Study of Activated Oxygen Production by Some Thiols Using Chemiluminescence

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Abstract. 2-3-dimercapto-1-propane sulfonic acid, D-penicillamine and meso-dimercapto succinic acid, drugs widely applied as antidota against metal poisoning, and cysteine and glutathione were studied with respect to their ability to generate and to scavenge superoxide anion radical. Superoxide production and scavenging were tested by means of luminol-dependent chemiluminescence. In presence of 1 μmol/l ADP-Fe^{3+} only cysteine and meso-dimercapto succinic acid induced chemiluminescence which could be inhibited by superoxide dismutase. 2,3-dimercapto-1-propane sulfonic acid, D-penicillamine and glutathione acted as O_2^- scavengers. These thiols inhibited O_2^--dependent lipid peroxidation thus acting as antioxidants, whereas cysteine and meso-dimercapto succinic acid accelerated peroxidation. It is suggested that the toxic side effects of thiols may be due to their ability to generate or to scavenge free radicals.

Key words: Thiols — Chemiluminescence — Superoxide radical — Lipid peroxidation

Abbreviations: DMPS — 2,3-dimercapto-1-propane sulfonic acid; D-pen — D-penicillamine; DMSA — meso-dimercapto succinic acid; SOD — superoxide dismutase; MDA — malondialdehyde, BHT — butylated hydroxytoluene; LDCL — luminol-dependent chemiluminescence

Introduction

Many thiols of low molecular weight possess a high affinity for heavy metal ions. Some of them, in particular DMSA, DMPS, D-pen and 2,3-dimercaptopropanol, have been used in the treatment of lead, mercury and copper intoxications (Chenoveth 1968; Boulding and Baker 1957; Lange 1975) as well as in Willson's disease (Walsh 1956). The same thiols have been successfully used as antidota against metal poisoning in animals (Soli et al. 1978; Aposhian 1983). However, many thi-

ols have been shown to be toxic and to exert undesirable side effects (Jellum et al. 1973). Thiyl radicals and "active oxygen" species are formed during the autoxidation of thiols, and it has been suggested that these species are responsible for tissue damage (Munday 1989). To the best of our knowledge, the possible production of "activated oxygen" by the above compounds has not yet been investigated.

In this work DMPS, DMSA, D-pen and two natural thiols - Cys and GSH were compared with respect to their ability to generate and to scavenge O_2^-.

Materials and Methods

GSH and ADP were obtained from Boehringer, Mannheim, Germany. Luminol (5-amino-2,3-dihydro-1,4-phtalazine dione) was purchased from Koch Light, England. L-Cysteine was supplied by Merck. D-pen and 5,5'-dithio-bis-(2-nitrobenzoic acid) were products of Sigma, St. Louis, Mo. DMSA and DMPS, sodium salt were products of Johnson and Johnson, and Heyl, Berlin, respectively. All other reagents were of the highest quality commercially obtainable, and water was deionized. SOD from bovine erythrocytes (3500 units/mg) was a product of Sigma.

Phospholipids were obtained from egg yolks as described by Clark and Switzer (1977). Liposomes were used at a final concentration of 2 mg phospholipid/ml buffered saline, pH 7.4.

The generation of O_2^- was tested in parallel by three different methods: cytochrome c reduction (Steinbrecher 1988), epinephrine oxidation (Misra and Fridovich 1972) and LDCL (Hodgson and Fridovich 1973). For chemiluminescence studies the sample cuvette contained 0.1 mmol/l luminol, 1 μmol/l ADP-Fe^{3+} and the thiol compound (in concentration indicated in Table and Figure legends) in 2 ml of 5 mmol/l phosphate buffer, pH 7.4. The rate of thiol compounds SH-groups oxidation was determined by the Ellman's test (Ellman 1959). In brief, 3 ml of the sample was mixed with 2.0 ml of 0.1 mol/l phosphate buffer, pH 8.0 and 5 ml deionized water. To 3 ml of this mixture in a photometer cell 0.02 ml of the Ellman's reagent were added and after 2 min of incubation the absorbance at 412 nm was measured. Ellman's reagent was freshly prepared by dissolving 39.6 mg of 5,5'-dithio-bis-(2-nitrobenzoic acid) in 10 ml of 0.1 mol/l phosphate buffer, pH 7.0.

