Induction of Superoxide Dismutase in Leukocytes by Paraquat: Correlation With Age and Possible Predictor of Longevity

By Yukie Niwa, Koichi Ishimoto, and Tadashi Kanoh

Reactive oxygen species (ROS) are thought to play a role in the aging process as well as in a number of human diseases states. Superoxide dismutase (SOD), an enzyme that scavenges the superoxide anion \((\mathbf{O}_2^-)\) is constitutively expressed in leukocytes and other tissues. When assayed in peripheral blood leukocytes (PBL), constitutive SOD activity shows little variation among individuals of different ages. We have found that significant induction of SOD activity occurs in PBL incubated in vitro with paraquat, an agent known to cause intracellular \(\mathbf{O}_2^-\) production. This induction was found to be highly age dependent; leukocytes from 36 healthy subjects aged 20 to 40 years showed an increase of 85% ± 10%, versus an increase of only 8% ± 1% for leukocytes from 30 healthy subjects aged 65 to 79 years (\(P < 10^{-4}\)). Forty subjects, aged 67 to 73 years, who were healthy at the time of assay of leukocyte SOD induction were followed up 5 years later. Nineteen of these subjects had died; all 19 had shown SOD induction of less than 10% (range, 0% to 7%; mean, 2.4%). In contrast, of the 21 survivors (range, 2.5% to 50%; mean, 21%), 12 had shown SOD induction greater than 10%, and 7 had shown SOD induction ≥35% (\(P < 10^{-3}\)). Thirteen of the 19 deaths were attributable to malignancy or cerebrovascular disease. Preservation of leukocyte SOD inducibility appears to correlate with longevity in elderly individuals and may be of value in predicting resistance to malignancy or fatal cardiovascular events.

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Ficoll-Hypaque (FH) gradient centrifugation under conditions previously shown to maintain maximum cell viability. Neutrophils or lymphocytes (3 x 10⁷/mL) suspended in RPMI medium containing 10% fetal calf serum (FCS) were incubated with 2.45 x 10⁻⁹ or 2.45 x 10⁻¹⁰ mmol/L paraquat (Wakojunyaku Kogyo Co, Osaka, Japan) at 37°C for 18 hours in a humidified 5% CO₂ atmosphere. Thereafter cells were harvested by centrifugation and sonicated, and the supernatants were obtained after centrifugation at 14,800g for 20 minutes.

For the assay of SOD activity, 0.2 mL of supernatant was added to the xanthine-xanthine oxidase O₂⁻ generation system. This system consisted of 16.5 mmol/L ferricytochrome c, 0.1 mmol/L hypoxanthine, and 1.25 mmol/L EDTA in total volume of 2 mL of 125 mmol/L phosphate buffer. After the addition of cell supernatant, 0.006 U/mL of dialyzed xanthine oxidase was added to generate O₂⁻. Under these assay conditions the amount of SOD required to inhibit the rate of reduction of cytochrome c by 50% (i.e., to a rate of 0.0125 absorbance at 550 mmol/L U/min) was defined as 1 U of activity. Since percent inhibition in leukocytes is not linear above approximately 1 U, it was adjusted according to Asada's formula.⁴ The SOD induction capacity of each leukocyte supernatant was calculated from the following formula:

\[
\% \text{ SOD Induction} = \left( \frac{b - a}{a} \right) \times 100,
\]

where a is SOD activity in the absence of paraquat and b is SOD activity in the presence of paraquat.

For the neutrophil phagocytosis assay, emulsions of paraffin oil containing oil red O were prepared as previously described, except that a lipopolysaccharide solution was replaced with normal human serum. Neutrophils (2 x 10⁶ cells/0.9 mL Krebs-Ringer phosphate buffer (KRP) were preincubated with paraquat for 6 or 18 hours and washed. Thereafter 0.1 mL of the opsonized emulsion was added to the neutrophil suspension, and the mixture was incubated for 5 minutes at 37°C. Then 9 mL ice-cold KRP was added to the solution to stop the reaction. The cells were washed three times with ice-cold KRP to remove the paraffin oil droplets that had not been ingested. Paraffin oil containing oil red O was extracted from the cells by the method of Bligh and Dyer⁴⁹ using chloroform and methanol (vol/vol, 1:2), and the optical density of the chloroform layer was determined at a wavelengths of 525 nm.

