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INTERACTIONS OF SULFHYDRYL AGENTS AND SOYBEAN LIPOXYGENASE INHIBITORS

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Abstract—The enzymic conversion of 5, 8, 11, 14-eicosatetraenoic acid to the corresponding hydroperoxy fatty acids by soybean lipoxygenase (lineolate: oxygen oxidoreductase E.C. 1.13.11.12.) was investigated and a simple selective extraction method was introduced. The known inhibition of the lipoxygenase pathway by phenidone, mercuric chloride, methylmercuric chloride, methylmercuric iodide, 1,5-dihydroxynaphthalene and acetone phenylhydrazone was influenced by thiol compounds in different ways. (1) A total reactivation of lipoxygenase activity was achieved when several thiol compounds, especially gluthatione, were preincubated with the inhibitor mercuric chloride and the enzyme. (2) A remarkable reduction of the inhibitory potency of phenidone against soybean lipoxygenase was seen when thiol compounds were preincubated with the enzyme before the addition of the inhibitor. When phenidone was preincubated with lipoxygenase first, sulfhydryl agents did not restore the enzyme activity. (3) No interaction was seen, when glutathione or other thiol compounds and the lipoxygenase inhibitors 1,5-dihydroxynaphthalene, nordihydroguaiaretic acid and acetone phenylhydrazone were tested against the enzyme.

Therefore, we suggest that soybean lipoxygenase inhibitors may act via different modes of action. It is important to study the mechanisms of lipoxygenase inhibitors, since mammalian lipoxygenase and their products are known to be involved in the inflammatory response.

INTRODUCTION

Unsaturated fatty acids, especially 5, 8, 11, 14-eicosatetraenoic acid (arachidonic acid) not only are important precursors for the biosynthesis of prostaglandins but they are also known to be oxidated to the corresponding hydroperoxy acids (HPETE) by lipoxygenases.

The most extensively investigated lipoxygenases were found in soybeans and other plants but during the last decade lipoxygenases also were discovered in mammalian tissues [1–4]. Besides, there is more and more evidence that the lipoxygenase products HPETE and its degradation product hydroxyeicosatetraenoic acid (HETE) as well as prostaglandins [5–8] are involved in the pathomechanism of inflammation. In contrast to prostaglandin biosynthesis until now only few compounds are known to inhibit the lipoxygenase pathway of the arachidonic acid cascade. In general most inhibitors of the prostaglandin synthetase are not active when tested against lipoxygenases, but today the development of compounds which are inhibitory against lipoxygenases has become of great interest. Thus, especially "selective" lipoxygenase inhibitors could be useful pharmacological tools which may aid our understanding of the biological role of lipoxygenases.

Known lipoxygenase inhibitors are the acetylenic arachidonic acid analogue 5, 8, 11 14-eicosatetraynoic acid [2, 9], phenidone (1-phenyl-3-pyrazolidone) [10, 11], 1, 5-dihydroxynaphthalene (1, 5-DHN) [12], nordihydroguaiaretic acid (NDHG) [13], acetone phenylhydrazone [14], BW 755 C [15] and benoxaprofen [16]. Most inhibitors are active both against plant and mammalian lipoxygenases [12]. There, however, exists little information concerning the mechanisms of lipoxygenase inhibitors.

Since free sulfhydryl groups may be essential for lipoxygenase-1 activity and since mercurials react readily with sulfhydryl groups, we investigated whether thiol compounds may influence not only mercurial inhibitors like methylmercuric iodide or chloride and mercuric chloride which exhibited strong anti-lipoxygenase properties but also well established lipoxygenase inhibitors. Because there exists no detailed information about the possible mechanisms of lipoxygenase inhibition these studies seemed to be justified.

We compared several compounds of different chemical structure which are known to be inhibitors of soybean or mammalian lipoxygenases against arachidonic acid peroxidation. To elucidate possible characteristic inhibition patterns we investigated the influence of phenidone, 1, 5-DHN, NDGH and acetone phenylhydrazone on the soybean lipoxygenase activity. Since according to Spaapen [17] methylated mercurials inhibit the enzyme by blocking sulfhydryl groups we compared methylmercuric chloride and methylmercuric iodide with mercuric chloride as to their lipoxygenase inhibiting properties.