Lipid peroxidation was assayed by the method of Asakawa and Matsushita (1980).

Superoxide was generated by the xanthine-oxidase system with acetaldehyde as the substrate. The concentration of the enzyme was 1 mU/ml, and that of acetaldehyde was 1 mmol/l.

In all experiments the concentration of the respective thiol compound was equimolar with respect to SH-groups.

The experiments were performed in triplicate and repeated at least three times. Results are presented as mean $\pm$ S.E.

Results

The aim of the preliminary experiments was to compare the thiols with respect to their ability to generate O_2^-. Using the chemical tests for O_2^- production we found that all thiols tested reduced cytochrome c. However, SOD did not inhibit the

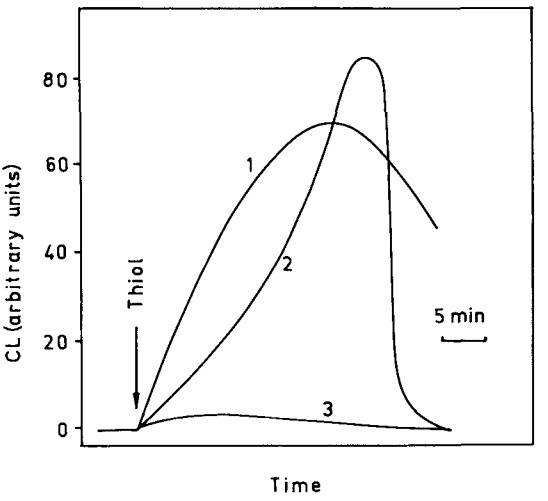

Figure 1. Thiol-induced chemiluminescence. The sample cuvette contained 0.1 mmol/l luminol, 1 μmol/l ADP-Fe^{3+} and one of the following thiol compounds: 0.5 mmol/l DMSA (curve 1); 1.0 mmol/l Cys (curve 2); 0.5 mmol/l DMPS, 1.0 mmol/l D-pen or 1.0 mmol/l GSH (curve 3) in 2 ml of 5 mmol/l phosphate buffer, pH 7.4.

reduction of cytochrome c, a finding which was in agreement with an earlier report (Tien et al. 1982). A similar result was obtained when epinephrine was used (data not given). As these results indicated direct interaction of thiols with cytochrome c and epinephrine, LDCL was used in further experiments to detect O_2^- production. Using this method in the presence of 1 μmol/l ADP-Fe^{3+} and pH 7.4, only DMSA and Cys induced measurable luminescence (Fig. 1). Addition of native SOB

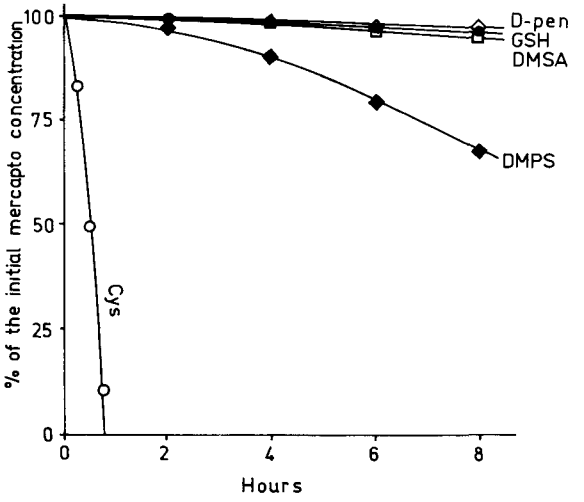

Figure 2. Time course of thiol compounds autoxidation. For incubation conditions see legend to Fig. 1. The content of SH-groups was assayed by the Ellman's test.

