Determination of true specific activity of superoxide dismutase in human erythrocytes

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(Received 1 February 1982; accepted 20 April 1982)

Summary

1. Activities, contents and true specific activities of superoxide dismutase (SOD) were determined in human erythrocytes of 105 normal healthy subjects.

2. The mean SOD activity assayed by the inhibition of xanthine autoxidation was 11.0 x 10^3 units/g of haemoglobin (Hb).

3. The mean SOD content assayed by an immunochemical method was 456 µg/g of Hb.

4. Both activity and content of SOD showed normal distributions, and no significant variations with regard to sex and age were detected.

5. A highly positive correlation between activities and contents of SOD was observed in normal healthy subjects (r = 0.77, P < 0.001).

6. True specific activity was calculated from levels of activity and content of SOD. The mean true specific activity of SOD in human erythrocytes was 23.7 units/µg of SOD.

7. There was no significant difference in true specific activity between young and old subjects.

Key words: superoxide dismutase, human erythrocytes, true specific activity, sex and aging.

Introduction

Superoxide dismutase (SOD) (EC 1.15.1.1) catalyses the dismutation of superoxide anion to oxygen and hydrogen peroxide. Its biological role is considered to serve as a cell defence reaction against the potentially harmful effects of superoxide anions generated by a wide variety of biological reactions. Since the significance of this enzyme was described by McCord & Fridovich [1], there have been many studies on its structure, properties and mode of action [2, 3]. Recently, this enzyme has also received attention in the field of practical medicine. In spite of its biological significance and the clinical importance, fundamental investigations on SOD in erythrocytes have not yet been performed in detail. In this report, the activity, content and true specific activity of SOD in human erythrocytes were simultaneously determined in 105 normal healthy subjects and the applicability of true specific activity of SOD is discussed.

Materials and methods

Experimental subjects and their blood samples

Blood samples of 124 normal healthy subjects were drawn by a heparinized vacuum syringe from cubital veins in the morning. Whole blood was separated by centrifugation into blood plasma and erythrocyte fractions. Erythrocyte fractions were washed with saline three times and stored in a freezer at -80°C until examination. These subjects were the members of some workshops, nurses of some hospitals and the members of a home for the aged; they were healthy and had not been taking any medications within 7 days before the time of blood sampling. The Hb content of each haemolysate was measured cyanmethemoglobin method [4].
Determination of SOD activity

SOD activity in red-blood-cell lysates was assayed in chloroform/ethanol extracts by the method described by Winterbourn et al. [5] with some modifications. The assay of activity was basically the same as that described by Beauchamp & Fridovich [6], and SOD activity was determined on logit paper adopted by Kobayashi et al. [7]. The assay was based on the inhibition of the conversion of Nitro Blue Tetrazolium by SOD into a blue tetrazolium salt, mediated by superoxide radicals which were generated by xanthine oxidase. The assay was performed in 3 ml of sodium carbonate buffer (50 mmol/l; pH 10.2) containing xanthine (0-1 mmol/l), Nitro Blue Tetrazolium (25 μmol/l) and xanthine oxidase (5-8 nmol/l). The amount required to inhibit the rate of reduction of Nitro Blue Tetrazolium by 50% was defined as 1 unit of activity. The assay was performed at 25°C and the rate of reduction was followed at 560 nm with a Hitachi (Tokyo, Japan) model 200-20 spectrophotometer.

Determination of SOD content

Human erythrocyte SOD was purified by the method described by McCord & Fridovich [11]. This preparation was confirmed to be homogeneous by polyacrylamide-gel electrophoresis. For raising antiserum in rabbits, the purified SOD was injected subcutaneously as a water-in-oil emulsion in Freund's complete adjuvant, and it was boosted 4 times, once a week. The amounts of purified SOD used for immunizing the rabbits were 1.6 mg per animal. The specificity of the antiserum obtained against cytoplasmic SOD was checked by the agar-gel double-diffusion test. This antiserum was used for the present investigation. SOD content was assayed on simple lysates of erythrocytes. A standard curve was obtained with purified SOD.

Determination of SOD true specific activity

True specific activity means the activity per μg of enzyme. We estimated it from the values of activity and content of SOD by the following calculation:

\[
\text{SOD true specific-activity units/μg of SOD} = \frac{\text{SOD activity (units/g of Hb)}}{\text{SOD content (μg/g of Hb)}}
\]

Inhibition of SOD by cyanide

Cyanide was added to samples at various concentrations (0.01-0.1 mmol/l); these samples were incubated for 30 min at 25°C, and then assayed by the two methods described above.

Results

Determination of SOD activity in human erythrocytes

SOD activities of human erythrocytes were determined in 124 normal healthy Japanese aged from teens to eighties. SOD activity showed a normal distribution, and no significant variations were detected with regard to sex and age (Figs. 1 and 2). Mean SOD activity was 11.0 × 10³ units/g of Hb, with SD 1.44 × 10³ units/g of Hb.

Determination of SOD content in human erythrocytes

SOD contents were determined in the same samples (104 samples out of 124) as those used for the determination of SOD activity. SOD content also showed a normal distribution, and no significant variations were detected with regard to sex and age (Figs. 1 and 2). Mean SOD content was 456 μg/g of Hb, with SD 45.7 μg/g of Hb.

Relationships between SOD activity and SOD content

A highly positive correlation was observed in normal healthy subjects between SOD activity and SOD content (\(r = 0.77, P < 0.001\)) (Fig. 3).