MATERIALS AND METHODS

Chemicals. [1-^{14}C]-Arachidonic acid (sp. act. 50 Ci/mole) was supplied by the Radiochemical Centre Amersham, U.K.). Soybean lipoxygenase-1 (130–150 U/μg protein), 1-phenyl 3-pyrazolidone

(phenidone), nordihydroguaiaretic acid, 1, 5-dihydroxynaphthalene, glutathione, L-cysteine, 2-mercaptoethanol, dithioerythritol (Sigma, Munich, West Germany), methylmercuric chloride, methylmercuric iodide (ICN-Pharmaceuticals, New York, NY), sodium hydrogensulfide (Fluka, Neu-Ulm), phenylhydrazine, mercuric chloride, L-ascorbic acid and all organic solvents (Merck, Darmstadt, West Germany) were commercially obtained. Acetone phenylhydrazone was synthesised in our laboratory according to standard procedures.

Reaction medium and incubations. Fatty acid lipoxygenation was carried out at 25° for 10 min in a mixture containing 50 mM potassium-phosphate buffer, pH 8,0, ethanol 1 per cent, [1-^{14}C]-arachidonic acid (3.75 nCi, 2.5 μM), lipoxygenase-1 (5 μg) and test drugs in a final volume of 300 μl. Ethanol was used to dissolve the arachidonic acid and had no effect on lipoxygenase activity. When test drugs were preliminarily pre-incubated at 25° with the enzyme for fixed time intervals, test drug concentrations are represented as those in the final fatty acid lipoxygenation assay. Pre-incubation procedures are described more detailed in the appropriate sections of the Results.

According to Hamberg and Samuelsson [18] lipoxygenase-1 catalyses the conversion of 5, 8, 11, 14-eicosatetraenoic acid (arachidonic acid) to its corresponding 15-hydroperoxy acid (15-HPETE). Besides 15-HPETE the more stable 15-hydroxyeicosatetraenoic acid (15-HETE) is the predominant lipoxygenation product.

(a) *Separation by thin-layer chromatography.* In order to separate [1-^{14}C]-arachidonic acid and its lipoxygenation products, thin-layer chromatography (t.l.c.) was applied.

First, the reaction was stopped by the addition of 50 μl N HCl to the incubation mixture and all the labelled eicosanoids were extracted three times into diethylether which then was taken to dryness under a stream of nitrogen. The residues were redissolved in 0.1 ml benzene/ethanol (1:1), quantitatively applied onto precoated silica gel t.l.c. plates (0.5 mm, E. Merck, Darmstadt, West Germany) and chromatograms were developed in ether/light petroleum/acetic acid (50:50:1). Precursor and products were located using a Berthold t.l.c. scanner (Wildbad, Germany), radioactive zones were scraped into scintillation fluid and quantified by standard liquid scintillation procedures (Tricarb 3385, Pico-Fluor 15, Packard Instruments). Lipoxygenation products (R_f = 0.15) were developed as a homogeneous zone distinct from arachidonic acid (R_F = 0.85) as shown in Fig. 1(a).

(b) *Separation by selective extraction procedures.* Secondly, we tried to separate arachidonic acid from its products HPETE and HETE by selective extraction of the fatty acid precursor after modification of the procedure described by Yanagi and Komatsu [19] for the prostaglandin synthetase assay. They found that 3 vols. *n*-hexane/ethyl acetate (2:1, v/v) were most suitable to extract selectively arachidonic acid from 1 vol. reaction medium under neutral pH-conditions leaving the more polar prostaglandins in the buffer phase. In order to prevent the lipoxygenation products HPETE and HETE which are less polar than prostaglandin E_2 but more polar than arachidonic acid from being extracted, we consecutively reduced the amount of the polar solvent ethyl acetate. Simultaneously the volume of the *n*-hexane/ethyl acetate extraction mixture was changed from 1 up to 5 times compared with the reaction medium. Finally these procedures were repeated under different pH-conditions (pH 5.5–9.0). The effectiveness of these extraction studies was compared with the established t.l.c. separation technique as described above.

