NADH OXIDATION AND OXYGEN REDUCTIONS BY THE GRANULE-RICH FRACTION ISOLATED FROM HUMAN POLYMORPHONUCLEAR BLOOD CELLS

Involvement of a cyclic chain reaction

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1. Introduction

When exposed to appropriate stimuli the PMNs (polymorphonuclear blood leukocytes) undergo an activation of their oxidative metabolism (reviewed [1]) The purpose of that appears to be the elaboration of reduced forms of O_2, including superoxide anion ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2). Reduced nicotinamides (NAD[P]H) are unanimously regarded as the electron donor of these reductions. The primary enzyme of the oxidative metabolism is thought to be a CN-insensitive NAD[P]H oxidase that catalyzes the conversion of O_2 into $O_2^{\cdot-}$ and H_2O_2 It has been reported that the granule-rich fraction (GRF), isolated from the PMNs shows NADH and NADPH oxidase activities and has consequently been proposed as the subcellular site in which H_2O_2 generation takes place [2–4]. Several pertinent studies [1] suggest that $O_2^{\cdot-}$ is generated first and gives rise to H_2O_2 according to one of the following two reactions

$$O_2^{\cdot-} + O_2^{\cdot-} + 2\,H^+ \longrightarrow O_2 + H_2O_2 \qquad (1)$$

$$O_2^{\cdot-} \xrightarrow{+\,1\,e} O_2^{2-} \xrightarrow{+\,2\,H^+} H_2O_2 \qquad (2)$$

If this was the case, the production of H_2O_2 should be half or equal to that of $O_2^{\cdot-}$. This, however, is not the case since during the GRF-mediated oxidative metabolism the amount of H_2O_2 produced is much larger than that of $O_2^{\cdot-}$ [1,5,6]. This apparent discrepancy suggests that a cyclic chain reaction between $O_2^{\cdot-}$, O_2 and NADH could account for the large H_2O_2 production. The purpose of our paper is to show that it does.

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2. Materials and methods

2.1. *Isolation of the GRF*

Human PMN were isolated from heparinized venous blood of normal healthy volunteers as in [4]. The erythrocyte were sedimentated, at room temperature, with Dextran T-500 (Pharmacia, Uppsala) in 0.9% NaCl. Contaminated erythrocytes in the leukocyte-rich plasma, were removed by NH_4Cl lysis. The cells collected by centrifugation were resuspended in 10% sucrose containing 0.04 M Tris–HCl (pH 7.4) (sucrose–Tris) to give a final 1×10^7 to 2×10^8 PMN/ml. Final preparation routinely contained 80–90% PMN. Suspended cells were stored overnight at −80°C.

After the suspended cells were thawed, the GRF was isolated as in [4]. The GRF isolation included the step of straining through a glass wool column, and of dialysis against a Ca^{2+}-free 0 1 M phosphate buffer (pH 5.5) (phosphate buffer), containing 10^{-4} M Na_2-EDTA, for 24 h at 4°C. After the dialysis, the GRF was recentrifuged at 15 000 × *g* and the pellet resuspended in its intial volume, in an EDTA-free phosphate buffer

2.2. *Assay of NADH oxidation activity*

NADH oxidation activity was measured by the production of NAD^+ which was measured fluorometrically as in [4,6]. In the assay medium, hereafter referred to as the standard assay medium, the following materials were incubated in final vol. 0.8 ml: 0.1 M citrate phosphate buffer (pH 5.0), 2 mM KCN (daily prepared), 0.1% bovine serum albumin, 2.5 mM NADH (extemporaneously prepared) and 0.1 ml (80 μg protein) of the GRF (or boiled GRF) or phosphate buffer

2 3. *Assay of O_2 uptake, H_2O_2 and $O_2^{\cdot-}$ formation*

O_2 uptake was measured as in [4], by polarographic method using a Gilson oxygraph fitted with a Clark (Yellow Springs Instruments) oxygen electrode, in a cylindrical 1.6 ml glass chamber, at 37°C. The standard assay medium was as above, except that 0.2 ml (160 μg protein) of the the GRF was used to keep all concentrations identical to those of the NADH oxidation assay. The H_2O_2 formed was calculated by the liberated O_2 (polarographically measured) after addition of 100 μg catalase into the incubation medium, before or after the measurement of O_2-uptake. This technique was as in [4]. $O_2^{\cdot-}$ production was measured by the superoxide dismutase (SOD)-inhibitable reduction of ferricytochrome *c* [7] as in [4]. Concentrations of ferricytochrome *c* used are given in the results

2.4. *Miscellaneous*

In some stated experiments $O_2^{\cdot-}$ was generated in the standard assay medium (whose modifications when used are specified) by photolysis of H_2O [8] in the chamber of the Gilson oxygraph which was illuminated with a mercury lamp (Zeiss Quartz Brenner). The $O_2^{\cdot-}$ generated was measured by SOD-inhibitable reduction of ferricytochrome *c* [7].

