SOD inside the cells.

3.5. \( \text{H}_2\text{O}_2 \) addition to activated macrophages causes conversion of DHR to rhodamine-1,2,3

\( \text{H}_2\text{O}_2 \) addition (33 \( \mu \text{M} \)) to macrophages (approximately 5.3 \( \times \) \( 10^6 \) cells) also caused conversion of DHR to rhodamine-1,2,3 and this fluorescence increase was inhibited by CN\(^-\) (10 mM), \( \text{H}_2\text{O}_2 \) (10 mM incubated for 30 min), or by urate (17 \( \mu \text{g/ml} \)) (Table 3).

3.6. \( \text{H}_2\text{O}_2 \) addition to activated macrophages causes cell death

\( \text{H}_2\text{O}_2 \) addition (4 aliquots of 100 \( \mu \text{M} \) in 60 min) to activated macrophages caused significant cell death at 30 and 60 min (approximately 18% cell death at 60 min) (Fig. 5). Macrophage cell death was significantly reduced when \( \text{H}_2\text{O}_2 \) was added to macrophages in the presence of the Cu/Zn-SOD inhibitor DETC (10 mM) or in the presence of the ONOO\(^-\) scavenger urate (100 \( \mu \text{g/ml} \)). Neither DETC (10 mM)

---

Table 3

\( \text{H}_2\text{O}_2 \) addition to macrophages expressing i-NOS causes conversion of DHR to rhodamine-1,2,3

<table>
<thead>
<tr>
<th>Condition</th>
<th>Rhodamine-1,2,3 fluorescence (nM) (after 210 s)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>i-NOS macrophages+( \text{H}_2\text{O}_2 )</td>
<td>267.5 ( \pm ) 10.8</td>
<td>5</td>
</tr>
<tr>
<td>i-NOS macrophages+CN(^-)+( \text{H}_2\text{O}_2 )</td>
<td>52.3 ( \pm ) 7.7</td>
<td>6</td>
</tr>
<tr>
<td>i-NOS macrophages+( \text{H}_2\text{O}_2 ) (10 mM preincubated for 30 min)+( \text{H}_2\text{O}_2 )</td>
<td>47.1 ( \pm ) 1.9</td>
<td>3</td>
</tr>
<tr>
<td>i-NOS macrophages+urate+( \text{H}_2\text{O}_2 )</td>
<td>46.7 ( \pm ) 5.8</td>
<td>3</td>
</tr>
</tbody>
</table>

Experiments were performed with approximately 5.3 \( \times \) \( 10^6 \) cells with DHR (50 \( \mu \text{M} \)) in Krebs-Hepes buffer (pH 7.4) at 37°C. Concentrations were: \( \text{H}_2\text{O}_2 \) (33 \( \mu \text{M} \)), CN\(^-\) (10 mM), and urate (17 \( \mu \text{g/ml} \)). Results are expressed as mean \( \pm \) S.D. of \( n \) experiments.
Fig. 4. (a) H$_2$O$_2$ addition to macrophages expressing i-NOS causes rapid NO breakdown. Experiments were performed with approximately $5 \times 10^6$ i-NOS macrophages in a Krebs-Hepes medium (pH 7.4) at 37°C. The addition of 1 μM oxyhaemoglobin removed all the NO. Trace is representative of three experiments. (b) H$_2$O$_2$ addition to i-NOS macrophages preincubated with DETC (10 mM for 30 min) does not cause NO breakdown. Experiments were performed with approximately $5 \times 10^6$ i-NOS macrophages in a Krebs-Hepes medium (pH 7.4) at 37°C. Trace is representative of three experiments.
alone nor urate (100 μg/ml) caused significant cell death. The same level of H₂O₂ addition to control macrophages (cells not expressing i-NOS and therefore not producing NO) did not produce significant cell death after 60 min; cell death was 6.0 ± 1.0% (n = 9) in these cells.

3.7. SOD, H₂O₂ and NO cause inhibition of liver mitochondrial respiration

ONOO⁻ has been shown to inhibit mitochondrial cis-aconitase and respiratory complexes I and II [15] and as they may be significant cytotoxic targets of ONOO⁻, we tested whether the addition of NO, H₂O₂ and SOD to isolated liver mitochondria could cause inhibition of respiration. Haemoglobin was added to the mitochondria after the SOD reaction but prior to measuring the O₂ consumption rate, in order to remove any reversible inhibition of cytochrome oxidase by NO itself. Cu/Zn-SOD, H₂O₂ and NO caused significant inhibition of liver mitochondrial uncoupled respiration and state 3 respiration when malate and pyruvate were used as substrate (Figs. 6 and 7, respectively). The state 3 inhibition caused by SOD, H₂O₂ and NO was completely prevented by adding urate to the mitochon-

