Polyamine inhibition of lipoperoxidation

The influence of polyamines on iron oxidation in the presence of compounds mimicking phospholipid polar heads

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Polyamines appear to inhibit peroxidation of vesicles containing acidic phospholipids. A correlation exists between polyamine binding to phospholipid vesicles and its protective effect. However, phosphatidylinositol-containing vesicles which bind spermine are not protected by the polyamine [Tadolini, Cabrini, Landi, Varani & Pasquali (1985) Biogenic Amines 3, 97–106]. In the present paper I tested the hypothesis that polyamines, in particular spermine, by forming a ternary complex with iron and the phospholipid polar head may change the susceptibility of Fe$^{2+}$ to autoxidation and thus its ability to generate free oxygen radicals. Different compounds mimicking phospholipid polar heads were studied, namely AMP, mimicking phosphatidic acid, CDP-choline, mimicking phosphatidylcholine, and glycerophosphoinositol, mimicking phosphatidylinositol. The results support the proposed hypothesis. In the presence of CDP-choline or of glycerophosphoinositol, spermine poorly affects Fe$^{2+}$ autoxidation, whereas a considerable inhibition is observed in the presence of AMP. The ability of other phosphorus-containing compounds (ATP, ADP, cyclic AMP, sodium phosphate) to affect Fe$^{2+}$ autoxidation in the presence of polyamines was also evaluated to understand the molecular mechanism of this phenomenon. It is proposed that polyamines may be part of the passive cellular defence mechanism against the oxidative damage caused by Fe$^{2+}$.

INTRODUCTION

The aliphatic polyamines spermine and spermidine and their diamine precursor, putrescine, are non-protein nitrogenous bases widely distributed in prokaryotic and eukaryotic cells (Bachrach, 1973). They are known to interact with anionic cell constituents, among which are acidic phospholipids in biomembranes (Tabor, 1960a; Tadolini, 1980; Schuber et al., 1983; Tadolini et al., 1984, 1985a). The phospholipid–polyamine complex appears to exert a stabilizing effect on the membranes (Tabor, 1960a; Jellinck & Perry, 1967), possibly through the inhibition of lipid peroxidation (Tabor, 1960b; Kitada et al., 1979, 1981; Tadolini et al., 1984, 1985b). We have demonstrated that protection from lipid peroxidation occurs only when spermine is bound to phospholipid vesicles (Tadolini et al., 1984, 1985b). In fact zwitterionic phospholipid vesicles, such as phosphatidylcholine vesicles, which do not bind spermine, are not protected by the polyamine. The effects of spermine on the peroxidation of vesicles containing different types and densities of acidic phospholipids are generally in agreement with the binding of spermine to the same vesicles (Tadolini et al., 1985b). However, when vesicles containing phosphatidylinositol instead of phosphatidic acid, phosphatidylserine and cardiolipin are used, these are not protected from peroxidation, although spermine binds the vesicles.

We have investigated the possible molecular mechanism by which spermine may inhibit lipid peroxidation. Spermine may compete with iron and mobilize it from the anionic sites of phospholipids. Metal interaction with target molecules appears to be essential for oxidative damage to arise (Hodgson & Fridovich, 1975; Van Hemmen & Meuling, 1977; Samuni et al., 1981). The experimental data, however, were only partially consistent with this hypothesis (Tadolini et al., 1985b).

The hypothesis that spermine bound to phospholipid vesicles may be able to interact with iron and lower its reactivity was also proposed (Tadolini et al., 1984). To verify the possible effect of polyamines bound to phospholipids on iron reactivity, we have studied whether and to what extent these amines affect Fe$^{2+}$ autoxidation in Mops buffer in the presence of compounds mimicking phospholipid polar heads. The compounds studied were AMP, CDP-choline and glycerophosphoinositol, which mimic respectively phosphatidic acid, phosphatidylcholine and phosphatidylinositol. Other phosphorus-containing compounds were studied to understand better the molecular mechanism of this phenomenon.

