SUPEROXIDE-DEPENDENT FORMATION OF HYDROXYL RADICALS IN THE PRESENCE OF THIOL COMPOUNDS

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Received 27 November 1981

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, systems generating $O_2^{\cdot-}$ have been observed to kill cells, inactivate enzymes and degrade DNA, cell membranes and polysaccharides [1,2,4]. Yet $O_2^{\cdot-}$ in aqueous solution is poorly reactive, acting mainly as a moderately strong reducing agent. Therefore, it has been suggested that the damaging effects of $O_2^{\cdot-}$-generating systems are due to the $O_2^{\cdot-}$-dependent formation of hydroxyl radicals, $OH^{\cdot}$, a reaction which also requires H_2O_2 and traces of non-protein-bound iron salts [5–8]. The overall reaction may be represented by the equation:

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

It may be the sum of the reactions:

$$Fe^{3+} + O_2^{\cdot-} \rightarrow O_2 + Fe^{2+} \qquad (2)$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\cdot} + OH^- \qquad (3)$$

although this is not certain [8]. Traces of 'free' iron salts are present in vivo [2,8,9,10] and so reaction (1) is a feasible explanation of $O_2^{\cdot-}$ toxicity in vivo.

However, this explanation has been criticised on the grounds that other reducing agents, such as GSH or ascorbic acid, that are present in vivo at greater concentrations than is $O_2^{\cdot-}$, would be more likely to reduce Fe^{3+} and so would obviate reaction (2) [11,12]. The likely effect of ascorbate has been discussed [8,13]. Here, we show that thiol compounds at physiological concentrations do not prevent $O_2^{\cdot-}$-dependent formation of $OH^{\cdot}$ radicals in the presence of iron salts, and indeed that they can themselves produce $OH^{\cdot}$ radicals under our reaction conditions. The monothiol GSH is present in animal and plant cells at mM levels [14,15].

2. Materials and methods

2.1. *Reagents*

Superoxide dismutase (spec. act. 2900 units/mg using the cytochrome *c* assay [16]), GSH, GSSG, cystamine, cysteamine, catalase (bovine liver, thymol free), hypoxanthine and albumin were obtained from Sigma. Catalase activity units (μmol H_2O_2 destroyed/min) were as measured in [17]. Xanthine oxidase activity was assayed using an O_2 electrode [7]; it was purchased from Boehringer. All other reagents were of the highest quality obtainable from BDH Chemicals Ltd.

2.2. *Assay methods*

Superoxide-dependent formation of $OH^{\cdot}$ radicals by the hypoxanthine/xanthine oxidase system was measured by a modification [18] of the salicylate hydroxylation method [7]. Solutions of iron salts were made up fresh just before use.

3. Results

3.1. *Effect of thiol compounds on $OH^{\cdot}$ generation by a hypoxanthine–xanthine oxidase system*

A mixture of hypoxanthine and xanthine oxidase generate $O_2^{\cdot-}$ and H_2O_2 which, in the presence of added

Published by Elsevier Biomedical Press
00145793/82/0000–0000/$02.75

iron salts, interact to form OH· radicals that can be detected by their reaction with salicyclic acid to yield diphenolic products [7,18,19]. Hydroxyl radical production is inhibited by catalase, superoxide dismutase and certain iron chelators [19]. Added Fe^{2+} or Fe^{3+} salts are equally effective in allowing OH· production [7].

Fig.1 shows the effect of various thiol compounds on OH· generating by the above system. Similar results were obtained using the physiological thiol GSH, thiourea or cysteamine, which has been used as a radioprotective agent. At high thiol concentrations the amount of OH· detected was decreased although GSH at 5 mM, higher than those of most tissues in vivo [14,15], inhibited by <40%. At low thiol concentrations a stimulation on OH· formation was observed. In all cases, OH· formation was inhibited by superoxide dismutase and catalase but not by bovine serum albumin, used as a protein control (table 1 shows some typical results). GSH and cysteamine at the concentrations used had no effect on xanthine oxidase. Thiourea has a slight inhibitory effect on this enzyme [7] which may mean that the stimulation shown in fig.1 should have been even greater. None of the thiols significantly affected the assay used to measure OH· formation.