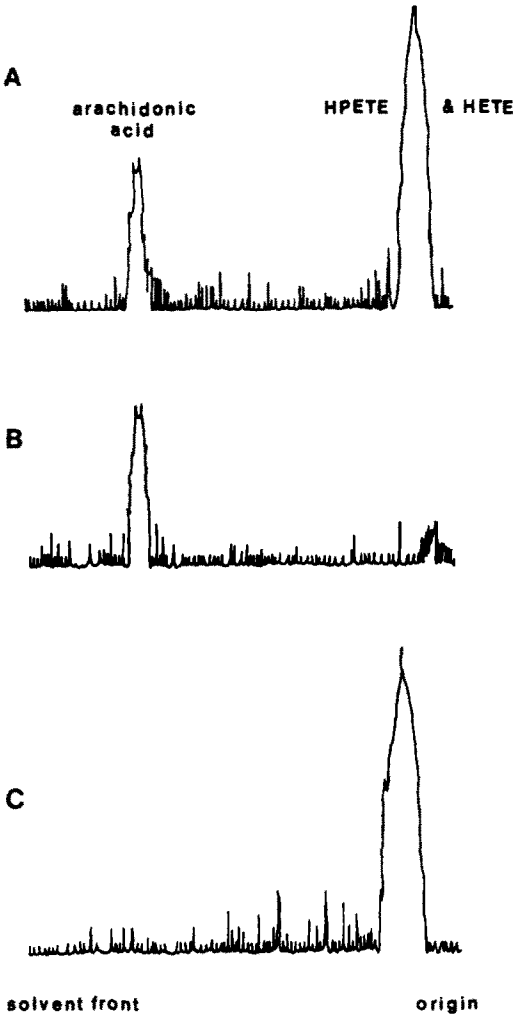

Fig. 1. Selective extraction of lipoxygenation products "HPETE" and "HETE" from the incubation medium. A = diethyl ether extract at pH 2.0. B = *n*-hexane extract (2.5 vol.) at pH 8.0. C = diethyl ether extract at pH 2.0 after extraction B. Chromatograms were developed with diethyl ether/light petroleum/acetic acid (50:50:1, v/v). Radioactive spots were localized by t.l.c. radio scanning. The reaction mixture contained 1-^{14}C-arachidonic acid (37.5 nCi. 2.5 μM), lipoxygenase-1 (5 μg), ethanol (1 per cent) in 50 mM potassium phosphate buffer (pH 8.0). Incubations were carried out at 25° for 10 min.

Best results were obtained if the reaction medium (pH 8.0) was extracted three times with 2.5 vols. *n*-hexane. Under these conditions all arachidonic acid was extracted into *n*-hexane and only small

amounts of radioactive products were extracted together with arachidonic acid into the organic solvent as shown in Fig. 1(b) by t.l.c. After acidification of the remaining aqueous phase with hydrochloric acid, more than 95 per cent of total lipoxygenation products could be extracted into diethylether following previous selective *n*-hexane-extraction of arachidonic acid (Fig. 1c). These results indicate that labelled arachidonic acid and its soybean lipoxygenation products HPETE and HETE can be separated quantitatively by simple means. We consider this method to be advantageous for lipoxygenase inhibition studies because of its good reproducibility especially when compounds are tested which interfere with the classic spectrophotometric lipoxygenase assays based on alterations in u.v.-absorbance of the unsaturated fatty acids.

Assay of lipoxygenase activity. After 10 min the reaction was stopped by the addition of 750 μl *n*-hexane and arachidonic acid was selectively extracted into the organic solvent. After centrifugation at 10,000 × *g* for 1 min the organic phase was discarded and extraction was repeated twice. After addition of 150 μl ethanol to the buffer phase containing the labelled soybean lipoxygenation products lipoxygenase-1 activity was quantified by liquid scintillation counting of the remaining ethanolic buffer phase.

Product formation was complete after 10 min, if enzyme and arachidonic acid concentrations were used as described above. For lipoxygenase inhibition studies enzyme and inhibitors were pre-incubated for 10 min unless otherwise stated.