MATERIALS AND METHODS

Mops, ATP, ADP, AMP, cyclic AMP, CDP-choline, glycerophosphoinositol, EDTA, spermine tetrahydrochloride, spermidine trihydrochloride and putrescine dihydrochloride were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). 1,10-Phenanthroline was obtained from Merck (Darmstadt, Germany). All procedures were carried out in plastic or acid-washed glassware, and solutions were prepared in Chelex-resin-treated water. The pH values of buffer were adjusted at room temperature. Stock solutions of Fe$^{2+}$ were prepared daily. Fe$^{2+}$ autoxidation was evaluated by determining the Fe$^{2+}$ content by the o-phenanthroline method (Mahler & Elowe, 1954). All incubations were carried out in 5 mM-Mops buffer, pH 7.2, at room temperature. The 1 ml samples to be analysed were prepared by the successive addition to the buffer of the phosphorus-containing compound and of the polyamine. When the accelerated Fe$^{2+}$ autoxidation was studied, 30 μM-H$_2$O$_2$, 30 μM-FeCl$_3$, and 15 μM-EDTA were first added to the
buffer. The reactions were started by addition of 150 μM-FeCl₃. At the time stated, the reactions were stopped by addition of 0.2 ml of 25 mM-1,10-phenanthroline, and A₃₁₅ was immediately read.

RESULTS

Effect of polyamines on Fe²⁺ autoxidation in the presence of AMP, CDP-choline and glycerophosphoinositol

When the rate of Fe²⁺ oxidation in Mops buffer is measured in the presence of 50 μM-spermine, this polycation only slightly affects the reaction. By contrast, spermine greatly decreases the rate of Fe²⁺ oxidation when the reaction is conducted in the presence of 10 μM-AMP (Fig. 1). I studied the effect of the concentrations of spermine, spermidine and putrescine on Fe²⁺ oxidation in the presence of an AMP concentration (10 μM) that is itself effective, but at an incubation time when its inhibition is over (25 min) (Fig. 2a). These amines are able to inhibit Fe²⁺ autoxidation almost completely, and the concentration causing half-maximal effect (C₀.₅) is 9 μM, 90 μM and 1.6 mM for spermine, spermidine, and putrescine respectively. Also, CDP-choline was shown to affect Fe²⁺ oxidation in Mops buffer, although higher concentrations were required to obtain a smaller effect (Tadolini & Sechi, 1987). I studied the effect of spermine in the presence of this compound. The inhibition exerted by 0.4 mM-CDP-choline is over in 25 min, whereas the small inhibition exerted by 40 μM of this nucleotide derivative lasts only 15 min. In both these conditions the spermine effect, also at the highest concentration tested (0.1 mM), is limited (Fig. 3).

Also, in the presence of glycerophosphoinositol (50 μM), which does not itself significantly affect Fe²⁺ oxidation, the spermine effect is limited and high concentrations of this amine are required (C₀.₅ > 0.1 mM) (Fig. 3).

Effect of polyamines on Fe²⁺ autoxidation in the presence of other adenine nucleotides and sodium phosphate

In order to clarify the molecular mechanism of the synergic effect of polyamines and AMP on the inhibition of Fe²⁺ oxidation, I studied the polyamine effect in the presence of other adenine nucleotides, namely ADP, ATP and cyclic AMP. Both ADP and ATP inhibit Fe²⁺ oxidation, whereas cyclic AMP does not significantly affect it (Tadolini & Sechi, 1987). As for AMP, the polyamine effect was studied at an incubation time when the eventual nucleotide effect was over (25 min for ADP and ATP, 15 min for cyclic AMP). The C₀.₅ values of spermine, spermidine and putrescine were respectively

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**Fig. 1.** Effect of spermine on the time course of Fe³⁺ autoxidation in 5 mM-Mops, pH 7.2, in the presence or absence of AMP

The disappearance of 150 μM-Fe³⁺ from the standard solution (○) and in the presence of 50 μM-spermine (□), 10 μM-AMP (△) or 50 μM-spermine+10 μM-AMP (■) was measured.

**Fig. 2.** Effect of polyamine concentration on Fe³⁺ autoxidation in 5 mM-Mops, pH 7.2, in the presence of AMP (a), ADP (b) or ATP (c)

The disappearance of 150 μM-Fe³⁺ from the standard solution containing 10 μM nucleotide and increasing concentrations of spermine (○), spermidine (△) and putrescine (■) was measured after 25 min incubation.

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2 \(\mu\)M, 90 \(\mu\)M and 1.4 mM in the presence of 10 \(\mu\)M-ADP (Fig. 2b) and 1.3 \(\mu\)M, 6.5 \(\mu\)M and 1.1 mM in the presence of 10 \(\mu\)M-ATP (Fig. 2c). When 50 \(\mu\)M-cyclic AMP was used, inhibition of Fe\(^{3+}\) oxidation was observed in the presence of spermine, although high concentrations of this amine were required \(C_{0.5} \approx 90 \mu\)M (Fig. 3). High concentrations of spermine \(C_{0.5} \approx 60 \mu\)M were also able to decrease Fe\(^{3+}\) oxidation in the presence of 20 mM-sodium phosphate (Fig. 3), a compound itself able to inhibit Fe\(^{3+}\) oxidation at low concentrations (Tadolini & Sechi, 1987).

