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PROTEIN-MEDIATED HYDROXYL RADICAL GENERATION - THE PRIMARY EVENT IN NADH OXIDATION AND OXYGEN REDUCTION BY THE GRANULE RICH FRACTION OF HUMAN RESTING LEUKOCYTES

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SUMMARY : The granule rich-fraction isolated from human resting polymorphonuclear leukocytes is capable of CN-insensitive NADH oxidation and O_2-uptake, accompanied by production of superoxide anion, hydroxyl radicals and H_2O_2. We showed that H_2O_2 initiates and maintains NADH oxidation and O_2-uptake but is also necessary for the formation of superoxide anion and hydroxyl radicals. It acts as a primary substrate for CN-insensitive protein-mediated formation of hydroxyl radicals, which in turn produce superoxide anions, probably through univalent oxidation of NADH as an intermediary.

When exposed to appropriate stimuli, PMN undergo CN-insensitive activation of their oxidative metabolism (1). The purpose of this appears to be the production of reduced forms of O_2, including $O_2^{\cdot-}$, H_2O_2, and HO·. NAD[P]H are unanimously regarded as the electron donors for these reductions. The primary enzyme in oxidative metabolism is thought to be a so called CN-insensitive NAD[P]H oxidase that catalyzes conversion of O_2 into $O_2^{\cdot-}$ and H_2O_2. The GRF isolated from PMN has been reported to exhibit NADH and NADPH oxidase activity and has consequently been suggested as the subcellular site at which H_2O_2 generation takes place (2,3). Generation of HO· was originally believed to occur through the Haber Weiss reaction (4) (Eq.1) :

$$H_2O_2 + O_2^{\cdot-} \longrightarrow HO\cdot + OH^- + O_2 \qquad \text{(Eq.1)}$$

However, recent work (5-7) has indicated that this reaction does not occur at significant rates and cannot compete with the spontaneous dismutation of $O_2^{\cdot-}$ (Eq.2) :

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

This suggests that a catalytic mechanism must be involved in HO· generation. On the other hand, we recently suggested (8,9) that HO· behaved like an early intermediate in aerobic NAD[P]H oxidation by GRF isolated from PMN.

Our purpose here is to show that generation of HO· 1) is protein-mediated, and 2) is the first metabolic event in GRF-mediated aerobic oxidation of NADH.

Abbreviations used : PMN, human polymorphonuclear blood cells ; $O_2^{\cdot-}$, superoxide anion radical ; HO·, hydroxyl radical ; H_2O_2, hydrogen peroxide ; GRF, granule-rich fraction ; PB, 0.1 M phosphate buffer (pH 5.5).

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EXPERIMENTAL SECTION

PMN were isolated from heparinized venous blood of normal healthy volunteers as previously described (9). Final preparation routinely contained 80-90 % PMN and 10-20 % mononuclear cells. Cells suspended in 10 % sucrose containing 0.04 M Tris-hydrochloride, pH 7.4, were stored overnight at minus 80°C. After thawing, the GRF was isolated as previously described (9). Such isolation included straining through a glass wool column, and dialysis against PB. GRF-mediated NADH oxidation, O_2-uptake, H_2O_2 and O_2^- formation were measured as previously described (9). CN-insensitive production of oxidizing agents was measured by CN-insensitive oxidation of ferro-cytochrome c (10), prepared according to Beauchamp and Fridovich (11). Experimental conditions were similar for all measurements, to allow comparison of the results. Incubation times were zero, 5 and 10 min. All parameters were linear with time during the incubation period. Protein concentrations were determined according to Lowry (12) with bovine serum albumin as a standard.

RESULTS AND DISCUSSION

As previously reported (9,13), the incubation medium described in the legends to Table I, contained H_2O_2 which was spontaneously formed upon preparation of NADH

TABLE I : H_2O_2 DEPENDENCE OF GRF-MEDIATED NADH OXIDATION O_2-UPTAKE AND H_2O_2 FORMATION.