$O_2^{\cdot-}$ generated by photolysis of H_2O was also measured after determination of the SOD apparent inhibitable O_2 consumption [9]. This technique relies on the ability of SOD to dismutase 2 $O_2^{\cdot-}$ according to the following reaction

$$2\,O_2^{\cdot-} \xrightarrow{+\,2\,H^+} H_2O_2 + O_2$$

and can be used if this reaction (which liberates one O_2 from 2 $O_2^{\cdot-}$) does not spontaneously occur (in the absence of SOD). This condition was verified in the incubation medium used. The $O_2^{\cdot-}$ generated by the photolysis of water in the absence of NADH and of the GRF (or in the presence of boiled GRF) as quantitated by the reduction of ferricytochrome *c* was ~0.9 $nmol.min^{-1}.ml^{-1}$. SOD (20 μg) completely inhibited this reduction. O_2 uptake, in the absence of ferricytochrome *c* was equal (>95%) on a molar basis, to the above-measured ferricytochrome *c* reduction. When SOD was added to the medium (ferricytochrome *c* omitted), O_2 uptake diminished by 50% as expected from the SOD-catalyzed dismutation of 2 $O_2^{\cdot-}$ into H_2O_2 and O_2 in its ground state. Addition of catalase (50 μg) did not modify the initial amount of ferricytochrome *c* reduced, while it diminished (in the absence of ferricytochrome *c*) the O_2 uptake by 25% as expected from its catalatic effects on the H_2O_2 formed. These results showed that the SOD-inhibitable O_2 uptake (in the absence of ferricytochrome *c*) could be used for the measurement of $O_2^{\cdot-}$ generated in our incubation medium. We realize that this technique does not measure in all conditions exactly all the $O_2^{\cdot-}$ generated. The $O_2^{\cdot-}$ measured will thus be equal to or lower than its real rate of formation.

Protein concentration was determined by the Lowry method [10], with bovine serum albumin as a standard.

All measurements were performed in duplicate with appropriate blanks and standards. Any additions or variations in the standard assay medium or in the experimental procedure are specified in the text and legends.

Chemicals NADH, NAD^+, NADPH, $NADP^+$, ferricytochrome *c* and catalase were purchased from Boehringer Mannheim; SOD, nitroblue-tetrazolium (NBT) and bovine serum albumin were supplied by the Sigma Chemical Co, St Louis. Other reagents were the best grade commercially available, and were used without further purification.

3. Results

Using the standard assay medium we measured the CN-insensitive GRF-mediated H_2O_2 formation, and O_2 uptake. Results showed that H_2O_2 was formed at a rate of 69 ± 6 $nmol.min^{-1}.mg\ protein^{-1}$ (mean ± 1 SD of 20 measurements) and that the stoichiometry

Table 1
GRF-mediated NADH oxidation O_2 uptake and H_2O_2 formation

Additions to the standard assay medium containing CN (2 mM)	NADH oxidation	O_2 uptake (nmol.min^{-1} ml^{-1})	H_2O_2 generated
Intact GRF (100 μg/ml)	7.23	6.61	6.88
+ SOD (62.5 μg/ml)	2 02	1.19	1.51
+ heat denatured SOD (62.5 μg/ml)	7.09	6.54	6.74
+ ferricytochrome *c* (0.1 mM)	3.48	3.04	2 96
+ NBT (0.1 mM)	2 68	2 51	2.48
+ KCl (0.1 mM)	7 21	6.64	6.91
Boiled GRF (100 μg/ml)	0.11	0.10	0.09
+ H_2O_2 (0 1 mM)	0.12	0.09	–
Phosphate buffer	0 10	0.08	0.11

For NADH oxidation measurement, protein content of the GRF (intact or boiled) added, was 80 μg for 0 8 ml incubation medium. For O_2-uptake and H_2O_2 generated measurements, protein content of the GRF (intact or boiled) added was 160 μg for the 1.6 ml incubation medium. In the results given, for intact GRF, the blanks (results obtained with boiled GRF) have been substracted. Results are the mean of at least 3 different experiments

between O_2 uptake and H_2O_2 formation was ~1 (table 1) for various incubation times and protein concentrations (results not shown). Addition of SOD, through itself inhibited by cyanide, inhibited 78% of the H_2O_2 formation, a value close to that of its effect on NADH oxidation and O_2 uptake (table 1). Ferricytochrome *c* and NBT inhibited 57% and 62% of the H_2O_2 formation, respectively. These inhibitions are also very close to those occuring on NADH oxidation and O_2 uptake (table 1).