**Effect of spermine on Fe\(^{3+}\) oxidation accelerated by \(\text{H}_2\text{O}_2\), \text{FeCl}_3\) and EDTA in the presence of ATP**

Spermine \((10 \mu\)M\) (Table 1) is able to increase the inhibition exerted by 15 \(\mu\)M-ATP on Fe\(^{3+}\) oxidation accelerated by either 30 \(\mu\)M-\(\text{H}_2\text{O}_2\) or 30 \(\mu\)M-\text{FeCl}_3. When Fe\(^{3+}\) oxidation is accelerated by a sub-stoichiometric concentration of EDTA \((15 \mu\)M\), both ATP \((10 \mu\)M\) and ATP plus spermine \((10 \mu\)M\) decrease the oxidation, although to a lesser extent.

**Table 1. Effect of spermine in Fe\(^{3+}\) oxidation accelerated by \(\text{H}_2\text{O}_2\), \text{FeCl}_3\) and EDTA in the presence of ATP**

<table>
<thead>
<tr>
<th>Addition</th>
<th>(\Delta A_{515})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.750</td>
</tr>
<tr>
<td>+ \text{H}_2\text{O}_2 (30 \mu)M)</td>
<td>1.247</td>
</tr>
<tr>
<td>+ \text{H}_2\text{O}_2 (30 \mu)M) + ATP (15 \mu)M)</td>
<td>0.580</td>
</tr>
<tr>
<td>+ \text{H}_2\text{O}_2 (30 \mu)M) + ATP (15 \mu)M) + spermine (10 \mu)M)</td>
<td>0.380</td>
</tr>
<tr>
<td>+ \text{FeCl}_3 (30 \mu)M)</td>
<td>1.250</td>
</tr>
<tr>
<td>+ \text{FeCl}_3 (30 \mu)M) + ATP (15 \mu)M)</td>
<td>0.252</td>
</tr>
<tr>
<td>+ \text{FeCl}_3 (30 \mu)M) + ATP (15 \mu)M) + spermine (10 \mu)M)</td>
<td>0.105</td>
</tr>
<tr>
<td>+ EDTA (15 \mu)M)</td>
<td>1.250</td>
</tr>
<tr>
<td>+ EDTA (15 \mu)M) + ATP (10 \mu)M)</td>
<td>0.762</td>
</tr>
<tr>
<td>+ EDTA (15 \mu)M) + ATP (10 \mu)M) + spermine (10 \mu)M)</td>
<td>0.431</td>
</tr>
</tbody>
</table>

From the data presented here, it is evident that spermine is capable of affecting Fe\(^{3+}\) autoxidation, but only in the presence of phosphorus-containing compounds. The \(C_{0.5}\) of spermine in the presence of different phosphorus-containing compounds, however, differs greatly. In fact, the \(C_{0.5}\) in the presence of 10 \(\mu\)M-AMP is 9 \(\mu\)M, compared with \(>0.1 \mu\)M when 0.4 mM-CDP-choline or 50 \(\mu\)M-glycerophosphoinositol is present. These compounds were chosen to mimic phosphatidic acid, phosphatidylcholine and phosphatidylinositol polar heads respectively. The results obtained show that there is a strong correlation between spermine inhibition of Fe\(^{3+}\) oxidation in the presence of the compound mimicking the phospholipid polar head and spermine inhibition of peroxidation of vesicles containing phospholipid with such a polar head (Tadolini et al., 1985b). These results suggest that spermine, in the presence of phospholipid polar heads, may affect Fe\(^{3+}\) oxidation and thus generation of oxy radicals. The studies conducted with other adenine nucleotides, to clarify the mechanism of the polyamine effect, show that the \(C_{0.5}\) depends on the charges and the structural features of nucleotides and also on the charges of polyamine molecules. Nakai & Glinsmann (1977) showed that polyamines bind ATP, ADP and AMP and that the interaction depends on the same parameters. Thus the inhibition by polyamine of Fe\(^{3+}\) oxidation appears to be due to the formation of a complex between the polyamine and the phosphorus-containing compound. We have previously proposed that the effect of phosphorus-containing compounds on Fe\(^{3+}\) oxidation in
Mops buffer may be due to the formation of a complex with iron (Tadolini & Sechi, 1987). Spermine interaction with the nucleotide, however, apparently does not displace iron from the phosphorus-containing compound. In this case iron would become free, the inhibition of its oxidation exerted by the phosphorus-containing compound would cease, and Fe²⁺ would be readily oxidized.