The lymphocytes that had been preincubated for 6 or 18 hours with paraquat were examined for their responses to PHA. Briefly, 3 x 10⁵ lymphocytes suspended in RPMI 1640 medium containing 20% heat-inactivated pooled human AB serum and 2 x 10⁵ mitomycin-C-treated monocytes were incubated 3 days at 37°C in a humidified 5% CO₂ atmosphere in the presence of 10 μg/mL PHA. Lymphocyte blastogenesis was assessed by the DNA uptake of tritiated thymidine (³H) Tdr (2 Ci/mmol/L, New England Nuclear, Boston, MA) during the last 24 hours of culture.

The results were expressed as the mean ± SD of replicate assay. Statistical significance was determined by χ² and by Student's t test.

### RESULTS

In preliminary experiments at paraquat concentrations of 2.45 x 10⁻³ and 2.45 x 10⁻² mmol/L and an incubation time of 18 hours, substantial induction of SOD activity was seen in both lymphocytes and neutrophils while cell viability remained greater than 90% (Figs 1 through 3). Lymphocytes showed a greater capacity for SOD induction than neutrophils, particularly with an 6-hour incubation period (Fig 1). For each individual, determinations of SOD induction were carried out with both neutrophils and lymphocytes, each at paraquat concentrations of 2.45 x 10⁻³ and 2.45 x 10⁻² mmol/L. For each individual subject, the percent induction reported in this study was the highest of the four separate assays.

In preliminary experiments, the addition of low concentrations of PHA to the lymphocyte culture during the incubation with paraquat increased the % SOD induction, but it did not significantly improve lymphocyte viability (Fig 2). Furthermore, the presence of PHA for longer periods (48 to 96 hours) reduced cell viability, as shown in Figs 2 and 3. Therefore in this study lymphocytes were incubated for 18 hours in the presence of 2.45 x 10⁻³ mmol/L or 2.45 x 10⁻² mmol/L paraquat without addition of PHA.

Lymphocytes from 36 subjects aged 20 to 40 years

<table>
<thead>
<tr>
<th>Cells</th>
<th>Paraquat Conc. (mmol/L)</th>
<th>Incubation Time</th>
<th>% Induction</th>
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<tbody>
<tr>
<td>neutrophils</td>
<td>2.45 x 10⁻⁴</td>
<td>6 hr</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.45 x 10⁻³</td>
<td>6 hr</td>
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</tr>
<tr>
<td></td>
<td>2.45 x 10⁻²</td>
<td>6 hr</td>
<td></td>
</tr>
<tr>
<td>neutrophils</td>
<td>2.45 x 10⁻⁴</td>
<td>18 hr</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.45 x 10⁻³</td>
<td>18 hr</td>
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<tr>
<td></td>
<td>2.45 x 10⁻²</td>
<td>18 hr</td>
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<tr>
<td>lymphocytes</td>
<td>2.45 x 10⁻⁴</td>
<td>6 hr</td>
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<td></td>
<td>2.45 x 10⁻³</td>
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<td>2.45 x 10⁻²</td>
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<td>lymphocytes</td>
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<td>2.45 x 10⁻³</td>
<td>18 hr</td>
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<tr>
<td></td>
<td>2.45 x 10⁻²</td>
<td>18 hr</td>
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Fig 1. Effect of paraquat concentration and time of incubation on percent SOD induction in neutrophils and lymphocytes from young adults. Leukocytes from 10 healthy donors, aged 20 to 40 years, were assessed in triplicate. Results are expressed as mean ± SEM.
LONGEVITY PREDICTED BY SOD INDUCIBILITY

