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EFFECTS OF SEMICARBAZIDE ON OXIDATIVE PROCESSES IN HUMAN RED BLOOD CELL MEMBRANES

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Summary

Semicarbazide can interfere with oxidative processes in the red blood cell membrane via different modes of action.

Treatment of human red blood cell membanes with O_3 results, among other effects, in cross-linking of membrane proteins and inhibition of glyceraldehyde-3-phosphate dehydrogenase activity. Semicarbazide inhibits these effects by acting as an O_3 scavenger. The effect of semicarbazide as an O_3 scavenger is complicated by the fact that ozonolysis of semicarbazide yields a product that causes inhibition of glyceraldehyde-3-phosphate dehydrogenase.

Glyceraldehyde-3-phosphate dehydrogenase inhibition can also be provoked by incubation of membrane suspensions with O_3-treated phospholipids. Semicarbazide prevented this effect by interaction with an inhibitory O_3-phospholipid reaction product.

Protoporphyrin-induced photodynamic cross-linking of membrane proteins is chemically distinct from O_3-induced cross-linking. Photodynamic cross-linking is also inhibited by semicarbazide, in this case via reaction with a histidine photooxidation product.

Introduction

Deleterious oxidative processes in the red blood cell membrane can be provoked by a wide variety of agents, like H_2O_2 [1, 2], [3—8], phenylhydrazines [9] and photosensitizing agents [10—12]. Such oxidative processes have several common structural and functional consequences, although the underlying pathochemical processes may be quite different. The oxidative hemolysis

caused by arylhydrazines, for instance, is presumably mediated by a primary reaction of the agent with hemoglobin, yielding ferrihemochrome and hydroxyl radicals [9]. The primary effect of H_2O_2 is presumably peroxidation of unsaturated fatty acid chains in the membrane bilayer [1], whereas protoporphyrin-induced photodynamic membrane damage involves a reaction of singlet oxygen with membrane proteins [10].

The mechanism of O_3-induced red cell membrane damage is not yet clear. Chan et al. [6] demonstrated inactivation of membrane-bound ($Na^+ + K^+$)-ATPase both by direct exposure of membranes to O_3 [6] and by incubation of membranes with ozonized phospholipids [7]. Based on the observation that semicarbazide ($NH_2CONHNH_2$) prevents ATPase inhibition in both situations, the authors suggested that similar mechanisms were involved. In this line of thought, ozonolysis of phospholipids in biomembranes would be a major cause of the toxicity of O_3 [7].

In a previous paper, we demonstrated O_3-induced cross-linking of spectrin in the absence of phospholipids and the protection against this cross-linking by semicarbazide [13]. These results contradict, to a certain extent, the conclusions of Kesner et al. [7]. Apparently, O_3 can affect membrane proteins not only indirectly, via ozonolysis of membrane lipids, but also directly. Moreover, the protective effect of semicarbazide against cross-linking of isolated spectrin cannot be ascribed to a reaction between this agent and toxic phospholipid ozonolysis products, as described by Kesner et al. [7].

To solve these apparent contradictions, the effects of semicarbazide were studied in more detail.

Materials and Methods

All reagents were analytical grade and used without further purification. ^{14}C-labeled semicarbazide was obtained from ICN Pharmaceuticals (Irvine). All other labeled compounds were from The Radiochemical Centre (Amersham).

Heparinized human blood was centrifuged and the red blood cells were washed three times in a solution containing 154 mM NaCl, 9.6 mM Na_2HPO_4 and 1.5 mM NaH_2PO_4. Red cell membranes were prepared by gradual osmotic lysis as described by Weed et al. [14]. Spectrin was eluted from ghosts according to the method of Bennett and Branton [15]. O_3 was generated with a Supelco Micro-Ozonizer in O_2 at a rate of 2.5 μmol/min. Photodynamic effects were studied by illuminating samples in the presence of protoporphyrin, as described before [16].

Glyceraldehyde-3-phosphate dehydrogenase activity was measured according to the method of Wu and Racker [17]. Malonaldehyde was measured with thiobarbituric acid, according to the method of Ottolenghi [18], using as a standard a malonaldehyde solution prepared to acid hydrolysis of 1,1,3,3-tetramethoxypropane, as described by Girotti [19].

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis was performed as described by Fairbanks et al. [20] after dissolving the samples in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 40 mM dithiothreitol and 1% SDS.