<table>
<thead>
<tr>
<th>Table 4</th>
<th>Succinate state 3 and uncoupled liver mitochondrial respiration is not inhibited by Cu/Zn-SOD, H₂O₂ and NO</th>
</tr>
</thead>
<tbody>
<tr>
<td>State 3 rate (nmol O/min/mg)</td>
<td>Uncoupled rate (nmol O/min/mg)</td>
</tr>
<tr>
<td>No addition</td>
<td>100.8 ± 1.1</td>
</tr>
<tr>
<td>+SOD+H₂O₂+NO</td>
<td>100.8 ± 1.1</td>
</tr>
<tr>
<td>+350 μM ONOO⁻</td>
<td>1.3 ± 0.5</td>
</tr>
</tbody>
</table>

O₂ consumption was measured after 10 min incubation of treatment in the presence of 10 μM rotenone at 37°C and after the addition of 5 μM oxyhaemoglobin. Concentrations used were: mitochondria, 1 mg; succinate, 2.5 mM; ADP, 1 mM (state 3); DNP, 10 μM (uncoupled); SOD, 50 μg/ml; H₂O₂, 5 additions of 100 μM in 10 min; NO, 5 additions of 1 μM in 10 min. Results are expressed as mean ± S.D. of three experiments.
dria and the inhibition of the uncoupled rate was partially prevented with urate. 350 μM ONOO$^-^-$ significantly inhibited both the state 3 rate and the uncoupled rate. No inhibition was observed in the presence of SOD and H$_2$O$_2$, SOD and NO, or H$_2$O$_2$ and NO. Neither SOD, nor H$_2$O$_2$, nor NO alone caused inhibition of respiration. Urate itself did not affect the state 3 or uncoupled rates. The reaction of Cu/Zn-SOD, H$_2$O$_2$ and NO caused no inhibition of the state 3 rate or the uncoupled rate when succinate was used as substrate (Table 4). Although both complexes I and II are known to be ONOO$^-^-$-sensitive [15], under the conditions we used, respiration on succinate was less sensitive to ONOO$^-^-$ produced from the reaction of SOD, H$_2$O$_2$ and NO.

4. Discussion

The present results indicate that SOD (Cu/Zn- and Mn-) with H$_2$O$_2$ causes an increase in the breakdown rate of NO with concomitant ONOO$^-^-$ formation, indicated by the conversion of DHR to rhodamine-1,2,3. Recently, the specificity of DHR as a sensitive indicator of ONOO$^-^-$ has been demonstrated [16], in that neither H$_2$O$_2$, O$_2^-$ nor physiological levels of NO are capable of significant DHR oxidation. We have expressed ONOO$^-^-$ formation as fluorescence of rhodamine-1,2,3, however the addition of 1 μM ONOO$^-^-$ caused the production of about 0.25 μM rhodamine-1,2,3, and we can use this to estimate how much ONOO$^-^-$ was produced from NO. Our results suggest an estimated maximal production of ONOO$^-^-$ of roughly 0.75 μM after 10 min from 1 μM NO addition to Cu/Zn-SOD (50 μg/ml) and H$_2$O$_2$ (100 μM). The observed increase in NO breakdown and subsequent ONOO$^-^-$ formation are inhibited by the addition of CN$^-^-$ (10 mM) or by incubating Cu/Zn-SOD with H$_2$O$_2$ – both inactivators of this form of SOD, suggesting involvement of the active enzyme in this reaction. DETC, a specific inhibitor of Cu/Zn-SOD, operates by removing the copper atom from the active site of the enzyme. No increased NO breakdown was recorded after H$_2$O$_2$ and NO addi-
tion to DETC treated Cu/Zn-SOD and this suggests the involvement of the copper atom of Cu/Zn-SOD in the present reaction. We could not include DETC in reactions where we measured fluorescence, as we found that DETC itself quenched rhodamine-1,2,3 fluorescence. However, CN⁻ and H₂O₂, which were used to inhibit SOD, had no effect on rhodamine-1,2,3 fluorescence. The zinc atom of Cu/Zn-SOD appears to play a structural stabilising role and is not involved with the dismutation reaction of this form of SOD [17] and probably has little or no function in the present reaction. The presence of urate, a scavenger of ONOO⁻, had no effect on the fluorescence of rhodamine-1,2,3 itself, but prevented the SOD-catalysed conversion of DHR to rhodamine-1,2,3, further indicating that ONOO⁻ is produced during the reaction of Cu/Zn-SOD, H₂O₂ and NO.

