SUBCELLULAR SUPEROXIDE DISMUTASE ACTIVITY IN PHAGOCYTOZING HUMAN BLOOD POLYMORPHONUCLEAR LEUCOCYTES

Christian AUCLAIR, Jacques HAKIM and Pierre BOIVIN
U 160 (INSERM), ERA 573 (CNRS), Université Paris VII and Hôpital Bichat, Paris, France

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1. Introduction

Both cyanide-sensitive and -insensitive superoxide dismutase (SOD) (Superoxide oxidoreductase EC 1.15.1.1) have been identified in human polymorphonuclear leucocytes (PMN) [1,2] and located both in the cytosol and in the granule fraction of the cells [3]. This enzyme catalyzes the dismutation of superoxide anion (O_2^-) into hydrogen peroxide (H_2O_2) and is thus thought to be protective against the highly reactive O_2^- in PMN as well as in other tissues of aerobic organism [2–5]. However, the functional activity of PMN depends on the production of O_2^- [6] and an inhibitory effect of SOD on the leukocytic bactericidal activity has been reported [7]. On the other hand, SOD shows a strong inhibitory effect on the O_2^--mediated chain reaction involved in the aerobic oxidation of NAD(P)H, likely responsible for the cyanide-insensitive burst of oxygen associated with phagocytosis [8–10]. These observations suggested that the function of SOD in PMN, could be more complicated than initially proposed, and needed to be further investigated. In this way, the present communication is concerned with the determination of the effect of phagocytosis on SOD activity in subcellular fractions isolated from human PMN.

Address for reprint requests: J. Hakim, Laboratoire Central d'Immunologie et d'Hématologie, Hôpital Bichat, 170 B Ney, 75877 Paris Cédex 18, France

2. Materials and methods

2.1. *Isolation of leucocytes*

Human leucocytes were isolated from heparinized venous blood, as previously described [11]. The final leucocyte pellet was resuspended in calcium-free Krebs Ringer phosphate buffer 0.01 M (KRP) (pH 7.4) containing 5.5 mM glucose and 10% AB serum. Isolated cells were adjusted at a concentration of 10^7 PMN/ml.

2.2. *Stimulation of leucocytes*

Zymosan (Sigma Chemical Co. St Louis, USA) suspended in KRP (10 mg/ml) was opsonized in the presence of 50% AB serum, during 30 min at 37°C. After centrifugation, the opsonized zymosan was resuspended at its initial concentration in KRP containing 10% AB serum. Isolated cells, at a concentration of 10^7 PMN/ml (9 vol.) were mixed with opsonized zymosan (1 vol.), after prior equilibration of the solutions at 37°C. Incubation time was 1 min. Stimulation was terminated by the addition of an equal volume of ice-cold 20% sucrose solution, containing 0.08 M Tris (pH 7.4). Suspension of resting cells were treated identically, except that zymosan was omitted in KRP.

2.3. *Preparation of chloroform–ethanol extract*

Resting and stimulated cells were ruptured by sonication during 50 s. Cells breakage was estimated by microscopy and found to be 100%. The disrupted cells were centrifuged at 350 × *g* for 10 min, to

remove large debris and zymosan particles. Aliquot of 350 × *g* supernate was centrifuged at 15 000 × *g* during 15 min and the resulting pellet was resuspended in the initial volume of KRP. Superoxide dismutase was extracted from the 350 g supernate, the 15 000 × *g* supernate and the resuspended 15 000 × *g* pellet, by chloroform–ethanol treatment essentially according to McCord and Fridovich [12]. Water–ethanol phase was used in the SOD assay procedure.

2.4. *Superoxide dismutase assay*

The assay relies on the ability of superoxide dismutase to inhibit the O_2^--mediated reduction of nitroblue tetrazolium dye (NBT) [13]. Photochemical reduction of riboflavin was used as O_2^--generating system. The incubation medium, for measurement of total SOD, contained in final vol. 1 ml: 0.025 M phosphate buffer (pH 7.8), 10^{-4} M EDTA, 0.15 mM NBT, 1 μM riboflavin and 0.1 ml water–ethanol phase. (NBT and riboflavin were from Sigma Chemical.) Tubes were placed in an aluminium box maintained at 25°C and illuminated with a mercury lamp (Carl Zeiss ST41). Reduced NBT was measured spectrophotometrically at 550 nm after 10 min exposure to light. 100% reduction of NBT was evaluated with water–ethanol phase extracted from heat denatured subcellular fraction. SOD activities were calculated by using a standard curve as represented in fig.1. This curve was constructed with commercial canine superoxide dismutase (Sigma Chemical Co.) which contained 3650 U/mg, according to the manufacturer specification. Cyanide-insensitive SOD was measured with 2 mM cyanide added to the reaction mixture to inhibit all cyanide-sensitive enzyme. Cyanide-sensitive SOD activity was calculated from the difference between the two assays: with and without cyanide in the incubation medium.