Analysis of ^{14}C-labeled semicarbazide reaction products was performed by

means of thin-layer chromatography on silica gel, with the solvent systems, *n*-butanol/acetic acid/water (4:1:1, v/v/v) and phenol/water (3:1, w/v) and subsequent autoradiography by placing the dried chromatograms in close contact with X-ray film for 7—14 days.

Photodynamic coupling of reagents to ghosts was measured by illumination of the ghosts in 10 mM phosphate buffer, pH 8.5, in the presence of 0.3 mM protoporphyrin and varying concentration of the labeled reagents, during 2 h, as described before [10]. Control experiments had shown that after this illumination period, photooxidation and photodynamic coupling were maximal. After illumination, the ghosts were spun down and the supernatant was analysed for residual, unbound radioactivity. The ghosts were washed five times in 50 vol. of buffer, to remove unbound reagent, and subsequently analysed for radioactivity. The number of binding groups per mg protein was estimated from plots of bound/free against bound reagent.

Protein was measured according to the method of Lowry et al. [21].

Results

Photodynamic effects

Photodynamic cross-linking of membrane proteins, with protoporphyrin as sensitizer, is shown in Fig. 1A. Cross-linking leads to accumulation of high molecular weight material on top of the gel after polyacrylamide gel electrophoresis. Spectrin (bands 1 and 2), band 4.1 and band 6 are more sensitive to cross-linking than the other membrane proteins. In previous papers, it has been shown that this photodynamic cross-linking is caused by direct photooxidation of sensitive amino acid residues of membrane proteins, followed by a secondary

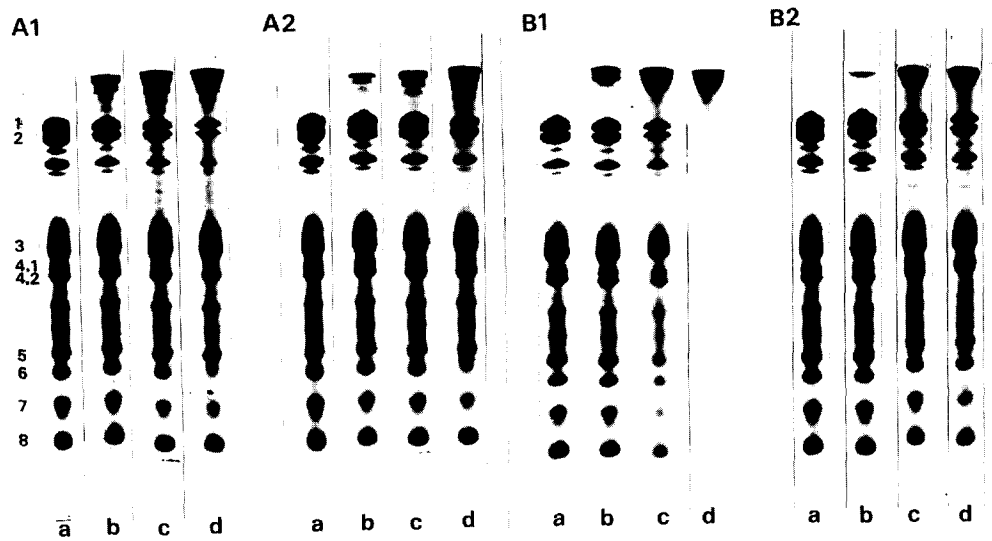

Fig. 1. Effect of semicarbazide on cross-linking of membrane proteins. Ghost suspensions: 3 ml, 1.1 mg protein/ml. (A) Photodynamic cross-linking. A1, without and A2, with 50 mM semicarbazide. Illumination times: o(a), 1(b), 2(c) and 5(d) min. (B) O_3-induced cross-linking. B1, without and B2, with 50 mM semicarbazide. Exposure times: 0(a), 2(b), 5(c) and 10(d) min.

reaction between free NH_2 groups and a photooxidation product of a sensitive amino acid residue, presumably histidine [10]. This photooxidation product of histidine is very sensitive to nucleophilic attack. In accordance, photodynamic cross-linking is inhibited by amino acids, primary amines and NaI, via competition of these agents with protein NH_2 groups and resulting in covalent binding of these agents to the membrane proteins [10]. Semicarbazide offered a similar protection against photodynamic cross-linking (Fig. 1A), with a concomitant covalent binding to membrane proteins. A quasi-Scatchard plot* of these binding experiments showed that semicarbazide was photo-