OH⁺ radicals can be produced by SOD, and therefore to test whether this radical is involved in the reaction, we included scavengers of OH⁺; formate, mannitol (k = 2.7 × 10⁹, pH 7.0 [18]) and arginine (k = 2.1 × 10⁹, pH 7.0 [19]), in the fluorimetric assay of ONOO⁻, and mannitol in the measurement of NO breakdown. The presence of these scavengers did not cause a decrease in the amount of rhodamine-1,2,3 fluorescence observed during the course of the reaction (Cu/Zn-SOD, H₂O₂ and NO in the presence of DHR), nor did mannitol decrease the SOD catalysed NO breakdown. These results therefore make OH⁺ an unlikely candidate for involvement in the present reaction. To exclude the involvement of free copper ions in this reaction, we prepared washed samples of Cu/Zn-SOD and with this enzyme preparation, H₂O₂ caused similar NO breakdown as with unwashed SOD. Also, the addition of 10 μM CuSO₄ and H₂O₂ did not cause any detectable in-

Fig. 7. Malate and pyruvate state 3 liver mitochondrial respiration is inhibited by Cu/Zn-SOD, H₂O₂ and NO. O₂ consumption was measured after 10 min incubation of each treatment at 37°C and after the addition of 5 μM oxyhaemoglobin (to ensure that all the NO was removed). Concentrations used were: mitochondria, 1 mg; malate and pyruvate, 5 mM; ADP, 1 mM (state 3); DNP, 10 μM (uncoupled); Cu/Zn-SOD, 50 μg/ml; H₂O₂, 5 additions of 100 μM in 10 min; NO, 5 additions of 1 μM in 10 min unless NO⁺ where the NO level was kept constant at 1 μM for 10 min; urate, 50 μg/ml; ONOO⁻, 350 μM. Results are expressed as mean ± S.D. of three experiments.
crease in NO breakdown. The addition of metal chelators EDTA and desferrioxamine had no effect on the conversion of DHR to rhodamine-1,2,3 during the reaction, suggesting that other trace metals are not involved in this reaction either.

We did not investigate the mechanism of NO breakdown and ONOO\(^-\) production. A plausible mechanism could involve a partial reversal of the dismutation reaction in the presence of SOD and H\(_2\)O\(_2\), causing reduction of the copper ion in Cu/Zn-SOD:

\[
\text{H}_2\text{O}_2 + \text{Cu}^{2+} \rightarrow \text{O}_2^\cdot + 2\text{H}^+ + \text{Cu}^+ \tag{1}
\]

This reaction of Cu/Zn-SOD is well characterised [20]. The O\(_2^\cdot\) so produced could then rapidly react with added NO:

\[
\text{O}_2^\cdot + \text{NO} \rightarrow \text{ONOO}^- \tag{2}
\]

In order for the reaction to be catalytic, the copper ion in Cu/Zn-SOD would have to be reoxidised, probably by NO itself:

\[
\text{Cu}^+ + \text{NO} \rightarrow \text{Cu}^{2+} + \text{NO}^- \tag{3}
\]

Metal-catalysed reduction of NO is well characterised [21]. The nitroxy anion (NO\(^-\)) so produced could either rapidly convert to nitrous oxide (N\(_2\)O), or that it might react with O\(_2\) to produce another molecule of ONOO\(^-\) [22]. However, we have shown that SOD and H\(_2\)O\(_2\) cause rapid NO breakdown even in the absence of O\(_2\) (Table 1), making ONOO\(^-\) formation from the reaction of NO\(^-\) and O\(_2\) unlikely.

In this work, we have shown that in the presence of approximately 3 \(\mu\)M Cu/Zn-SOD, subsequent additions of H\(_2\)O\(_2\) continued to cause breakdown of added NO (up to twelve additions of 1 \(\mu\)M NO) at a rate significantly higher than the NO breakdown caused by O\(_2\) alone and we can estimate that a minimum of 6 \(\mu\)M NO (of the total 12 \(\mu\)M NO added) was broken down by 3 \(\mu\)M Cu/Zn-SOD alone, suggesting that the reaction is in fact catalytic. An increase in the breakdown rate of NO and subsequent production of ONOO\(^-\) may have important clinical implications, especially in conditions with elevated NO and H\(_2\)O\(_2\) levels, e.g., in inflammatory conditions or after ischaemia [23]. Recently NO has been shown to inhibit catalase [24] and glutathione peroxidase [25], which might cause elevated H\(_2\)O\(_2\) levels and a subsequent increase in ONOO\(^-\) production. ONOO\(^-\) itself is an inhibitor of many cellular enzymes, including aconitase [26] and glutathione reductase [27].