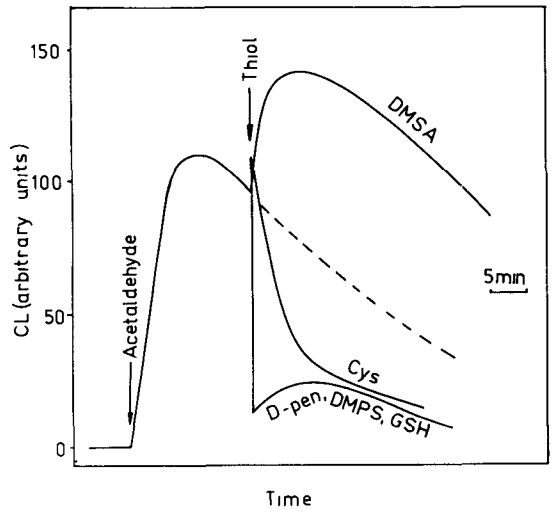

Figure 3. Effects of thiols on xanthine-oxidase/acetaldehyde-induced luminol-dependent chemiluminescence. The sample cuvette contained 1 mU/ml xanthine oxidase, 1 mmol/l acetaldehyde, 0.1 mmol/l luminol and the respective thiol at concentration as indicated in Fig. 1.

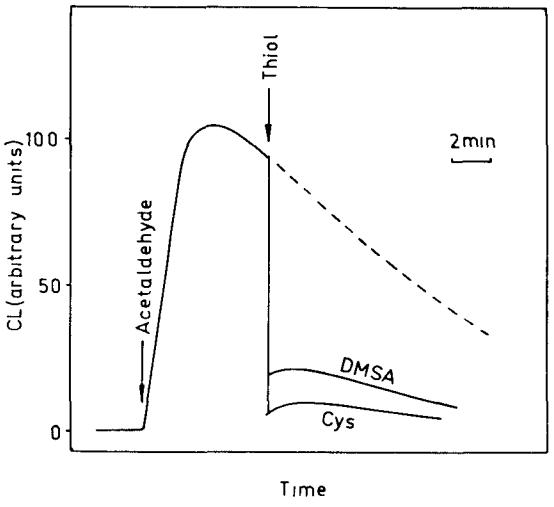

Figure 4. Effects of oxidized thiols on xanthine-oxidase/acetaldehyde-induced luminol-dependent chemiluminescence. For experimental conditions see legend to Fig. 3, the only exception being that DMSA and Cys SH-groups were completely oxidized before the addition of the thiols into the sample cuvette.

(10 U/ml) to the reaction system almost completely inhibited LDCL whereas denatured SOD was without effect. There was no luminescence signal when air over the sample was replaced by argon. No light was observed when DMSA and Cys were previously oxidized. These results may indicate that O_2^- generated during the oxidation of thiols is the main factor inducing LDCL. It may be suggested that the oxidation of DMSA and Cys proceeds much faster than that of the other thiols

tested, thus producing measurable amount of O_2^-. However, this assumption was not confirmed when the rates of the thiol oxidation were measured (Fig. 2): DMPS autoxidation was much faster than that of DMSA, which is in agreement with the report of Aposhian et al. (1982).

Thiols have been shown to interact with O_2^- (Ross et al. 1985). This interaction may be the reason for the inability of some of the thiols tested to induce LDCL. To test this possibility we investigated the effects of thiols on xanthine oxidase/acetaldehyde-induced LDCL. O_2^--induced LDCL was quenched by DMPS, D-pen, GSH and Cys, whereas DMSA caused an increase in light emission (Fig. 3).

Since autoxidation of thiols may change their ability to interact with O_2^-, we investigated also the effects of the products of thiol oxidation on O_2^--induced LDCL. Fig. 4 shows that disulfides inhibited LDCL stronglier than did the respective thiols. Even DMSA inhibited LDCL when oxidized.

In additional experiments the effects of thiols on O_2^--induced peroxidation of phospholipid liposome membranes were studied. Two of the thiols tested (DMSA and Cys) were found to accelerate lipid peroxidation whereas DMPS, D-pen and GSH inhibited peroxidation (Table 1).

Table 1. Effect of thiols on superoxide-induced lipid peroxidation

Addition to the incubation mixture	MDA (pmol/min/ml)
–	195.8 ± 1.3
DMPS	153.3 ± 0.8
DMSA	266.3 ± 2.1
D-pen	52.7 ± 0.6
Cys	273.4 ± 1.1
GSH	101.5 ± 1.5
SOD	28.8 ± 0.4
BHT	15.1 ± 0.4

Liposome suspension (2 mg phospholipid/ml) containing the respective thiol compound in concentration indicated (Fig. 1), 1 μmol/l ADP-Fe^{3+}, 1 mU/ml xanthine-oxidase and 1 mmol/l acetaldehyde was incubated at 37 °C. The concentration of SOD was 10 U/ml and that of BHT was 1 mmol/l. Values are means ± S. E. (n=9).