RESULTS

Similar to cyclo-oxygenase inhibitors [19] the inhibition of soybean lipoxygenase activity proved to be enhanced by pre-incubation of enzyme and test compound. In our assay system maximum inhibition of lipoxygenase-1 activity was achieved when inhibitors were pre-incubated for 10 min with the enzyme. Longer pre-incubation time intervals had no further effect. After pre-incubation for 10 min with lipoxygenase-1 phenidone, 1, 5-DHN, NDGA and acetone phenylhydrazone exhibited a known concentration-dependent inhibition of arachidonic acid peroxidation by the plant enzyme. Also methylmercuric chloride and methylmercuric iodide which according to Spaapen [17] interact with free sulfhydryl groups of the lipoxygenase enzyme protein were weak inhibitors after 10 min of pre-incubation. Even after prolonged pre-incubation times (up to 60 min), however, the methylated mercurials did not result in a 50 per cent inhibition of fatty acid lipoxygenation when the inhibitors were tested in concentrations up to 1 mM in our assay system which differs in substrate and enzyme conditions from those applied by the Spaapen group. However, in contrast to the methylated mercurials, mercuric chloride proved to be inhibitory against soybean lipoxygenase comparable in its potency to phenidone. The IC_{50}

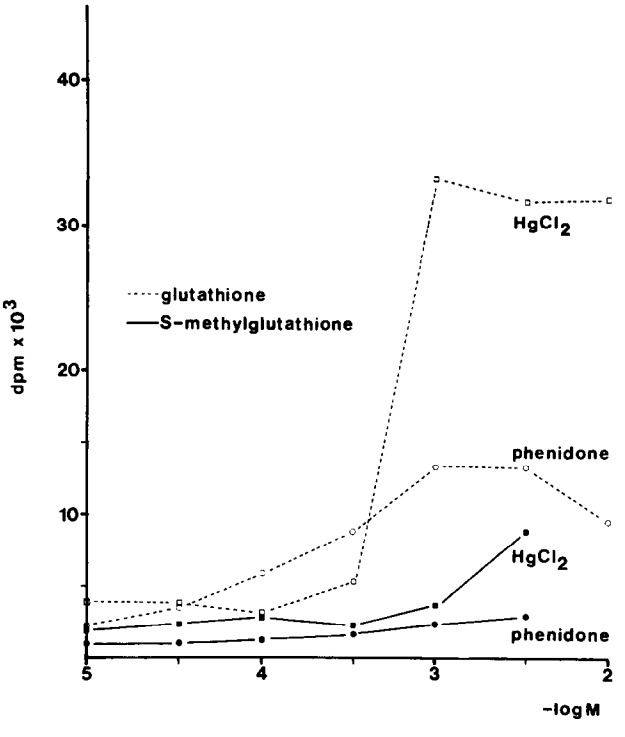

Fig. 2. Concentration-dependent effect of reduced glutathione and *S*-methylglutathione, respectively, on the efficacy of the lipoxygenase-inhibitors phenidone and $HgCl_2$. Glutathione or *S*-methylglutathione were preincubated for 5 min with the enzyme before the inhibitors (1 mM) were added to the preincubation mixture. Lipoxygenase activity is expressed as relative yield of radioactive lipoxygenation products. Arachidonate and enzyme concentrations as given in Fig. 1. Each value is the mean of four observations.

Table 1. IC_{50}-values and relative potencies of soybean lipoxygenase inhibitors. Each value is the mean of four observations

Inhibitors	IC_{50} (μM)	Relative potency
Phenidone	51	1.0
CH_3HgJ	$\geqslant$1000	—
CH_3HgCl	1470	0.035
1, 5-dihydroxynaphthalene	117	0.44
$HgCl_2$	72	0.71
Nordihydroguaiaretic acid	72	0.71
Acetone phenylhydrazone	0.3	170

values (concentration of inhibitor resulting in 50 per cent inhibition of arachidonic acid lipoxygenation) and the corresponding relative potencies of the inhibitors in comparison with phenidone are shown in Table 1.