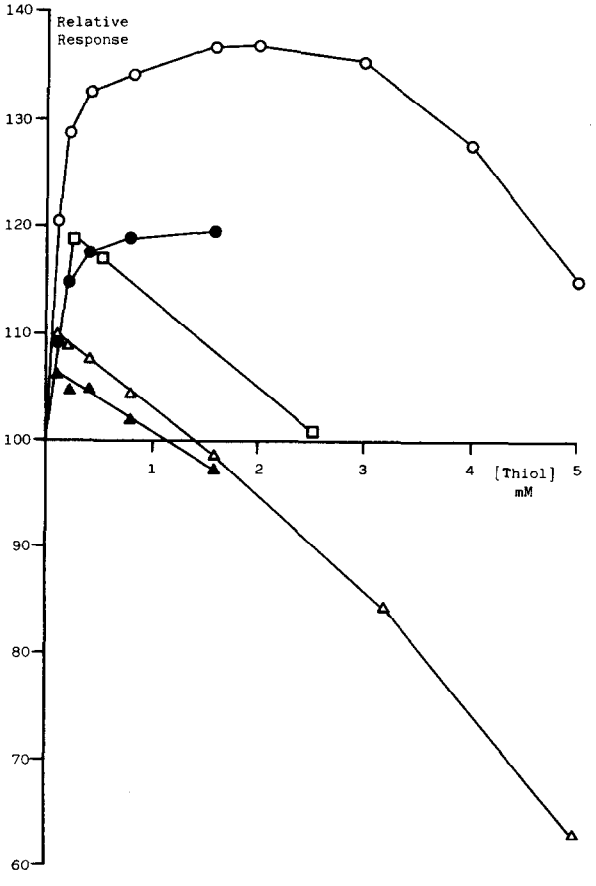

Fig.1. Effect of thiol compounds on OH• generation in the hypoxanthine/xanthine oxidase system. Results are shown relative to those obtained in the absence of added thiol (100%). At 10 and 100 μM Fe^{3+}–EDTA the 100% values correspond to 85 and 188 nmol hydroxylated product, respectively: (o---o) 100 μM Fe^{3+}–EDTA plus cysteamine; (•---•) 10 μM Fe^{3+}–EDTA plus cysteamine; (□---□) 100 μM Fe^{3+}–EDTA plus thiourea; (△---△) 100 μM Fe^{3+}–EDTA plus GSH; (▲---▲) 10 μM Fe^{3+}–EDTA plus GSH.

3.2. *Production of hydroxyl radicals from thiols and iron salts*

It was found that thiol compounds themselves in the presence of iron salts and added H_2O_2 generate OH· radicals at significant rates, thus accounting for the stimulatory effects observed in fig.1. Table 2 shows the results obtained with 100 μM GSH, although similar effects were found with cysteine and cysteamine The rate of hydroxyl radical generation was constant for ~1 h (fig.2). For maximum rates of OH· production H_2O_2, Fe^{2+} and GSH were required. Fe^{3+} could

Table 1
Effect of thiol compounds on hydroxyl radical formation by hypoxanthine–xanthine oxidase in the presence of iron salts

Thiol added	Other addition to reaction mixture	OH· production (nmol diphenol produced/h)
None	–	188
None	10^3 units SOD	55
None	500 units catalase	48
None	BSA (1 mg/ml)	190
GSH (100 μM)	–	208
GSH (100 μM)	10^3 units SOD	53
GSH (100 μM)	500 units catalase	40
GSH (100 μM)	BSA (1 mg/ml)	200
GSH (5 mM)	–	118
GSH (5 mM)	10^3 units SOD	47
GSH (5 mM)	500 units catalase	41
GSH (5 mM)	BSA (1 mg/ml)	120

Abbreviations: BSA, bovine serum albumin; SOD, superoxide dismutase

Experiments were carried out and units of enzyme activity defined as in section 2. Reaction mixtures contained $FeCl_2$ (100 μM) but no EDTA. GSSG (100 μM) or cysteamine (100 μM) had no significant effect on OH· production

Table 2
Production of hydroxyl radicals from GSH and iron salts

Reaction mixture	Addition to reaction mixture	OH· production (nmol diphenol produced/h)
Complete	–	32
–GSH	–	4
$-H_2O_2$	–	3
$-FeCl_2$	–	0
$-FeCl_2$	$FeCl_3$ (100 μM)	0
$-FeCl_2$	$FeCl_3$–EDTA (100 μM)	31
Complete	BSA (1 mg)	34
Complete	Superoxide dismutase (10^3 units)	6
Complete	Catalase (500 units)	1
Complete	Mannitol (10 mM)	8
Complete	Urea (10 mM)	28
Complete	Sodium formate (10 mM)	6
Complete	Thiourea (10 mM)	0

Reaction mixtures contained, in a total volume of 2 ml, 100 μM $FeCl_2$, 100 μM GSH, 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 and assayed as in [18]

not replace Fe^{2+}, although Fe^{3+}–EDTA could (table 2, fig.2).