	NADH oxidation	O_2-uptake	H_2O_2 generated
	(nmol min^{-1} ml^{-1})		
Intact GRF (100 µg/ml)	6.66	6.04	6.31
+ CN (2 mM)	7.22	6.61	6.88
+ Catalase (62.5 µg/ml)	0.18	0.20	
+ CN (2 mM) + catalase (62.5 µg/ml)	0.34	0.28	
Boiled GRF (100 µg/ml)	0.11	0.11	0.11
+ CN (2 mM)	0.11	0.10	0.09
+ Catalase (62.5 µg/ml)	0.10	0.08	
+ CN (2 mM) + H_2O_2 (0.1 mM)	0.12	0.09	
Phosphate buffer	0.11	0.08	0.10
+ CN (2 mM)	0.10	0.10	0.11

The incubation medium contained 0.1 M citrate-phosphate buffer, pH 5.0, 0.1 % bovine serum albumin, 2.5 mM NADH and 80 µg (NADH oxidation) or 160 µg (O_2-uptake and H_2O_2 generated) of GRF-protein (boiled or intact). Final volume : 0.8 ml (NADH oxidation) or 1.6 ml (O_2-uptake and H_2O_2 generated). KCN was prepared daily. Cyanide remained effective in the incubation medium throughout the time course of the reactions, as proved by its complete blockage of the catalatic activity of 1 µg catalase/ml, even after 20 min preincubation in the medium used. NADH oxidation was measured after zero, 5 and 10 min incubation time. O_2-uptake was measured for 10 min (during which it was linear with time), and the H_2O_2 generated was measured after measurement of O_2-uptake. In 6 experiments, the H_2O_2 generated was measured after only 5 min measurement of O_2-uptake, and was half the amount found after 10 min incubation. Large amounts of catalase were used in order to circumvent its inhibition by cyanide. Consequently less than 50 % of the catalase activity was inhibited by CN at pH 7.0 and 5.0. When catalase was added before initiation of the reaction, 15 $\pm$ 1.5 nmol (mean $\pm$ 1 SD of 6 experiments) of H_2O_2 were present in 1.0 ml standard assay medium. In the results for intact GRF, the blanks (results obtained with boiled GRF) have been subtracted. Results are the mean of at least three different experiments.

stock solution. In all subsequent experiments, we standardized at 15 μM the H_2O_2 initially present in the incubation medium containing 2.5 mM NADH, prepared extemporaneously. Additional autooxidation of NADH associated with H_2O_2 formation and O_2-uptake in the incubation medium containing boiled GRF, was less than 0.12 nmol min^{-1} ml^{-1}, measured either polarographically (O_2 and H_2O_2) or fluorimetrically (NAD^+).

Stoichiometry for CN-insensitive NADH oxidation (72 ± 5 nmol min^{-1} mg^{-1}) was 0.92 ± 0.05 (mean ± 1 SD of 6 paired measurements) with O_2-uptake, and 0.96 ± 0.04 (mean ± 1 SD of 20 paired experiments) with H_2O_2 formation (Table I). All these parameters were proportional to incubation time and protein concentration (9). Reactions were inhibited (8,9 and Table I-III) by any one of the following : catalase leading to an H_2O_2-free medium, ascorbate [a redogenic substance], SOD, scavengers of $O_2^{\cdot-}$ [ferricytochrome c or NBT] or scavengers of HO· [Tris, sodium benzoate, mannitol or ferrocytochrome c, known to react with HO· and not with $O_2^{\cdot-}$ or H_2O_2 (9,11,14-19)]. SOD is a specific enzyme (20) which dismutates two $O_2^{\cdot-}$ into H_2O_2 (21,22) more rapidly than when dismutation occurs spontaneously (23). The SOD preparation had no catalytic effect on H_2O_2 and reciprocally, catalase was free of SOD activity.

Up to this point we only confirmed and extended previous experiments (8,9), showing that GRF-mediated aerobic NADH oxidation was associated with equimolecular O_2-uptake and H_2O_2 formation, heat-labile, and dependent on H_2O_2, $O_2^{\cdot-}$ and HO·. It was therefore important to establish the sequence of metabolic events leading to aerobic oxidation of NADH.