In biological systems, ONOO\(^-\) will rapidly react with and nitrate phenolic compounds such as tyrosine, salicylic acid (2-hydroxybenzoic acid) and 4-hydroxyphenylacetic acid. In fact, tyrosine nitration is used as a marker of reactive nitrogen-centred oxidant production, but specifically as an ONOO\(^-\) footprint in vivo. Tyrosine nitration can be found from lung biopsy samples from patients with sepsis, pneumonia or adult respiratory distress syndrome [28,29]. ONOO\(^-\) does not inactivate Cu/Zn SOD, but can react to produce the highly reactive and toxic nitrogen ion (NO\(_2^+\)). Once in the active site, ONOO\(^-\) forms a cuprous adduct which can donate NO\(_2^+\) to phenolics. SOD-catalysis of nitration of neurofilaments has been suggested as a cause of motor neuron disease and this may contribute to the role of SOD in causing amyotrophic lateral sclerosis [2]. In fact, a specific ALS-mutation for Cu/Zn-SOD can lead to enhancement of free radical formation due to a decrease in the \(K_m\) for H\(_2\)O\(_2\) [30]. We observed nitration of phenol when NO was added to phenol in the presence of Cu/Zn-SOD and H\(_2\)O\(_2\) but we do not know whether this nitration was caused directly by ONOO\(^-\) or whether it was catalysed by SOD.

i-NOS can be induced in a variety of cell types and here we have shown that H\(_2\)O\(_2\) addition to activated J774 macrophages (which contain SOD) causes rapid breakdown of the NO they produce and that this breakdown is inhibited by 10 mM CN\(^-\) or by incubating the cells with 10 mM DETC. The further addition of isolated Cu/Zn-SOD, which is not permeable to the cells, caused further NO breakdown, suggesting that the present reaction may operate inside the cells, but also extracellularly as well. We have also shown the conversion of DHR to rhodamine-1,2,3 when H\(_2\)O\(_2\) is added to these cells and that this reaction is inhibited by incubating the cells with either 10 mM CN\(^-\) or with 10 mM H\(_2\)O\(_2\), or by including urate which is an ONOO\(^-\) scavenger. The amount of rhodamine-1,2,3 fluorescence equates to approximately 1 \(\mu\)M ONOO\(^-\) production suggesting then that most, if not all the NO produced by the cells is oxidised to ONOO\(^-\). We have also demon-
strated that H$_2$O$_2$ addition to activated macrophages can cause significant cell death within 60 min and that this death can, in part, be prevented by treating the cells with the Cu/Zn-SOD inhibitor DETC or by including the ONOO$^-$ scavenger urate. These results suggest that cellular ONOO$^-$ production involving SOD may be of pathological importance under conditions of elevated NO and H$_2$O$_2$ levels.

ONOO$^-$ is known to be an inhibitor of the mitochondrial electron transport chain [15] and here, we have also shown that the reaction of SOD, H$_2$O$_2$ and NO can cause significant inhibition of the O$_2$ consumption of liver mitochondria respiring on malate and pyruvate. Both the state 3 rate and uncoupled rate are inhibited by this reaction and the presence of urate partially prevents the inhibition. We do not know whether this mitochondrial inhibition contributes to the cell death we observed when H$_2$O$_2$ was added to i-NOS macrophages. Respiration on succinate, which is a FAD-linked substrate, was not significantly affected by SOD, H$_2$O$_2$ and NO. Thus the reaction of SOD, H$_2$O$_2$ and NO appears to be inhibiting either mitochondrial complex I or some component of the citric acid cycle. Since mitochondria are a primary cytotoxic target of the ALS mutants [31], these findings are consistent with this reaction contributing to the pathology of familial ALS, and possibly ALS generally.

High levels of SOD have been shown to be cytotoxic in a variety of in vitro systems [3,32]. An extra copy of the Cu/Zn-SOD gene has been suggested to contribute to Down’s syndrome [33]. A small proportion of patients with motor neuron disease have a variety of mutations in SOD which cause the disease [6] and these mutations, in general, do not affect the dismutation activity of SOD [34], but rather cause it to gain a cytotoxic function which has been suggested to involve SOD catalysed protein tyrosine nitration by ONOO$^-$ [5]. The findings presented here, that SOD catalyses ONOO$^-$ production, suggest the possibility that the cytotoxicity of SOD may be due to this reaction.

In conclusion, our findings suggest the possibility that increased breakdown of NO and concomitant production of ONOO$^-$ in the presence of SOD may be of significant importance pathologically under conditions of elevated H$_2$O$_2$ and NO levels.

Acknowledgements

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References