SUPEROXIDE-DEPENDENT FORMATION OF HYDROXYL RADICALS FROM NADH AND NADPH IN THE PRESENCE OF IRON SALTS

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1. Introduction

Superoxide dismutase plays a vital role in the protection of aerobic cells against the toxic effects of O_2 [1,2]. Since this enzyme is specific for the superoxide radical, $O_2^{\cdot-}$, as substrate [3] it follows that $O_2^{\cdot-}$ must be a dangerous species. Indeed, $O_2^{\cdot-}$-generating systems have been observed to kill cells, inactivate enzymes and degrade DNA, cell membranes and polysaccharides [1,2,4]. Many of these damaging effects have been attributed to the $O_2^{\cdot-}$-dependent formation of the hydroxyl radical $OH^{\cdot}$, a highly-reactive species [1,5–7]. Formation of $OH^{\cdot}$ also requires H_2O_2 and traces of non-protein-bound iron salts and may be represented by the overall equation:

$$O_2^{\cdot-} + H_2O_2 \xrightarrow[\text{catalyst}]{\text{Fe-salt}} O_2 + OH^{\cdot} + OH^- \qquad (1)$$

Traces of free iron salts are present intra-cellularly [8,9] and in some extracellular fluids [10], whilst $O_2^{\cdot-}$ and H_2O_2 can be produced by the action of several enzymes and by activated phagocytes as well as by the autoxidation of compounds such as thiols [2]. Exposure of physiological concentrations of reduced glutathione, GSH, to Fe(II) salts gives rise to hydroxyl radicals in a $O_2^{\cdot-}$- and H_2O_2-dependent reaction [11] and have suggested that this reaction is a significant source of $OH^{\cdot}$ radicals in vivo. It therefore occurred to us to see if a similar reaction could be detected using other physiological reducing agents, and we here report the results of experiments using NADH and NADPH.

2. Methods

2.1. *Reagents*

Superoxide dismutase (spec. act. 2900 units/mg protein as assayed by the method in [12], $NADP^+$, NADPH, NAD^+, NADH, catalase (bovine liver, thymol-free) and GSH were obtained from Sigma. Catalase activity units (μmol H_2O_2 destroyed/min) were measured as described in the Sigma catalogue.

2.2. *Assay methods*

Superoxide-dependent formation of $OH^{\cdot}$ radicals was measured by a modification [13] of the salicylate hydroxylation method [6]. Solutions of iron salts were made up fresh immediately before use. Reaction mixtures contained, in a total volume of 2 ml, 100 μM $FeCl_2$, NADPH at the concentration stated, 100 μM H_2O_2, 150 mM KH_2PO_4, adjusted to pH 7.4 with KOH, and 2 mM salicylate. After incubation at 25°C for 90 min, 80 μl conc. HCl was added and the diphenolic products extracted into ether and assayed as above [13].

3. Results

Hydroxyl radicals can be detected easily by their reaction with aromatic compounds such as salicylate to give hydroxylated products that can be measured by a colorimetric reaction [6,13]. Reaction of $FeCl_2$ with H_2O_2 produces some $OH^{\cdot}$ radicals, but inclusion of GSH in the reaction mixture greatly increases the number of $OH^{\cdot}$ radicals detected (fig.1), in agreement with [11]. Fig.1 shows the results obtained when GSH was replaced by NADH or NADPH at physiological concentrations, and it may be seen that increased amounts of $OH^{\cdot}$ are again detected at pH 7.4.