Production and sequential formation of both HO· and O_2 were shown by measuring their production rate and by inhibiting each of them with scavengers of either $O_2^{\cdot-}$ or HO·. When NADH was omitted and H_2O_2 added, ferrocytochrome c was oxidized (Table IV). This oxidation was inhibited by catalase or HO· scavengers but not by SOD. The fact that ferrocytochrome c is a better scavenger of HO· than benzoate (Table III) explains the slight inhibitory effect of benzoate (3.6 nmol min^{-1} mg $protein^{-1}$). NADH (2.5 mM) or Tris were more potent inhibitors of ferrocytochrome c oxidation than 40 mM benzoate. When, in the absence of NADH, ferricytochrome c replaced ferrocytochrome c, it was not reduced. This means that the GRF causes H_2O_2-dependent formation of HO·. We also showed that H_2O_2 was consumed in the standard assay medium, in which NADH was replaced by 0.6 mM ferrocytochrome c + 40 μM of H_2O_2. H_2O_2 uptake was difficult to measure precisely and was between 8 and 15 $nmol^{-1}$ min^{-1} mg $protein^{-1}$ GRF.

The reduction of ferricytochrome c (0.1 or 0.4 mM) in the complete standard assay medium was inhibited by approximately 50 % when 40 mM of benzoate were added, i.e. the ferricytochrome c reduction rate decreased by 2.2 nmol min^{-1} mg $protein^{-1}$ (from 4.3 to 2.1 with 0.1 mM of ferricytochrome c) and by 3.0 nmol (from 6.1 to 3.1 with 0.4 mM ferricytochrome c). This latter value (3.0 nmol) is of the same order of magnitude as the value for benzoate inhibition of ferrocytochrome c oxidation (i.e.

TABLE II : EFFECT OF RADICAL SCAVENGERS ON GRF-MEDIATED NADH OXIDATION AND O_2-UPTAKE.

	NADH oxidation	O_2-uptake	H_2O_2 generated
	(in % of intact GRF + 2 mM KCN)		
Intact GRF (100 µg/ml)			
+ CN (2 mM)	100	100	100
+ Ascorbate (0.5 mM)	0	0	0
+ Ascorbate (0.5 mM) + CN (2 mM)	0	0	0
+ SOD (31.25 µg/ml)	3	4	3
+ SOD (62.5 µg/ml)	0	0	0
+ heat denatured SOD (62.5 µg/ml)	98	99	98
+ SOD (62. 5 µg/ml) + CN (2 mM)	28	18	22
+ Ferricytochrome c (0.1 mM)	48	46	43
+ NBT (0.1 mM) + CN (2 mM)	37	38	36
+ KCl (0.1 mM)	100	100	100
+ KCl (0.1 mM) + CN (2 mM)	100	100	100
Boiled GRF (100 µg/ml)			
+ CN (2 mM)	1.6	1.5	1.3

For experimental conditions, see legends to Table I.

TABLE III : EFFECT OF HO· SCAVENGERS ON NADH-OXIDATION AND O_2-UPTAKE.

Additions to the incubation medium	NADH oxidation	O_2-uptake
	(in % of the controls)	
Intact GRF (100 µg/ml) with CN (2 mM)	100	100
+ Tris 10 mM	27	25
+ Benzoate 10 mM	-	86
+ Benzoate 20 mM	72	71
+ Benzoate 40 mM	-	42
+ Benzoate 60 mM	28	27
+ Benzoate 20 mM (NADH was 1.25 mM instead of 2.5)*	56	58
+ Benzoate 20 mM (NADH was 0.8 mM instead of 2.5)*	48	43
+ Mannitol 20 mM	-	84
+ Mannitol 40 mM	70	68
+ Ferrocytochrome c 0.1 mM	92	93
+ Ferrocytochrome c 0.24 mM	-	64
+ Ferrocytochrome c 0.6 mM	32	31
+ KCl 60 mM	100	100

For experimental conditions, see legends to Table I.

* Results for the two experiments with a lower NADH concentration (1.25 and 0.8 mM) are expressed in % of control experiments, performed in the absence of benzoate with the same NADH concentrations (1.25 and 0.8 mM respectively). The H_2O_2 initially present was adjusted to 15 µM.

TABLE IV : CN-INSENSITIVE FERROCYTOCHROME c OXIDATION BY GRF.