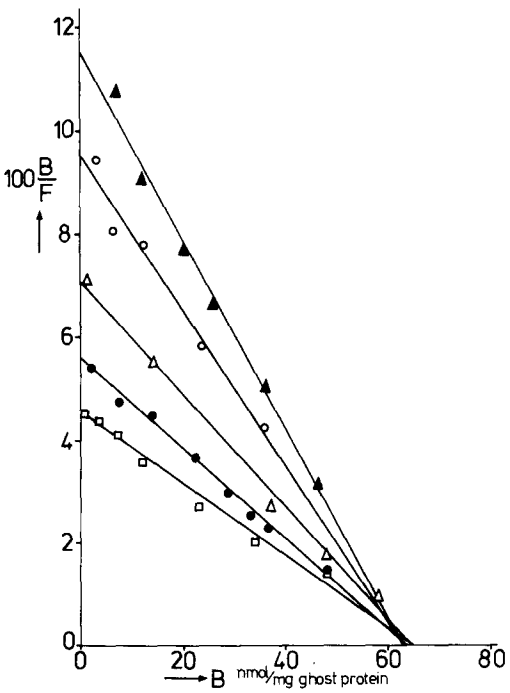

Fig. 2. Quasi-Scatchard plot of photodynamic binding of reagents to ghosts. (▲) I^-; (○) lysine; (△) ethylamine; (●) semicarbazide and (□) glycine.

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* This quasi-Scatchard plot is based on the following considerations. If a group, X, can either react irreversibly with a reagent, A, or be converted to Y:

$$A + K \xrightarrow{k_1} AX,\ v_1 = k_1\,[A][X]$$

$$X \xrightarrow{k_1} Y,\ v_2 = k_2\,[X]$$

$X_t = AX + Y$, in the final situation.

$$\frac{[AX]}{[A]} = \frac{k_1}{k_2}\,([X_t] - [AX])$$

In terms of the quasi-Scatchard plot:

$[AX] = B$(bound); $[A] = F$(free)

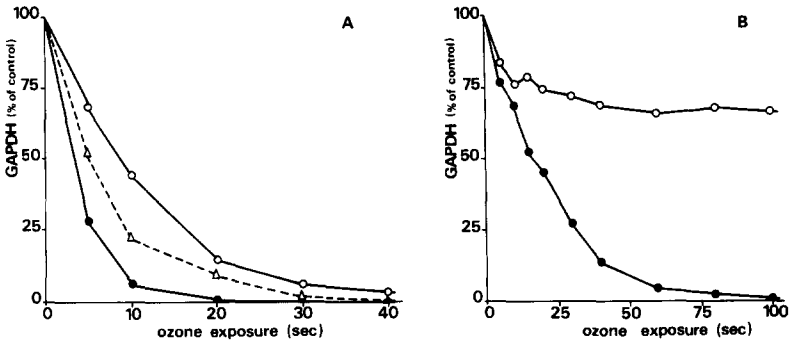

Fig. 3. Effect of semicarbazide on O_3-induced activation of erythrocyte membrane glyceraldehyde-3-phosphate dehydrogenase. (A) Direct treatment of ghosts (3 ml, 1.1 mg protein/ml) with O_3. (•) In the absence of semicarbazide. (△) 100 mM semicarbazide present during O_3 treatment. (○) 100 mM semicarbazide, added after O_3 treatment. After O_3 treatment, the samples were incubated during 15 min at 37°C, prior to assay of enzyme activity. (B) Treatment of ghosts with ozonized phospholipids. Egg phosphatidylcholine (5 ml, 5 mg/ml) was exposed to O_3 and then incubated for 15 min at 37°C either with (○) or without (•) 100 mM semicarbazide prior to addition to the ghost suspension (5 mg phospholipid/mg membrane protein).

dynamically linked to the same number of binding groups as found with other protecting agents (Fig. 2).