When lipoxygenase-1 was pre-incubated with reduced glutathione for 5 min before the inhibitor was added to the pre-incubation mixture, this resulted in a dose-dependent decrease of lipoxygenase inhibition by phenidone (Fig. 2). The same phenomenon of decreased inhibition of lipoxygenase activity was seen when the enzyme was pre-incubated with mercuric chloride after prior addition of glutathione to the pre-incubation medium (Fig. 2). In the latter case glutathione exhibited a concentration-dependent effect on mercuric chloride-inhibition of fatty acid lipoxygenation, too. Lipoxygenase inhibition by mercuric chloride could be completely abolished when reduced glutathione was incubated for 30 min with the mercuric chloride-inactivated enzyme, whereas the phenidone-treated enzyme was not reactivated by the addition of glutathione. In order to elucidate whether the free sulfhydryl groups of glutathione are responsible for the decrease of inhibitory action of mercuric chloride and phenidone, the enzyme was pre-incubated with *S*-methylglutathione instead of reduced glutathione before inhibitors were added to the pre-incubation mixture. As shown in Fig. 2, *S*-methylated glutathione did not decrease lipoxygenase-1 inhibition by phenidone and mercuric chloride in concentrations up to 1 mM. The decrease of lipoxygenase inhibition by phenidone and mercuric chloride provoked by *S*-methylglutathione in concentrations higher than 1 mM may be due to contamination of the *S*-methyglutathione stock with traces of reduced glutathione. When lipoxygenase was pre-incubated with *S*-methylglutathione and phenidone or mercuric chloride respectively, there was no difference whether the inhibitors were pre-incubated with the enzyme before or after *S*-methylglutathione was added to the pre-incubation mixture.

As to mercuric chloride, there is a total reactivation of lipoxygenase activity by glutathione when added in concentrations equimolar or higher than the concentration of the inhibitor mercuric chloride (Fig. 2). Thus mercuric chloride may be trapped by equimolar amounts of glutathione so that no free mercury is left to inhibit the enzyme. Compared to mercuric chloride, the interactions between glutathione and the lipoxygenase inhibitor phenidone seem to be different: Pre-incubation of lipoxygenase with glutathione does not fully restore the enzyme activity when phenidone is added instead of mercuric chloride, even if glutathione is used in concentrations far exceeding the concentration of the inhibitor phenidone (Fig. 2).

To investigate a possible regeneration of the phenidone- or mercuric chloride-modified enzymic activity, several thiol compounds were tested in comparison to reduced glutathione. When lipoxygenase was pre-incubated for 5 min with 2-mercaptoethanol, cysteine, dithioerythritol or sodium hydrogensulfide before the addition of mercuric chloride, a complete recovery of the enzyme activity could be obtained with all thiol compounds. The most potent reactivators were glutathione (1.3 mM), sodium hydrogensulfide (5 mM) and 2-mercaptoethanol (4 mM). As reported above for glutathione, the other thiol compounds only partly restored lipoxygenase activity when phenidone was added to the preincubation mixture afterwards. In this case, too, glutathione was the favourable reactivator among the thiols tested.

Since glutathione can be considered to be an inhibitor of lipoxygenase inhibition by phenidone, various concentrations of phenidone were tested against lipoxygenase activity with and without the addition of a fixed amount of the antagonist glutathione. Figure 3 shows the dose–response curves of phenidone and phenidone +0.1 mM GSH. The corresponding double-reciprocal plot is given in Fig. 4. The addition of glutathione results in a parallel shift of the dose-response curve to higher concentrations of phenidone without lowering the maximum inhibition magnitude of the lipoxygenase inhibitor phenidone.

To investigate a possible influence of thiol compounds like glutathione on other inhibitors of the lipoxygenase pathway, lipoxygenase-1 was pre-incubated for 5 min with 1.3 mM glutathione before varying concentrations of inhibitors were added to the pre-incubation medium. Figure 5 shows the concentration-dependent inhibition of lipoxygenase-1 by nordihydroguaiaretic acid, 1, 5-DHN and acetone phenylhydrazone with and without a fixed amount of glutathione. In contrast to the data obtained for mercuric chloride and phenidone, neither glutathione nor other thiol compounds decreased or enhanced the inhibitory efficacy of NDGA, 1, 5-DHN or acetone phenylhydrazone significantly no matter if thiol compounds or inhibitors were added first to the pre-incubation mixture. In order to investigate whether molecular oxygen can modify the activity of the lipoxygenase-1 which is