Fig 2. Correlation of cell viability with paraquat concentration. (A) Lymphocytes. Cell viability as assessed by trypan blue exclusion (——) and proliferative response to 10 μg/mL PHA (----). ○, 6-hour incubation; ●, 18-hour incubation at 37°C with the indicated concentrations of paraquat. (B) Neutrophils. Cell viability by trypan blue (——) and by phagocytosis using paraffin oil (----). ○ and ● are as in A. Donors were as in Fig 1. Results are expressed as mean ± SEM (N = 10).

incubated with $2.45 \times 10^{-2}$ mmol/L paraquat showed 85.1% ± 10.3% SOD induction, whereas lymphocytes from 30 subjects aged 65 to 79 years incubated with the same concentration of paraquat showed only 8.0% ± 0.9% induction (Table 1, $P < .001$). Similarly SOD induction in neutrophils was significantly higher in the younger adults (28.4% ± 3.6%) than in the elderly subjects (3.5% ± 0.3%; $P < .01$). As shown in Fig 4, SOD induction declined with

Fig 3. Correlation of incubation time to leukocyte viability (N = 6). (A) Lymphocyte viability after incubation for the indicated periods of time at 37°C with paraquat, $2.45 \times 10^{-3}$ mmol/L (O) or $2.45 \times 10^{-2}$ mmol/L (●). (B) Neutrophil viability after incubation for the indicated periods of time at 37°C with paraquat, $2.45 \times 10^{-3}$ mmol/L (O), or $2.45 \times 10^{-2}$ mmol/L (●). —— and —— are as defined in legend for Fig 2; donors were as in Fig 1.
Table 1. Percent SOD Induction of Neutrophils and Lymphocytes From Asymptomatic Younger and Aged Individuals

<table>
<thead>
<tr>
<th>Group/N</th>
<th>Lymphocytes</th>
<th>Neutrophils</th>
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<tbody>
<tr>
<td>Younger adults (20-40 yrs)/36</td>
<td>85.1 ± 10.3</td>
<td>28.4 ± 3.6</td>
</tr>
<tr>
<td>Aged individuals (65-79 yrs)/30</td>
<td>8.0 ± 0.9</td>
<td>3.5 ± 0.3</td>
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Lymphocytes and neutrophils were incubated at 37°C for 18 hours with 2.45 × 10^{-2} mmol/L paraquat, respectively, and assessed for SOD induction as described in Methods.

age beginning in the fifth decade, increasing slightly in those over 80 years old.

Forty subjects, 67 to 73 years of age and in good health upon entry into the study, were assessed for SOD induction. Over the next 5 years, 19 of these subjects died, while 21 survived. The 19 nonsurvivors had originally shown markedly lower SOD induction (mean 2.4%) compared with both young adult controls (P < .0001) and the 21 survivors (mean 21.1%, P < .01; Tables 1 and 2). None of the nonsurvivors showed SOD induction above 7%; in contrast, one third of the 21 survivors (cases 3, 6, 8, 11, 14, 17, 19) showed potent SOD induction (35% to 50%). As shown in Table 3, the number of survivors who showed SOD induction above 10% was far greater than that of nonsurvivor (P < .001). Among the nonsurvivors, seven cases died of malignancies (cases 23, 24, 27, 28, 30, 31, 34) and five of cerebrocardiovascular disease (cases 33, 37, 38, 39, 40; two with myocardial infarction, two with strokes and one with Burger's disease; Table 2). As shown in Table 1 and Figs 1 and 2, SOD induction in neutrophils was qualitatively similar to that in lymphocytes but quantitatively lower.

DISCUSSION

In this study, we describe a reliable technique for assessing the induction of SOD activity in leukocytes subjected to oxygen toxicity caused by the presence of the drug paraquat, which had previously only been reported to induce SOD activity in bacteria. It has been reported that in bacteria and human cancer cell lines, only Mn-SOD is induced under oxidative toxicity or inducers such as TNF or IL1. However, in our study not only Cu, Zn-SOD, but also Mn-SOD has been induced to the similar extent (submitted for publication).

Application of this assay of SOD induction using paraquat to normal human subjects demonstrated a marked age-related decline in SOD induction beyond 40 years of age. Interestingly there was a slight increase in SOD induction in those over 80 years of age compared with those in the eighth decade. This finding suggested that preservation of the capacity for SOD induction might be correlated with increased longevity. A similar pattern has been observed in studies of neutrophil chemotaxis in the elderly.