Ozone effects

Treatment of human red blood cell membranes with O_3 also caused covalent cross-linking of membrane proteins, as shown in Fig. 1B. As contrasted with photodynamic cross-linking, all membrane proteins are about equally sensitive, with no clear difference in the velocity of disappearance of the individual protein bands. A similar cross-linking was observed after treatment of solubilized, lipid-free spectrin with O_3. Both with ghosts and with solubilized spectrin, cross-linking was inhibited by semicarbazide (Fig. 1B), but not by the other agents that inhibited photodynamic cross-linking. In studies with ^{14}C-labeled semicarbazide, it appeared that this inhibition was not attended by binding of semicarbazide to the ghosts, as distinct from the results on inhibition of photodynamic cross-linking. Addition of semicarbazide after O_3-treatment of the ghosts did not cause any decrease in cross-linking.

O_3-treatment of red cell membranes led to a rapid inactivation of glyceraldehyde-3-phosphate dehydrogenase (Fig. 3A). After 20 s, when glyceraldehyde-3-phosphate dehydrogenase activity had vanished, the malonaldehyde concentration in the sample was 0.02 μM. The inhibition of glyceraldehyde-3-phosphate dehydrogenase is partly reversed by subsequent incubation with semicarbazide. The effect of semicarbazide when present during O_3 treatment was much less (Fig. 3A). With purified, lipid-free glyceraldehyde-3-phosphate dehydrogenase similar results were obtained.

Effects of ozonized phospholipids

To investigate whether these O_3-effects could be mimicked by O_3-treated phospholipid suspensions, the procedure described by Kesner et al. [7] was

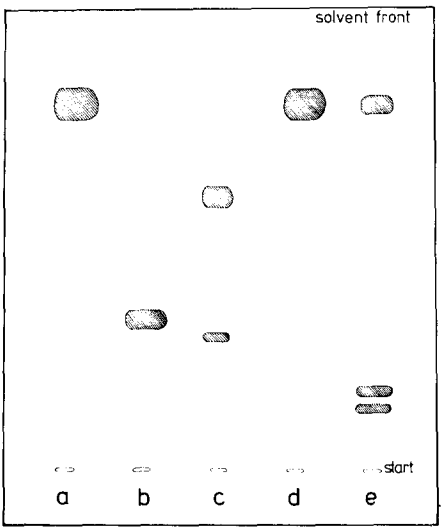

Fig. 4. Autoradiography of silica gel thin-layer chromatograms of ^{14}C-labeled semicarbazide. Solvent system: phenol/water (3:1, w/v). (a) Untreated semicarbazide; (b) after incubation with O_3-treated phosphatidylcholine; (c) after O_3 treatment; (d) after illumination in the presence of protoporphyrin; (e) after illumination with histidine in the presence of protoporphyrin.

followed. Incubation of ghosts with an excess of O_3-treated phospholipids resulted in inhibition of glyceraldehyde-3-phosphate dehydrogenase (Fig. 3B). With the phospholipid sample, exposed to O_3 for 40 s, an almost 90% inhibition was reached, at a malonaldehyde concentration in the incubation medium of 2.2 μM. The inhibitory effect was almost completely reversed by incubation of the O_3-treated phospholipid suspensions with semicarbazide, prior to addition to the ghost sample (Fig. 3B).

Incubation of isolated spectrin with O_3-treated phospholipids led to a very limited covalent cross-linking, that was prevented by incubation of the O_3-treated phospholipids with semicarbazide.

Model experiments with semicarbazide

To elucidate the effects of semicarbazide in these various processes, this agent was studied in a number of model systems. When ^{14}C-labeled semicarbazide is incubated with O_3-treated phospholipids, a new product is formed, apparently by a reaction between semicarbazide and a phospholipid oxidation product (Fig. 4b). This phospholipid oxidation product was not malonaldehyde: after incubation of ^{14}C-labeled semicarbarzide with malonaldehyde, no reaction product could be detected by thin-layer chromatography.

O_3 treatment of a pure semicarbazide solution yielded at least two oxidation products (Fig. 4c). These oxidation products are apparently generated by an interaction between O_3 and semicarbazide, as no oxidation products were found in a control experiment in which the semicarbazide was only exposed to O_2.

Illumination of a semicarbazide solution in the presence of protoporphyrin had no effect (Fig. 4c). Apparently, semicarbazide is not sensitive to direct photooxidation in this system. When a solution of semicarbazide and histidine

was illuminated in the presence of protoporphyrin, coupling products of photooxidized histidine and semicarbazide were visible (Fig. 4e). These products were not formed after illumination in the absence of protoporphyrin or after incubation in the dark.

