Formation of hydroxyl radicals from hydrogen peroxide and iron salts by superoxide- and ascorbate-dependent mechanisms: relevance to the pathology of rheumatoid disease

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(Received 18 June/15 November 1982; accepted 10 December 1982)

Summary

1. Superoxide and hydrogen peroxide are formed by activated phagocytes and react together in the presence of iron salts to form the hydroxyl radical, which attacks hyaluronic acid. Ascorbic acid also interacts with hydrogen peroxide and iron salts to form hydroxyl radical in a reaction independent of superoxide. Since iron salts, ascorbate and activated phagocytes are present in the rheumatoid joint, experiments were designed to see whether ascorbate-dependent or superoxide-dependent formation of hydroxyl radicals would be more important in vivo.

2. In the present study, addition of ascorbate to a superoxide-generating system at concentrations of 100 μmol/l provoked a superoxide-independent formation of hydroxyl radicals for a short period. Lower concentrations of ascorbate did not do this. It is therefore suggested that the superoxide-dependent reaction is probably more important.

3. It is further suggested that destruction of ascorbate by oxygen radicals formed by activated phagocytes accounts for the previously, reported low concentrations of this compound in the serum and synovial fluid of rheumatoid patients.

Key words: ascorbate, hydrogen peroxide, hydroxyl radicals, iron, rheumatoid disease, superoxide, synovial fluid.

Introduction

In inflammatory joint diseases such as rheumatoid arthritis, phagocytes accumulate in the joints. When activated by immune complexes, they produce the superoxide radical (O$_2^-$) and hydrogen peroxide [1], which, in the presence of the traces of iron salts found in synovial fluid [2, 3], interact to form the highly reactive hydroxyl radical (OH$_{-}$). Hydroxyl radical will attack and destroy most biological molecules, e.g. it will degrade hyaluronic acid and thus reduce synovial fluid viscosity [4–6] and also induce lipid peroxidation, products of which accumulate in rheumatoid disease [7].

Formation of OH$_{-}$ from O$_2^-$ and H$_2$O$_2$ can probably be represented by the following reactions [8]:

$$\text{Fe}^{3+} + \text{O}_2^- \rightarrow \text{Fe}^{2+} + \text{O}_2 \quad (1)$$

$$\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^- + \text{OH}^- \quad (2)$$

Net $\text{O}_2^- + \text{H}_2\text{O}_2 \xrightarrow{\text{Fe catalyst}} \text{OH}^- + \text{OH}^- + \text{O}_2 \quad (3)$

i.e. O$_2^-$ is serving merely to reduce Fe$^{3+}$ to Fe$^{2+}$. It has been pointed out [9, 10] that ascorbic acid is also capable of reducing Fe$^{3+}$ to Fe$^{2+}$ [11] and can thus give rise to a $\text{O}_2^-$-independent formation of OH$_{-}$ radicals from H$_2$O$_2$ [12]. Since ascorbate is present in synovial fluid [10, 13], it has been suggested that ascorbate-dependent formation of OH$_{-}$ radicals would be more important than $\text{O}_2^-$-dependent formation of OH$_{-}$ in vivo [9, 14]. If this were the case, then the production of O$_2^-$ by activated phagocytes would be irrelevant to
the pathology of rheumatoid disease, a conclusion not in agreement with previous work [1–7], and attempts to treat the disease by injection of superoxide dismutase (SOD) into the joint would be misplaced. Other biological reducing agents, such as reduced glutathione (GSH) or NAD(P)H, do not appear to reduce Fe3+ in competition with O2−, however [15, 16].

However, the effect of ascorbate is not simple. Although it certainly does reduce Fe3+ [9–12], it also reacts rapidly with O2− [17] and even more rapidly with OH− [18], in both cases being oxidized to dehydroascorbate. This product is unstable and decomposes to a complex mixture of other molecules. The total amount of ascorbic acid plus dehydroascorbate in the serum [19] and synovial fluid [13] of rheumatoid patients is below normal, and the ratio of ascorbate to dehydroascorbate appears abnormally low [10]. Ascorbate can either stimulate or inhibit lipid peroxidation depending on its concentration and on the amount of iron present [20].