To test the hypothesis that SOD induction might be correlated with survival, we performed a longitudinal study of 40 initially healthy elderly subjects whose SOD induction was assayed upon entry into the study. There was a striking difference with respect to paraquat-induced SOD induction between the 21 subjects who survived 5 years and the 19 subjects who died during this follow-up period. Of the 19 deaths, 13 were due to either malignancies or cardiovascular disease, disease processes for which there may be a pathogenetic role for ROS and lipid peroxides. The results of the 5-year longitudinal study thus are consistent with the earlier cross-sectional study, suggesting that the preservation of SOD induction capacity is correlated with survival to advanced age. Furthermore, these studies raise the possibility that measurement of SOD induction in an individual subject may serve as a predictor of longevity as well as of predisposition to malignancy and cardiovascular disease.

There has been a controversial report that by SOD...
enzyme, oxidative damages in the cells and organs will be increased instead of being reduced; increasing the SOD content of erythrocytes predisposed these cells to greater oxidant damage because SOD will push superoxide into hydrogen peroxide, which is more cytotoxic than superoxide. However, one of the main principles of SOD action mechanism is considered to be as follows: superoxide radical that is scavenged by SOD directly inactivates various enzymes, including catalase and glutathione peroxidase. In this connection, in the presence of sufficient amount of SOD, H₂O₂ is also well quenched with catalase (and glutathione peroxidase), which are protected from being degraded or inactivated by superoxide radical.

After many preliminary experiments, paraquat was chosen to induce oxygen stress in preference to direct addition of O₂⁻, ROS, or lipid peroxides because the latter additions produced unacceptable reduction in cell viability. The mechanism of SOD induction by paraquat is presumed to be the generation of intracellular O₂⁻, with subsequent stimulation of SOD synthesis, perhaps by the action of one or more as yet undetermined intermediate compounds.

Although the lack of induction of SOD activity in response
to paraquat is most likely due to a defect in the steps leading to enzyme synthesis, we have not excluded the possibility that lack of induction of SOD activity in some cell preparations in response to paraquat may be due to an inhibition of enzyme synthesis itself induced by paraquat toxicity. This explanation may be applicable in cases in which induction was seen at a paraquat concentration of \(2.45 \times 10^{-3}\) mmol/L but not at \(2.45 \times 10^{-2}\) mmol/L. Preliminary evidence in support of this hypothesis has been obtained in intrinsic labeling experiments using \(^1\)H leucine uptake into the SOD enzyme and other proteins\(^3\) in our laboratory (Niwa et al, unpublished data, November 1989).

Alternatively lack of induction may reflect a constitutive level of intracellular SOD sufficient to quench the ROS generated by the concentration of paraquat used. This explanation may be applicable in cases in which induction was seen at a paraquat concentration of \(2.45 \times 10^{-3}\) mmol/L but not at \(2.45 \times 10^{-2}\) mmol/L. Further experiments will be necessary to investigate in more detail the intracellular mechanisms involved in paraquat-induced SOD induction. In addition, it is required to examine in future studies whether paraquat causes a generalized increase in protein synthesis in treated cells. However, regardless of the mechanism, our studies indicate a striking correlation between age and longevity on the one hand and the response of leukocyte SOD activity to paraquat on the other hand. It is well known\(^{15,14}\) that paraquat mediates \(O_2^-\) production, and it is surmised that this leads to OH - production with resultant nuclear chromosome aberration and DNA injury and that long use of this cytotoxic herbicide will be the cause for carcinogenesis and malformation. Investigation of this correlation of aging with SOD induction using paraquat, along with previously reported correlations of aging with neutrophil chemotaxis and serum-lipid peroxide levels,\(^6\) should provide new and important insight into the nature of the aging process and possibly carcinogenesis in humans.

REFERENCES

Induction of superoxide dismutase in leukocytes by paraquat: correlation with age and possible predictor of longevity

Y Niwa, K Ishimoto and T Kanoh