In the absence of SOD, increasing ferricytochrome *c* increased its inhibitory effect on O_2 uptake and H_2O_2 formation. The amount of ferricytochrome *c* reduced was, however, 7–8-times smaller than the inhibition it caused on the O_2 uptake (fig.1): 0.1 mM ferricytochrome *c* inhibited the O_2 uptake by ~32 nmol.min^{-1}.mg protein^{-1} while only 4.3 nmol. min^{-1}.mg protein^{-1} were reduced, and 0.4 mM ferricytochrome *c* inhibited the O_2 uptake by about 51 nmol.min^{-1}.mg protein^{-1} while only 6.1 nmol. min^{-1}.mg protein^{-1} were reduced. In the presence of boiled GRF or in the absence of NADH no reduction of ferricytochrome *c* occurred. These results indicate that trapping 1 nmol. of $O_2^{\cdot-}$ with ferricytochrome *c*, inhibited the oxidation of about 8 nmol of NADH and associated O_2 uptake. We thus assumed that a cyclic chain reaction involving $O_2^{\cdot-}$ could be essential for the production of H_2O_2. The assumed chain reaction would be made up of the 2 following known reactions [11]:

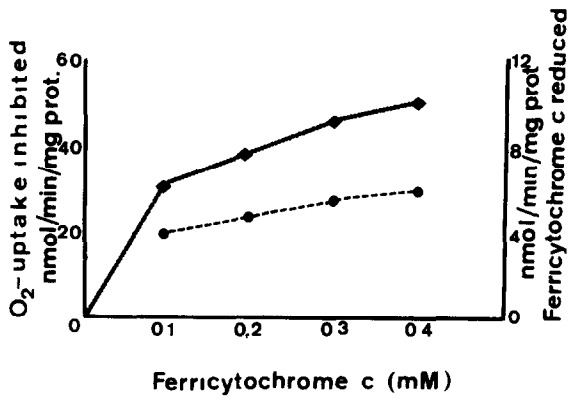

Fig.1. Inhibition of GRF (PMN granules) O_2 uptake by ferricytochrome *c* and SOD inhibitable reduction of ferricytochrome *c*. Experiments were performed in the oxygraph chamber using the standard assay medium and increased amounts of ferricytochrome *c*. O_2 uptake inhibited (♦.... ...♦), ferricytochrome *c* reduced (●——●) in the presence of increased amounts of ferricytochrome *c*.

Table 2
Effect of O_2^- generated by photolysis on O_2 uptake and H_2O_2 generation

Additions to	O_2 uptake (nmol $min^{-1}.ml^{-1}$)	H_2O_2 generated
I NADH and GRF-free standard assay medium		
+ phosphate buffer	0.90	0.92
+ boiled GRF (100 μg/ml)	0.90	0.93
SOD + boiled GRF (or phosphate buffer)	0.45	0.00
+ catalase + boiled GRF	0 66	–
II GRF-free standard assay medium (containing 2 5 mM NADH)		
+ phosphate buffer	1.88	–
+ boiled GRF	6.10	–
+ catalase + boiled GRF	1.46	–
+ catalase + boiled or intact GRF	4.61	–
+ intact GRF	13.32	–
+ intact GRF + 40 mM benzoate	9.64	–

O_2^- was generated by photolysis of water (see section 2) in a 1 ml chamber thermostated at 37°C. Reduction of ferricytochrome *c* was 0.9 $nmol^{-1}$ $min^{-1}.ml^{-1}$, was completely inhibited by SOD and not inhibited by catalase (see text). Additions were SOD (20 μg), catalase (50 μg), GRF (0.1 mg), NADH (2.5 mM) and benzoate (40 mM) SOD (20 μg) lowered the O_2 uptake in all the experiments below 0.50 nmol $min^{-1}.ml^{-1}$

$$NADH^{\cdot} + O_2 \longrightarrow HO_2^{\cdot}$$

$$(\text{protonated form of } O_2^{\cdot-}) + NAD^+ \qquad (3)$$

$$HO_2^{\cdot} + NADH + H^+ \longrightarrow H_2O_2 + NADH^{\cdot} \qquad (4)$$

Reaction (3) is known to occur with a second order rate constant of 1.9×10^9 $M^{-1}.s^{-1}$ (12). $HO_2^{\cdot}$ in reaction (4), is a more powerful oxidant than $O_2^{\cdot-}$. The half-cell potentials for the one electron reduction of $HO_2^{\cdot}$ and $O_2^{\cdot-}$ are of 1.7 V and 1.0 V, respectively, [13].