Determination of SOD true specific activity

SOD true specific activities were determined in the same samples (\(n = 105\)) as those used for the determination of SOD levels. The mean true specific activities were: male 23.4 ± 0.29, female 24.1 ± 0.31, and in total 23.7 ± 0.22 units/μg of SOD (means ± SE) (Table 1).

Discussion

The average value of SOD activity (+SD) determined by the method described was 11.0 (±1.14) × 10³ units/g of Hb. This method has such a high sensitivity and specificity that we can measure small changes in SOD value. This method therefore seems to be more suitable for clinical work than those reported previously [1].
**Human erythrocyte superoxide dismutase**

**FIG. 1.** Distribution of the activity (a) and content (b) of superoxide dismutase (SOD) in human erythrocytes of normal healthy subjects. The means, standard deviations and numbers of subjects (n) are shown.

**FIG. 2.** Superoxide dismutase (SOD) activity (a) and content (b) in human erythrocytes of normal healthy subjects in each age group: O, male; ●, female. The means and standard errors are shown. Black points in parentheses are values obtained in one subject.

**FIG. 3.** Correlation between SOD activity and SOD content in human erythrocytes of normal healthy subjects (n = 105).

The average value of SOD content (456 μg/g of Hb) in the present paper is very close to that (461.4 μg/g of Hb) determined by Michelson *et al.* [9] and that (500 μg/g of Hb) by Winterbourn *et al.* [5], but it is lower than that (700 μg/g of Hb) reported by Stansell & Deutsch [10] and that (850 μg/g of Hb) by Del Villano & Tischfield [11].

In our experiments, we used packed red cells and we express both activity and content of SOD per g of Hb. In some experiments previously reported [12, 13], whole blood was used for the assay of blood cell enzymes. This procedure does not seem to provide accurate values, since Hb...
TABLE 1. True specific activity of superoxide dismutase in human erythrocytes of normal healthy subjects in each age group

Mean values ± SE; range and numbers of subjects (n) are shown. *: Mean values, range and numbers of subjects (n) are shown.

<table>
<thead>
<tr>
<th>Age group (years)</th>
<th>True specific activity (units/µg of SOD)</th>
<th>Male</th>
<th>Female</th>
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<tbody>
<tr>
<td>10–19</td>
<td>23.2 ± 1.54 (16.0–26.1) (n = 6)</td>
<td>23.8</td>
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<tr>
<td>20–29</td>
<td>23.2 ± 0.58 (19.0–26.1) (n = 14)</td>
<td>24.6 ± 0.39 (21.9–26.2) (n = 10)</td>
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<td>30–39</td>
<td>25.4 ± 0.55 (23.9–27.7) (n = 6)</td>
<td>24.7 ± 0.39 (22.9–27.4) (n = 9)</td>
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<tr>
<td>40–49</td>
<td>23.0 ± 0.53 (17.7–25.2) (n = 14)</td>
<td>23.0 ± 0.83 (18.6–25.7) (n = 10)</td>
<td></td>
</tr>
<tr>
<td>50–59</td>
<td>22.9 ± 0.74 (17.9–26.8) (n = 11)</td>
<td>23.2</td>
<td>(20.6–25.9)* (n = 4)</td>
</tr>
<tr>
<td>60–69</td>
<td>24.0 (23.2–24.8)* (n = 2)</td>
<td>26.2</td>
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<tr>
<td>70–79</td>
<td>24.0 ± 0.79 (22.0–25.7) (n = 5)</td>
<td>23.8 ± 0.39 (17.4–27.0) (n = 6)</td>
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<tr>
<td>80–89</td>
<td>24.0 (22.4–25.6)* (n = 2)</td>
<td>25.4</td>
<td>(24.5–26.8)* (n = 4)</td>
</tr>
<tr>
<td>Total</td>
<td>23.4 ± 0.29 (n = 60)</td>
<td>24.1 ± 0.31 (n = 45)</td>
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<tr>
<td>Grand total</td>
<td>23.7 ± 0.22 (n = 105)</td>
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Fig. 4. Inhibitory effects of cyanide on superoxide dismutase activity and content in human erythrocytes. Activity (○) and content (□) are expressed as percentages of that in controls without cyanide. Cyanide was added to samples, and they were incubated for 30 min at 25°C, and then assayed by the two methods described in the text.

Hb content for catalase estimation. As to the determination of SOD levels, since Hb itself is understood to be the principal source of superoxide anion, we took Hb contents into consideration and we expressed SOD levels in terms of Hb concentration. But the use of true specific activity seems to be ideal, since it is not influenced by the effect of Hb.

Reiss & Gershon [16] demonstrated a considerable decrease in SOD specific activity in the liver, a very small decrease in specific activity in the heart, and no decrease in the brain, in aged rats and mice. Furthermore, they also showed that the true specific activity of the 'old' cytoplasmic SOD, i.e. obtained from livers of old rats, amounts to about 40% of the 'young' enzyme in purified enzyme fractions [17]. In human erythrocytes, we confirmed no significant difference in true specific activity between young and old subjects.

Fig. 4 showed the effect on SOD activity and content after the addition of KCN to the sample. With KCN at 0.1 mmol/l approximately 90% of SOD activity was inhibited, while the content of SOD determined by our method was unaffected. Some red-cell enzymes lose their activity in diseases despite normal enzyme contents. Shapira et al. [18] discovered enzymically inactive carbonic anhydrase B in red blood cells in a family with renal tubular acidosis. Our normal values for SOD true specific activity in erythrocytes could be applied to the study of such inactive forms of the enzyme in various diseases.

References

Human erythrocyte superoxide dismutase