In order to clarify some of these points, we have investigated the effect of ascorbate at physiological concentrations on the rate of production of OH. radicals by an O2−-generating system.

Materials and methods

Reagents

Superoxide dismutase (specific activity 2900 units/mg of protein as measured by the cytochrome c assay [21]), xanthine oxidase (grade 1, EDTA-free), ATP, catalase and hypoxanthine were obtained from Sigma, ascorbic acid (AnalaR) from BDH Chemicals Ltd. Solutions of ascorbic acid were made up fresh and adjusted to the required pH with KOH immediately before use. Units of catalase (μmol of H2O2 destroyed/min) were measured as described in the Sigma catalogue.

Assay methods

Formation of OH. radicals was measured by a modification [22] of the salicylate hydroxylation method [23]. This method measures the ability of OH. radicals to attack salicylic acid to produce 2,3-dihydroxybenzoate, which is then measured colorimetrically. Final reaction mixtures contained reagents at the concentrations referred to in the legends to the Figures and Table 1, in a total volume of 2 ml of potassium phosphate buffer (0.15 mol/l), pH 7.4. Incubations were carried out at 25°C in a shaking water bath.

Results

A mixture of hypoxanthine and the enzyme xanthine oxidase produces H2O2 and O2− radicals, which, in the presence of iron salts, interact to form OH−, which can be detected by its reaction with aromatic compounds to give hydroxylated products [5, 22, 23]. Fig. 1 shows that OH. production by this system is almost completely inhibited by superoxide dismutase or by catalase, proving that both O2− and H2O2 are essential for the OH. production. The amount of OH. detected is also decreased by compounds that 'scavenge' this radical, such as mannitol, sodium formate and thiourea [22, 23]. A mixture of ascorbate, iron salt and H2O2 also produces OH. radicals; the amount of radicals detected can be decreased by catalase or known scavengers of hydroxyl (mannitol, formate, thiourea) but not by superoxide dismutase or by bovine serum albumin, added to check for nonspecific protein effects (Table 1). Hence ascorbate-dependent OH. formation does not require the O2− radical.

When ascorbate (100 μmol/l) was added to a hypoxanthine/xanthine oxidase system the
Ascorbate, superoxide and rheumatoid disease

Table 1: Effect of radical scavengers on hydroxyl radical production by systems containing ascorbic acid and iron salts

<table>
<thead>
<tr>
<th>Addition to reaction mixture</th>
<th>Rate of OH. production (as nmol of hydroxylated product in 90 min)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>98.4</td>
<td>—</td>
</tr>
<tr>
<td>Superoxide dismutase (100 units/ml)</td>
<td>101.6</td>
<td>0</td>
</tr>
<tr>
<td>Catalase (1000 units/ml)</td>
<td>2.6</td>
<td>97</td>
</tr>
<tr>
<td>Sodium formate (10 mmol/l)</td>
<td>32.6</td>
<td>67</td>
</tr>
<tr>
<td>Mannitol (10 mmol/l)</td>
<td>41.6</td>
<td>58</td>
</tr>
<tr>
<td>Thiourea (10 mmol/l)</td>
<td>20.6</td>
<td>79</td>
</tr>
<tr>
<td>Albumin (1 mg/ml)</td>
<td>97.4</td>
<td>1</td>
</tr>
</tbody>
</table>

Fig. 2. Time course of hydroxyl radical production by systems containing Fe(III)-EDTA (100 μmol/l), hypoxanthine (200 μmol/l) and xanthine oxidase (0-02 unit/cm³) in the presence of various concentrations of ascorbate (μmol/l as indicated to the right of the curves). Open symbols show the effect of superoxide dismutase at 100 units/cm³.

Discussion

Activated phagocytes in the rheumatoid joint will release large quantities of $O_2^-$ and $H_2O_2$ into their surrounding environment [1]. Since iron catalysts are present [2, 3, 24, 26] OH radicals can be formed by a reaction of Fe$^{3+}$ with $H_2O_2$ (eqn. 2). To keep the reaction going Fe$^{3+}$ must be recycled to Fe$^{2+}$. This could be achieved either by the $O_2^-$ radical [5] or by ascorbate [9, 10]. Which will be the more important in vivo?