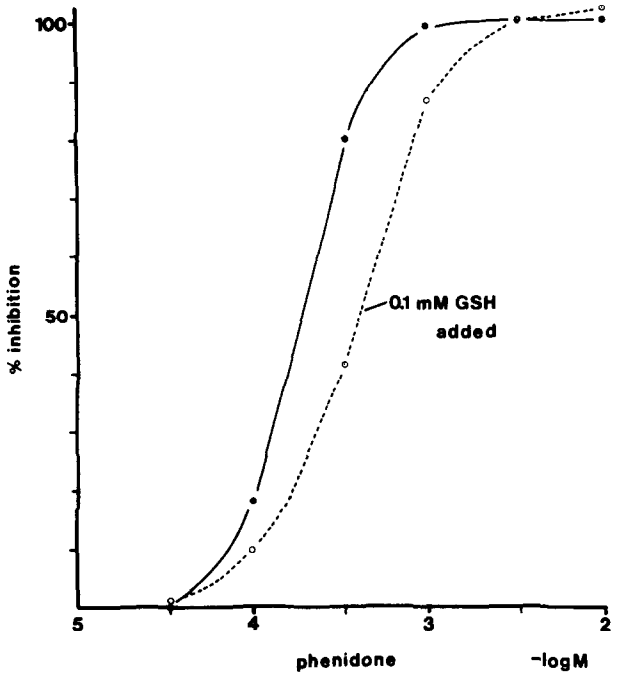

Fig. 3. Concentration–response curves for phenidone against soybean lipoxygenase inhibition in the presence or absence of glutathione. Lipoxygenase was preincubated for 5 min with a fixed amount of 0.01 mM glutathione or buffer before various concentrations of phenidone were added to the preincubation medium. Incubation conditions as given in Fig. 1. Each point is the mean of at least four observations.

known to contain sulfhydryl groups during pre-incubation with inhibitors, the pre-incubation procedures were performed under aerobic and strictly anaerobic conditions for several time intervals (1–10 min) before the fatty acid substrate and oxygen were added to the mixture to start the enzyme reaction. Then, phenidone and the other inhibitors were tested against lipoxygenase activity under aerobic and strictly anaerobic conditions. Under these conditions there were no significant differences in the efficacy of the inhibitors. In both cases lipoxygenase inhibition reached a maximum after 10 min of pre-incubation in our assay system. Thus the actions of thiol compounds cannot be explained by merely protecting the enzyme from being oxygenated during pre-incubation before the inhibitor phenidone is added to the reaction mixture. Pre-incubation of lipoxygenase with glutathione for 5 min in concentrations of up to 1.3 mM did not alter the time-dependent yield of lipoxygenase products. Thus glutathione does not seem to interact directly with the enzyme so that an interference with the self-inactivating process is rather improbable.

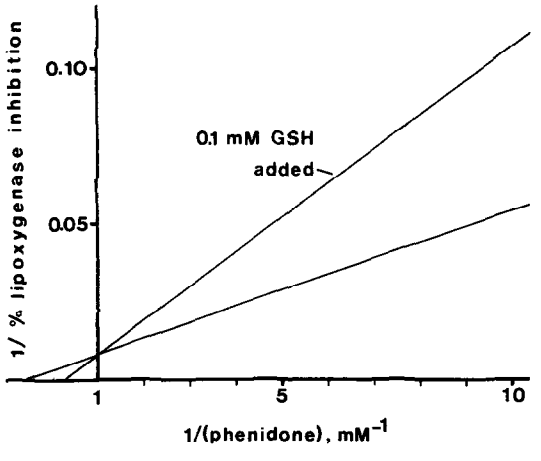

Fig. 4. Lineweaver-Burk plots showing that glutathione competively inhibits the inhibitory effect of phenidone on soybean lipoxygenase. The plots are derived from values represented in Fig. 3.

Finally we investigated what kind of lipoxygenase inhibition pattern a combination of phenidone and an inhibitor like 1, 5-DHN which cannot be influenced by thiol compounds would exhibit. Figure 6 shows the concentration-dependent inhibition of soybean lipoxygenase by phenidone with and without the addition of a fixed concentration of 1, 5-DHN. The addition of 1, 5-DHN results in a parallel shift of the dose-response curve to lower concentrations of phenidone, suggesting at least an additive inhibitory effect of phenidone and 1, 5-DHN against the soybean lipoxygenase. In order to elucidate whether the lipoxygenase inhibitors investigated and their interactions with sulfhydryl compounds influence the time-course of arachidonic acid peroxidation fatty acid, enzyme and test compounds were incubated for 1, 2, 3, 5 and 10 min. Reactions were stopped by selective extraction of arachidonic acid into *n*-hexane as usual. As shown in Figs. 7–9 lipoxygenase product yield was complete after 10 min, when $HgCl_2$ (1, 0.1 mM), phenidone (1, 0.1 mM) and acetone phenylhydrazone (0.1, 0.001 mM) were tested with and without 1.3 mM glutathione against the enzyme. These studies revealed the same kinetics for the