Table 1 shows that NADPH-dependent formation of $OH^{\cdot}$ requires H_2O_2 and is significantly inhibited by catalase and by superoxide dismutase, implying that $O_2^{\cdot-}$ is also required. Addition of the $OH^{\cdot}$ radical scav-

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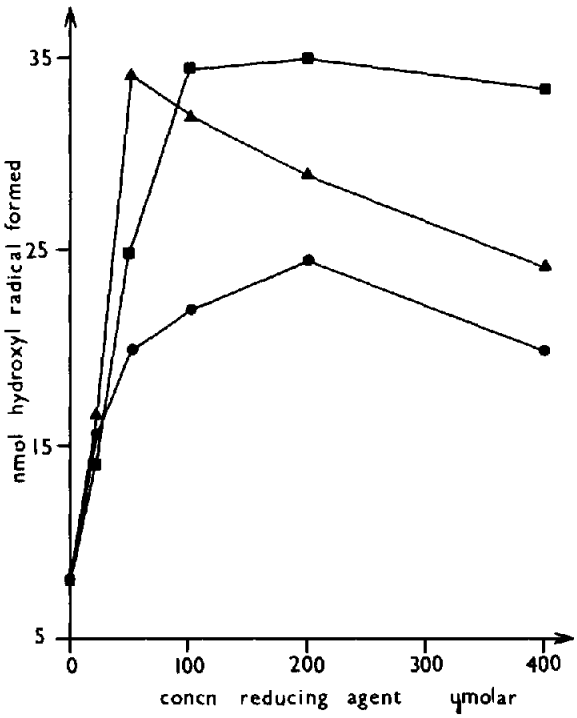

Fig.1. Hydroxyl radical generation by reducing agents in the presence of ferrous iron and hydrogen peroxide. Reaction mixtures contained 100 μM $FeCl_2$, 100 μM H_2O_2, 2 mM salicylate and various concentrations of the reducing agent in a total volume of 2 ml 0.15 M potassium phosphate (pH 7.4). Incubation was carried out at 25°C for 90 min. The reaction was terminated by the addition of 80 μl conc. HCl. Diphenolic products were extracted and assayed as in [13]: (•) NADH; (■) NADPH; (▲) reduced glutathione.

engers mannitol, thiourea or formate decreased the amount of $OH^{\cdot}$ detected whereas urea, which reacts only slowly with $OH^{\cdot}$ [14] had little effect. Bovine serum albumin, used as a control for non-specific protein effects, also had little effect. $NADP^+$ and NAD^+ could not replace their reduced counterparts.

Fe^{2+} could be replaced by Fe^{3+}–EDTA giving similar results, but not by Fe^{3+} alone (not shown).

4. Discussion

Both NADH and NADPH at physiological concentrations have been shown to interact with iron salts and H_2O_2 to produce $OH^{\cdot}$ radicals at physiological pH. Since iron salts are present in vivo (section 1) and H_2O_2 is generated by some enzymes and by activated phagocytes [15], this reaction is perfectly feasible

Table 1
Production of hydroxyl radicals from NADPH and iron(II) salts

Reaction mixture	Addition to reaction mixture	$OH^{\cdot}$ production (nmol salicylate hydroxylated/h)
Complete	–	34.8
Omit H_2O_2	–	5.0
Omit NADPH	–	8.2
Omit NADPH	$NADP^+$ (100 μM)	10.4
Omit $FeCl_2$	–	0
Complete	Mannitol (10 mM)	18.6
Complete	Thiourea (10 mM)	4.6
Complete	Sodium formate (10 mM)	16.2
Complete	Urea (10 mM)	28.2
Complete	Catalase (500 units)	6.0
Complete	Superoxide dismutase (300 units)	10.8

Assays of $OH^{\cdot}$ formation using 100 μM NADPH were carried out as in section 2. Reagents were added to give the final concentrations stated. Similar results were obtained with NADH

in vivo. The reaction is inhibited by superoxide dismutase; it may be that $O_2^{\cdot-}$ produced by oxidation of NADPH induced by the iron salt [16] recycles Fe^{3+}, produced during the oxidation, to Fe^{2+} and so allows both the oxidation and reaction [1] to continue. An important physiological role of superoxide dismutase in vivo may be that of decreasing $O_2^{\cdot-}$-dependent formation of $OH^{\cdot}$ from GSH, NADH and NADPH in the presence of iron salts.

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