Discussion

The oxidation of thiols in the presence of transition metals has been believed to proceed with concomitant formation of O_2^- (Munday 1989):

$$RS^- + Me^{n+} \longrightarrow RS + Me^{(n-1)+} \quad (1)$$

$$Me^{(n-1)+} + O_2 \longrightarrow Me^{n+} + O_2^- \quad (2)$$

$$RS^- + RS \longrightarrow RS^-S\,R \quad (3)$$

$$RS^-S\,R + O_2 \longrightarrow RSSR + O_2^- \quad (4)$$

The involvement of the thiolate anion in the rate-limiting step of oxidation implies that thiols will undergo rapid oxidation only in conditions under which they are appreciably ionized. The rate of oxidation of Cys (pK 8.5) is greater than that of GSH (pK 9.2) (Munday 1989). Steric hindrance at the reaction site decreases the rate of thiols oxidation. For example, oxidation of D-pen proceeds considèrably slowlier than that of Cys and DMPS. This seems to be due to the bulky CH_3-groups in the D-pen molecule, making the formation of the disulphide difficult (Jellum and Skrede 1976).

According to reactions 1–4, the amount of O_2^- generated is proportional to the rate of autoxidation of thiols. However, we could find no correlation between LDCL and the rate of oxidation of thiols. It has been found that excitation of luminol requires its oxidation to semiquinon radical, the subsequent recombination of which with O_2^- results in light emission (Merenyi et al. 1985). In the absence of O_2^-), reactions yielding "dark products" prevailed (Merenyi et al. 1990). O_2^-, however, is always generated in the presence of luminol radicals and molecular oxygen (Merenyi et al. 1990). Therefore, the effect of SOD says nothing at all about whether and how much O_2^- is produced in a luminol-free system (Vilim and Wilhelm 1989).

In addition to O_2^- sulfur centered free radicals are formed in the course of thiols oxidation (reactions 1 and 3). These radicals have been shown to be good oxidants (Schoneich et al. 1989; Dunster and Willson 1990), and they very likely are able to oxidize luminol to produce semiquinon radical. This is sufficient to induce light emission even if O_2^- is not generated during oxidation of thiols. Nevertheless, DMPS, D-pen and GSH did not cause LDCL, but quenched O_2^--induced LDCL. As in our additional experiments xanthine oxidase was not inactivated it was concluded that the quenching was due to O_2^- scavenging. The greater O_2^--scavenging activity of the disulphides seems to be associated with their ability to act in aqueous media as reductants rather than oxidants (Braugler et al. 1986). The scavenging of O_2^- by DMPS, D-pen and GSH seems to be a reasonable explanation for their inability to induce LDCL. It is interesting that Cys induces LDCL itself but quenches O_2^--induced LDCL. This could be explained if cystine is a better scavenger of O_2^- than is Cys. In our experiments Cys-induced LDCL dropped sharply long before Cys was completely oxidized (Fig. 1). When an additional source of O_2^- is present, Cys oxidizes more rapidly. Sufficient amounts of cystine accumulated and scavenged O_2^-, thus quenching LDCL (Fig. 3). Further studies will be required to check the above assumption. The rate constant of the direct reaction of O_2^- with Cys reported so far have varied from less than 100 $mol^{-1}.s^{-1}$ (Finkelstein et al. 1984)

to 2.7×10^6 $mol^{-1}.s^{-1}$ (Asada and Kanematsu 1976).

We could show in the present work that the thiol compounds tested differ considerably with respect to their ability to scavenge O_2^- and to affect lipid peroxidation. Some of them (DMPS and D-pen) are scavengers of O_2^- and are similar to GSH, whereas DMSA produces free radicals and accelerates lipid peroxidation similarly as does Cys. Whether or not these properties of the thiols employed as antidotes against metal poisoning relate to their toxic side effects remain to be established, but they may be of interest when new antidotes are designed.

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