The data obtained suggest that the polyamine, by contrast, participates in the formation of the complex. The polyamine may form, for example with AMP and iron, a ternary complex more efficient than the binary complex AMP–iron in decreasing Fe²⁺ oxidation. It may be also able to transform an inefficient binary complex (for example cyclic AMP–iron) into an efficient ternary complex (cyclic AMP–iron–spermine). The concentrations of spermine (C₉₋₁₀ 1–10 μM) and effective nucleotide (10 μM) that are able to decrease the oxidation of 150 μM Fe²⁺ lead one to wonder about the nature of the iron taking part in the formation of the complex.

Fe²⁺ oxidation in Mops buffer is characterized by a lag phase (Lambeth et al., 1982; Tadolini, 1987a) that is decreased by Fe⁴⁺ addition (Tadolini, 1987b). A rate-limiting reaction producing a Fe⁴⁺ catalyst thus appears to occur. Phosphorus-containing compounds are able to decrease Fe²⁺ oxidation, and their C₀₉ is related to the concentration of Fe²⁺ in the assay (Tadolini & Sechi, 1987). We proposed that phosphorus-containing compounds might decrease Fe²⁺ oxidation by complexing Fe³⁺ and thus decreasing its reactivity as a catalyst. The demonstration that spermine increases the inhibition by ATP of Fe²⁺ oxidation accelerated by Fe⁴⁺, either added or generated by Fe⁴⁺ oxidation by H₂O₂ and EDTA, indicates Fe⁴⁺ as the iron form participating in the formation of the proposed ternary complex. In this complex, Fe⁴⁺ would greatly decrease its catalytic activity and Fe²⁺ oxidation would be inhibited. This hypothesis is also supported by the time course of the inhibition of Fe²⁺ oxidation by AMP, either alone or complexed with spermine. An increased lag phase is observed, corresponding to the binding to the inhibitor of Fe²⁺ generated by the rate-limiting step. This is followed by an increased rate of Fe²⁺ oxidation, corresponding to the production of a concentration of the Fe⁴⁺ catalyst exceeding the inhibitor concentration. The hypothesis is further supported by the observation that higher concentrations of AMP, either alone or complexed with spermine, proportionally increase the lag phase (results not shown).

In summary, the results presented in this paper are consistent with the hypothesis that the inhibition of lipoperoxidation by polyamine is due to its ability to form a ternary complex with the phospholipid polar head and the Fe⁴⁺ catalyst of Fe²⁺ oxidation. The decreased Fe²⁺ oxidation would result in a decreased generation of oxygen free radicals. The lack of spermine inhibition of lipoperoxidation of certain vesicles types may be due either to a low affinity of the polyamine for the polar head of such phospholipids or to a low ability of the polyamine–acidic-phospholipid complex to bind and/or affect the Fe⁴⁺ catalyst. The lack of inhibition by spermine of the peroxidation of phosphatidylincholine-containing vesicles appears to belong to the former case, as these vesicles were shown not to interact significantly with spermine (Schuber et al., 1983; Tadolini et al., 1985a). On the contrary, phosphatidylinositol-containing vesicles bind spermine (Tadolini et al., 1985a), but this complex does not interfere with Fe⁴⁺–dependent peroxidation. The result obtained by studying the glycerophosphoinositol effect on Fe⁴⁺ autoxidation (Tadolini & Sechi, 1987) indicates that, in this case, it is the interaction between the phospholipid polar head and iron that does not appear to be suitable in affecting the Fe⁴⁺ catalyst.

The results presented, besides suggesting a possible molecular mechanism for the protection by polyamines of lipid peroxidation, proposed polyamines as part of the passive cellular defence mechanism against the oxidative damage caused by Fe⁴⁺. When bound to suitable phosphorus-containing compounds, they may decrease Fe²⁺ oxidation and thus oxygen free-radical production.

This work was supported by grants from the Italian C.N.R. and M.P.I. I am extremely grateful to Dr. N. Seiler for helpful comments and to Miss A. Franzò for secretarial assistance.

REFERENCES