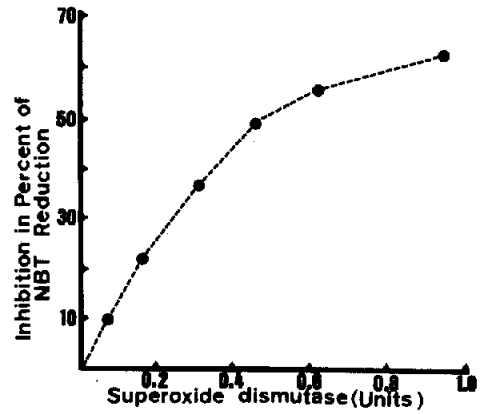

Fig.1. Calibration curve performed with commercial canine SOD (Sigma Chemical Co.). Units are those specified by the manufacturer. Assays were performed as described in methods, KCN omitted from the incubation medium. The maximal inhibition varied between 70% and 80% for canine SOD. Maximal NBT reduction rate seen in the presence of boiled SOD was 0.021 *A*/min.

3. Results

Results are summarized in table 1. In resting cells, cyanide-insensitive SOD activity was recovered both in the cytosol and in the granule fraction (15 000 × *g* pellet). The 15 000 × *g* supernate contained about 20% of total cyanide-insensitive SOD activity

Table 1
Cyanide-insensitive superoxide dismutase activity

	Resting cells (Sigma units/10^6 PMN)	Stimulated cells	Significant differences
350 × *g* Supernate	0.59 ± 0.11	0.18 ± 0.09	$p < 0.001$
15 000 × *g* Supernate	0.15 ± 0.06	0.04 ± 0.03	$p < 0.01$
15 000 × *g* Pellet	0.55 ± 0.19	0.50 ± 0.15	$p > 0.5$

Effects of phagocytosis on cyanide-insensitive SOD activity. SOD activity was assayed as described in Materials and methods. Values are the means ± 1 SD of six different experiments. Significance of the differences was calculated by the students *t*-test for unpaired data.

recovered. A cyanide-sensitive SOD activity was also recovered. It was found (data not indicated) that about 60% of the SOD activity of the 15 000 × *g* supernate was cyanide-sensitive, whereas in 15 000 × *g* pellet, more than 80% of SOD activity was cyanide-insensitive. In phagocytozing cells, values indicated in table 1 show that the stimulation of the cells by opsonized zymozan induced a significant decrease in the SOD activity of the 350 × *g* supernate and of the 15 000 × *g* supernate. In contrast, no significant decrease in SOD activity was observed when measured in the isolated 15 000 × *g* pellet. The presence of the 15 000 × *g* supernate is thus required for the inhibition of the 15 000 × *g* sedimentable SOD. It was verified that SOD activity of the 15 000 × *g* pellet isolated from resting cell was inhibited when resuspended in 15 000 × *g* supernate isolated from stimulated cells (data not indicated).

4. Discussion

In our experiments, the larger part of cyanide-insensitive SOD activity was found in the 15 000 × *g* pellet as reported by Rister and Baehner in guinea pig PMN [14]. The utilisation of NBT as an electron acceptor in the SOD assay, avoids the possible interference by peroxidase which is present at a high level in this fraction.

The inhibition of SOD in the activated PMN could be important as an explanation of the metabolic activation associated with phagocytosis. Salin and McCord have pointed out the hypothesis that, during phagocytosis, the hydrogen peroxide produced could be responsible of the inactivation of SOD [3]. They, however, did not confirm their hypothesis as they did not observe a decrease in the SOD activity after 30 min of phagocytosis. In our experiments, a short stimulation by opsonized zymosan, induced a large decrease in SOD activity suggesting the possible participation of this phenomenon in the burst of oxygen consumption and associated metabolic events. The following evidence support this hypothesis: granule fraction contains for a large part the enzyme responsible of the oxidative metabolism and is likely the site of production of the superoxide anion [15,16]

On the other hand, O_2^- is both a product and a necessary intermediate of the oxidase reaction. It has, also, been demonstrated that the aerobic oxidation of NADH and NADPH responsible for the production of H_2O_2 and O_2^- during phagocytosis was strongly inhibited by superoxide dismutase [9,17]. The very strong inhibitory effect of SOD on oxidase reaction is justified by the fact that the process of O_2^--mediated NAD(P)H oxidation revolves around enzymatic [10,17] or non-enzymatic chain reaction [9]. In this occurrence, a slight decrease in SOD activity induces a strong activation of the chain reaction. Since SOD is present in the granule fraction as well as in the other fractions of the cell, a high level of the enzymatic activity should be incompatible with the oxidative burst associated with phagocytosis unless its activity is inhibited. The mechanism of SOD inhibition remains to be elucidated.

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