The reality of this chain reaction was verified in our standard assay medium (table 2). In the absence of NADH and GRF (or in the presence of boiled GRF), we generated $O_2^{\cdot-}$ by the photolysis of water. The $O_2^{\cdot-}$ generated measured by the reduction of ferricytochrome *c* or by the SOD-inhibitable O_2 consumption was of about 0 9 nmol.min^{-1}.ml^{-1}. The latter technique was used for the further measurement of $HO_2^{\cdot}$ produced since it would not interfere with the chain reaction we were looking for.

Generation of radicals by photolysis of water in our standard assay medium, in the presence of catalase and 2.5 mM NADH (GRF omitted) (table 2) resulted in more than a 2-fold increase of O_2 uptake as compared to its uptake in the absence of NADH. Further addition of either intact or boiled GRF (0.1 mg) increased O_2 uptake 3-fold. Thus, 1 mol of $O_2^{\cdot-}$ generated, yielded ~7 nmol O_2 uptake, in the presence of catalase, NADH (2.5 mM) and the GRF (intact or boiled). In the absence of catalase, with the same amount of $HO_2^{\cdot}$ generated by photolysis of water, NADH (2.5 mM) increased, to the same extent as above, the O_2 uptake, whether in the absence of GRF or in the presence of boiled GRF. The O_2 uptake by intact GRF illuminated, was approximately equal to its spontaneous O_2 uptake plus the O_2 uptake measured in the presence of boiled GRF, both being approximately equal. SOD almost completely inhibited O_2 uptake (0.5 nmol.min^{-1}.ml^{-1}) in all the experiments. These results indicated that the chain length was of 6–7 cycles, i.e., each $O_2^{\cdot-}$ radical generated, perpetuated the oxidation of 6–7 NADH and O_2-associated uptake, as an average, in our experimental conditions.

4. Discussion

Some investigations have shown that CN-insensitive NADH oxidation associated with an O_2 uptake and an H_2O_2 generation occur in PMN extracts [1]. The enzyme activity responsible for that is thought to be that of an 'NADH oxidase'. This enzyme, has, however never been clearly identified in the PMNs, and the initial steps leading to $O_2^{\cdot-}$ production are still a matter of controversy [1,4,14]. Whatever are these steps, there is a general agreement that $O_2^{\cdot-}$ further produces H_2O_2.

If $O_2^{\cdot-}$ directly produced H_2O_2 then their production rates should be equal (reaction (1)) or that of H_2O_2 be half that of $O_2^{\cdot-}$ (reaction (2)). It, however, was not the case, and we found that a larger amount of H_2O_2 was produced in our experimental conditions. We thus examined the possibility that a cyclic chain reaction involving reactions (3) and (4) could account for the resultant H_2O_2 formation. This cyclic chain reaction was indeed shown through the comparison of the reduction rate of ferricytochrome *c* and its inhibitory effect on O_2 uptake and H_2O_2 formation. Scavenging 1 mol $O_2^{\cdot-}$ with ferricytochrome *c* inhibited the uptake of 7–8 mol O_2 and the formation of 7–8 mol H_2O_2. Furthermore, a standard production of $O_2^{\cdot-}$ by water photolysis induced an equimolecular O_2 uptake and H_2O_2 formation in the absence of NADH and GRF, while addition of NADH alone increased O_2 uptake 2-fold and H_2O_2 formation. Further addition of GRF (intact or boiled) increased O_2 uptake and H_2O_2. formation 7-fold with the same standardized production of $O_2^{\cdot-}$. These results were taken as evidence for the reality of the chain reaction since none of these increments were observed when SOD was added.

The GRF-mediated cyclic chain reaction was heat-insensitive and probably not mediated by an enzyme [6,11,15] since boiling the GRF, did not modify the activity. It was also CN-insensitive. The responsible molecule for the GRF-mediated cyclic chain reaction remains to be identified. On the other hand, the cyclic chain reaction was shown to be inhibited by SOD This suggests that SOD may play an important role in the mechanism of the oxidative burst of the PMNs since we have shown [16] that SOD activity decreases in the stimulated PMNs. Decrease in SOD activity upon PMN stimulation could release the cyclic chain reaction and induce the oxidative reduction of O_2, i.e., the oxidative burst.

In conclusion, CN-insensitive NADH oxidation and associated O_2 uptake with formation of H_2O_2 occurred with the GRF of the PMNs. Our results show that, when $O_2^{\cdot-}$ is formed, a non-enzymatic cyclic chain reaction occurs between $HO_2^{\cdot}$, NADH and O_2. This chain reaction is enhanced by the GRF, and produces a large amount of H_2O_2. The release of the SOD-mediated inhibition of this cyclic chain reaction may play an important role in the mechanism of the PMN oxidative burst.

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