The amount of OH· detected from the GSH/H_2O_2/Fe^{2+} system was decreased at high thiol concentrations (e.g., 10 mM thiourea, table 2), by addition of catalase or superoxide dismutase or by scavengers of the hydroxyl radical such as formate and mannitol. By contrast, bovine serum albumin or urea, which reacts only slowly with OH· [20], had little effect.

4. Discussion

Glutathione is present within animal, plant and some bacterial cells at mM levels [14]. Although thiol compounds react readily with the OH· radical [20], physiological concentrations of GSH decreased OH· formation from $O_2^{\cdot-}$ and H_2O_2 (fig.1) by <40%, nor did they change the mechanism of the reaction in that superoxide dismutase and catalase were still inhibitory (table1). Thus suggestions that GSH would prevent reaction (2) in vivo are not substantiated by our data. The results in fig.1 appear to be produced by a combination of two effects: scavenging of OH· at high

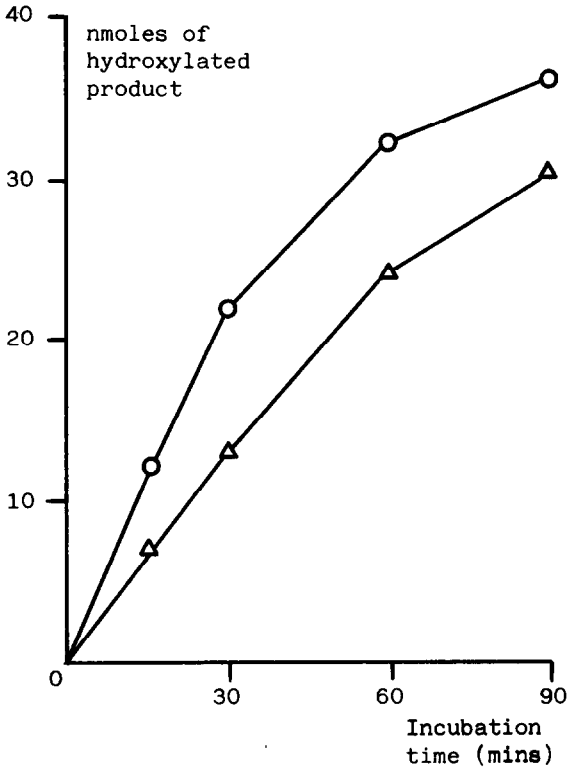

Fig.2. Time course of OH· generation by thiol compounds in the presence of FE(III)–EDTA and H_2O_2. Reaction mixtures contained, in 2 ml total vol., 100 μM GSH or cysteamine, 100 μM $FeCl_3$, 100 μM EDTA, 100 μM H_2O_2, 2 mM salicylate and 150 mM KH_2PO_4 adjusted to pH 7.4 with KOH. Incubation was carried out at 25°C. The reaction was stopped at various time periods by the addition of 80 μl conc. HCl and the diphenolic products extracted and assayed as in [18]. Results were identical if Fe^{2+} was used instead of Fe^{3+}–EDTA (table 2). (▲) GSH; (●) cysteamine.

thiol concentrations and production of OH· from GSH and Fe^{2+} in the presence of H_2O_2 generated during oxidation of hypoxanthine by xanthine oxidase.

In some body tissues (e.g., lung, brain, muscle) and in extracellular fluids the GSH concentrations are much lower, e.g., 20–30 μM in plasma [14,21]. Since some extracellular fluids contain traces of iron salts, both normally and in pathological conditions [10,22], another source of OH· in vivo will be the reaction described in table 2. Hydroxyl radical production by GSH and Fe^{2+} requires H_2O_2 (produced perhaps by extracellular enzymes, leakage from cells or activated neutrophils [23]) and is inhibited by superoxide dismutase. Presumably $O_2^{\cdot-}$ produced by oxidation of GSH induced by iron salt [24] recycles Fe^{3+} to Fe^{2+},

permitting both $OH^{\cdot}$ generation by reaction (3) and GSH oxidation to continue. The inability of Fe^{3+} to replace Fe^{2+} suggests strongly that GSH itself is incapable of reducing trivalent iron, hence the requirement for $O_2^{\cdot-}$. Fe^{3+}–EDTA will replace Fe^{2+}, although this has no physiological significance.

Acknowledgement

We are grateful to the Cancer Research Campaign for financial support.

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