In the hypoxanthine–xanthine oxidase system, which produces $O_2^-$ and $H_2O_2$ and thus serves as a model for activated phagocytes [1, 4], iron reduction is achieved by $O_2^-$ and thus OH formation is almost completely inhibited by superoxide dismutase. If, by contrast, ascorbate is the reductant, this enzyme has no effect (Table 1).

When ascorbate was added to the hypoxanthine–xanthine oxidase system in the presence of Fe$^{3+}$ at 100 μmol/l the rate of OH. amount of OH. radicals detected increased (Fig. 1) but OH. radical formation was still almost completely prevented by catalase. Fig. 1 shows the effect of superoxide dismutase under the presence of ascorbate; there was little inhibition of OH. formation in the first 30 min of the reaction but an almost complete inhibition subsequently.

The concentration of 'total ascorbate' in the plasma and synovial fluid of rheumatoid patients varies in the range 14–49 pmol/l [13], and the effect of a series of ascorbate concentrations within this range on OH. production by a mixture of hypoxanthine and xanthine oxidase was examined (Fig. 2). At 10 or 25 μmol of ascorbate/l, OH. production was largely inhibited by superoxide dismutase even at the early stages of the reaction; at 50 μmol of ascorbate/l this enzyme inhibited by about 36% over the first 5 min of the reaction but much more effectively at later stages. The concentration of iron salt in these reaction mixtures was 100 μmol/l, in order to maximize production of OH. radicals [23]. Similar results were obtained if the concentration of iron salt in the reaction mixture was lowered to 2-5 μmol/l, except that it took slightly longer for the reaction to become fully inhibitable by superoxide dismutase. For example, with 50 μmol of ascorbate/l and 2.5 μmol of iron salt/l superoxide dismutase inhibited OH. production by 30% in a 1 h incubation; with 20 μmol of ascorbate/l and 2.5 μmol of iron salt/l the inhibition was 53%. In all systems EDTA could be replaced as the chelating agent by ATP without affecting the results.
generation increased slightly. With ascorbate at concentrations up to 25 \( \mu \text{mol/l} \), the OH. generation was still largely inhibited by superoxide dismutase, indicating that \( \text{O}_2^- \) was still the major reducing agent. At 50 \( \mu \text{mol of ascorbate/l} \) there was only partial inhibition by superoxide dismutase for the first 15 min of the reaction, and virtually complete inhibition at later stages. At 100 \( \mu \text{mol of ascorbate/l} \) complete inhibition by dismutase was not seen until 30 min had elapsed. Thus high ascorbate concentrations can partially inhibit superoxide dismutase, with time. It should be noted also that the fraction of OH. production not inhibited by the fraction of \( \text{O}_2^- \) production will fall and \( \text{O}_2^- \) becomes relatively more important as a reducing agent. This explains the increasing effect of superoxide dismutase, with time. It should be noted also that the fraction of OH. production not inhibited by superoxide dismutase is actually an overestimate of ascorbate-dependent OH. generation: this enzyme, by removing \( \text{O}_2^- \), will not only prevent \( \text{O}_2^- \)-dependent OH. generation but it will also prevent the oxidation of ascorbate by \( \text{O}_2^- \) and so allow the ascorbate to remain for a longer period.

The ‘total ascorbate’ in the synovial fluid of rheumatoid patients is in the range 14–49 \( \mu \text{mol/l} \). It is clear from the above results that \( \text{O}_2^- \)-dependent formation of OH. will be more important in vivo. It may well be that the lowered ascorbate levels of rheumatoid patients are due to its oxidation by \( \text{O}_2^- \) and OH. Hence therapy directed against oxygen radicals such as \( \text{O}_2^- \) would still seem to be relevant in the management of rheumatoid disease. In these experiments the iron salt concentration was 100 \( \mu \text{mol/l} \) so that the maximum flux of OH. radicals could be obtained [23]. At more physiological concentrations OH. radicals were still produced and essentially similar results were obtained. Over the very long period in which phagocytes are activated in the rheumatoid joint, superoxide-dependent formation of OH. will be a major damaging mechanism [24–26].

Acknowledgments

We are grateful to the Cancer Research Campaign and CIBA-Geigy Pharmaceuticals for financial support.

References

[23] Halliwell, B. (1978) Superoxide-dependent formation of