Additions to the incubation medium	Oxidation of ferrocytochrome c nmol min^{-1} mg $protein^{-1}$
Intact GRF (100 µg/ml) + H_2O_2 + Ferrocytochrome c + CN (2 mM)	15.2
+ 10 mM Tris	8.1
+ 40 mM benzoate	11.6
+ 50 µg SOD	14.9
+ 50 µg catalase	2.2
- H_2O_2 (omitted)	2.1
+ 2.5 mM NADH	6.1
+ 60 mM KCl	15.3
Boiled GRF (100 µg/ml) + H_2O_2 + Ferrocytochrome c + CN (2 mM)	3.4
+ 40 mM benzoate	3.1
+ 50 µg SOD	3.3

Experiments were performed in the incubation medium indicated in Table I, except that 40 µM H_2O_2 and 0.6 mM ferrocytochrome c were added instead of NADH. When 2.5 mM NADH were added, the supplementary H_2O_2 was adjusted to a final concentration of 40 µM. The extinction coefficient used for calculation (reduced minus oxidized) was 15.5 mM^{-1} cm^{-1}. Values are given without subtraction of blanks (boiled GRF). Incubation time was 10 min. Ferrocytochrome c oxidation was proportional to incubation time.

3.6 nmol) (Table IV). Since benzoate does not itself scavenge $O_2^{\cdot-}$, and SOD does not inhibit the amount of HO· trapped by ferrocytochrome c (Table IV), these results suggest that the HO· produced from H_2O_2 leads to $O_2^{\cdot-}$ formation.

The following results showed that NADH competed with benzoate for the HO· radical and suggested that HO· might have reacted with NADH as an intermediate step in $O_2^{\cdot-}$ production : 1) Lowering the concentration of NADH to 1.25 and 0.8 mM in the standard assay medium increased the inhibitory effect of 20 mM of benzoate on NADH oxidation and O_2-uptake (Table III). 2) O_2-uptake was observed in the presence of NADH but not in its absence. 3) Ferrocytochrome c oxidation in the complete assay medium (including 2.5 mM NADH + 40 µM H_2O_2) was 40 % of its oxidation in the absence of NADH (Table IV). These results suggest that once HO· is formed from H_2O_2, it oxidizes NADH univalently, with secondary formation of $O_2^{\cdot-}$. However, this still requires more direct demonstration.

As measured by ferrocytochrome c oxidation in our standard assay medium (NADH omitted and replaced by 0.6 mM ferrocytochrome c + 80 µM of H_2O_2), HO· formation progressively increased in a linear fashion with the incubation time and for protein concentrations ranging from 40 to 100 µg GRF. The Km H_2O_2 of the activity was not measured, but preliminary experiments showed that the ferrocytochrome c oxidation

rate was at its maximum for an H_2O_2 concentration of 8×10^{-6} M. We may therefore infer that the apparent Km for H_2O_2 is fairly low.

Several investigators have shown that CN-insensitive NADH oxidation associated with O_2-uptake and H_2O_2 generation occurs in PMN extract (1). The enzyme activity responsible for this process is thought to be a so called NADH oxidase. However, such an enzyme has never been clearly identified in the PMN. The results reported here show that the only catalytic activity isolated from the GRF which, under our experimental conditions, could result in apparent NADH oxidase activity primarily catalyzes formation of HO· from H_2O_2. We also provided evidence for the following sequence of metabolic events leading to aerobic oxidation of NADH :

$$\text{Protein (reduced form)} + H_2O_2 \longrightarrow \text{Protein (oxidized form)} + HO\cdot + OH^- \quad \text{(Eq.3)}$$

$$HO\cdot + \text{NADH} \longrightarrow NAD\cdot + H_2O \quad \text{(Eq.4)}$$

$$NAD\cdot + O_2 \longrightarrow O_2^{\cdot-} + NAD^+ \quad \text{(Eq.5)}$$

$$O_2^{\cdot-} + \text{NADH} + H^+ \longrightarrow NAD\cdot + H_2O_2 \quad \text{(Eq.6)}$$

In reaction 3, the action of the protein in the production of HO· might be similar to that of the iron-EDTA complex recently described by McCord and Day (24).

In conclusion, the GRF of the PMN is responsible for CN-insensitive NADH oxidation, O_2-uptake and H_2O_2 formation. Our results show that the first step in this process is the protein-mediated production of HO· from H_2O_2. To our knowledge, this has never yet been shown with biological material. The second step is the reaction of the HO· with NADH to produce NAD·, which in turn reacts with O_2 to produce $O_2^{\cdot-}$. $O_2^{\cdot-}$ then generates H_2O_2. The generation of a large quantity of H_2O_2 is very probably due to a cyclic chain reaction occurring between $O_2^{\cdot-}$, NADH and O_2 as previously reported (25).

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