Finally, the influence of semicarbazide oxidation products on glyceraldehyde-3-phosphate dehydrogenase activity was investigated. A solution of semicarbazide in 50 mM phosphate buffer, pH 7.4, and buffer solution without semicarbazide were treated for varying periods with O_3. These solutions were subsequently added to ghost suspensions, to give the same final concentrations as in the experiments described above. It appeared that semicarbazide solutions, exposed to O_3 during ½ and 2 min, caused a 75 and 97% inhibition of enzyme activity, respectively. O_3-exposed buffer solutions did not cause inhibition.

Discussion

Although inhibition of glyceraldehyde-3-phosphate dehydrogenase and protein cross-linking can be caused by ozonized phospholipids (Figs. 1B and 3B), it seems improbable that this mechanism plays a significant role in enzyme inactivation by direct exposure of membranes to O_3. The amount of ozonized phospholipids, utilized in these experiments, represents a 6-fold excess with reference to the amount of phospholipid in the membrane sample. Complete inhibition of glyceraldehyde-3-phosphate dehydrogenase is reached at a malonaldehyde concentration in the incubation mixture of 0.02 μM with direct exposure and of 2.2 μM with O_3-treated phospholipids. Even taking into account the 6-fold higher phospholipid concentration and the longer O_3-exposure time (see Fig. 3), these results show that phospholipids in suspension are much more reactive with respect to O_3 than phospholipids in the membrane bilayer. This is in accord with conclusions reached by Freeman et al. [8] who found liposomes more reactive to O_3 than phospholipids in biomembranes.

Furthermore, both O_3-induced cross-linking of spectrin and inhibition of glyceraldehyde-3-phosphate dehydrogenase were observed in lipid-free systems with about the same velocity as in membrane preparations.

The main argument for the suggestion that O_3 inhibited membrane-bound ($Na^+ + K^+$)-ATPase via ozonolysis of membrane lipids was the protective effect of semicarbazide in both the direct and the indirect experiments [7]. This argument would lose its conclusive force, however, if the protective effects were to appear to be based on different modes of action. The results presented in this paper indicate that this is the case.

The protection against protein cross-linking and glyceraldehyde-3-phosphate dehydrogenase inhibition induced by ozonized phospholipids can be explained easily by the interaction between semicarbazide and phospholipid oxidation products, as shown in Fig. 4b. The protection of isolated spectrin against cross-linking, however, can only be ascribed to the effect of semicarbazide as an O_3 scavenger, as shown in Fig. 4c. It seems highly probable that the protection of membrane-bound ($Na^+ + K^+$)-ATPase against inactivation by direct exposure to O_3, as described by Kesner et al. [7], is also based on this mode of action.

As shown in Results, O_3 treatment of semicarbazide resulted in the formation of one or more products, that strongly inhibit glyceraldehyde-3-phosphate dehydrogenase. This explains the, at first sight surprising, observation that semicarbazide, despite its activity as an O_3 scavenger, hardly protected glyceraldehyde-3-phosphate dehydrogenase against O_3 inactivation, whereas incubation with semibarcazide subsequent to O_3 treatment led to a more pronounced restoration of activity (Fig. 3A). The mechanism of reactivation of O_3-treated glyceraldehyde-3-phosphate dehydrogenase by semicarbide is not yet clear.

Photodynamic and O_3-induced cross-linking differ not only in minor aspects like different sensitivities of membrane proteins but also apparently in the chemical nature of the cross-links. As shown previously, photodynamic cross-linking is caused by a secondary reaction between NH_2 groups and a photo-oxidation product of, presumably, histidine and is inhibited by binding of certain protecting agents (including semicarbazide) to the histidine photo-oxidation product [10]. O_3-induced cross-linking, on the other hand, is inhibited by semicarbazide, but not by the other agents that inhibit photodynamic cross-linking. Furthermore, this inhibition is not attended by semicarbazide binding, but can be explained satisfactorily by the O_3-scavenging effect of the reagent, depicted in Fig. 4c. The chemical nature of O_3-induced cross-links is the subject of present investigations in our laboratory.

In conclusion, these experiments have shown that semicarbazide can interfere with oxidative processes in red cell membranes via different modes of action, depending on the experimental conditions. This should be considered when evaluating the effects of this reagent in various forms of oxidative stress.

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