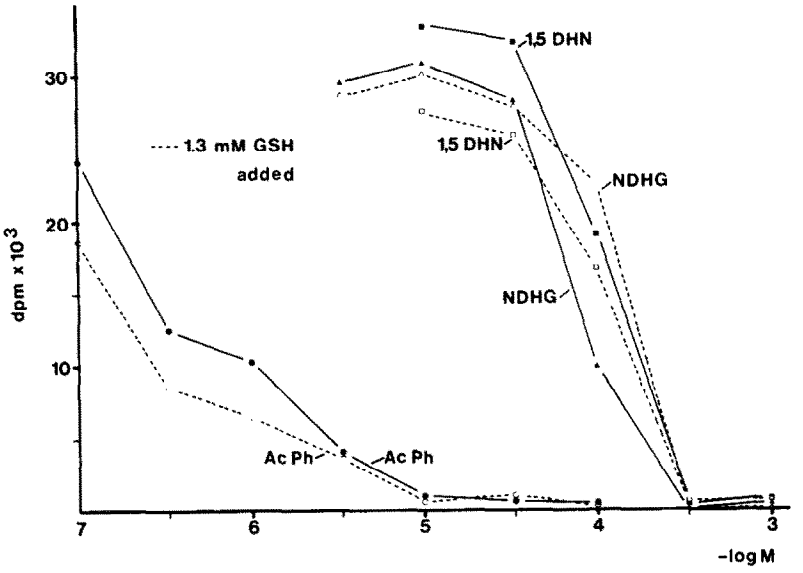

Fig. 5. Effect of a range of concentrations of acetone phenylhydrazone (AcPh), 1, 5-dihydroxynaphthalene (1, 5-DHN) and nordihydroguaiaretic acid (NDHG) on soybean lipoxygenase activity with and without the addition of glutathione. Lipoxygenase was preincubated for 5 min with a fixed amount of glutathione (1.3 mM) or buffer before various concentrations of inhibitors were added to the preincubation mixture. Lipoxygenase activity is expressed as relative yield of radioactive lipoxygenation products. Incubation conditions as in Fig. 1. Each point is the mean of four observations.

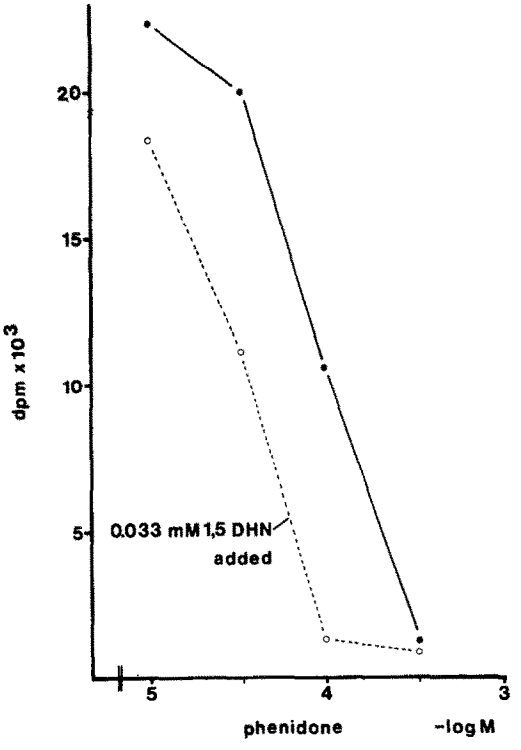

Fig. 6. Concentration-dependent inhibition of soybean lipoxygenase activity by phenidone and a combination of phenidone and a fixed amount of 1,5-dihydroxynaphthalene (0.033 nM). Lipoxygenase activity is expressed as relative yield of radioactive lipoxygenation products. Lipoxygenase and inhibitors were preincubated for 10 min. Incubation conditions as described in Fig. 1. Each point is the mean of four observations.

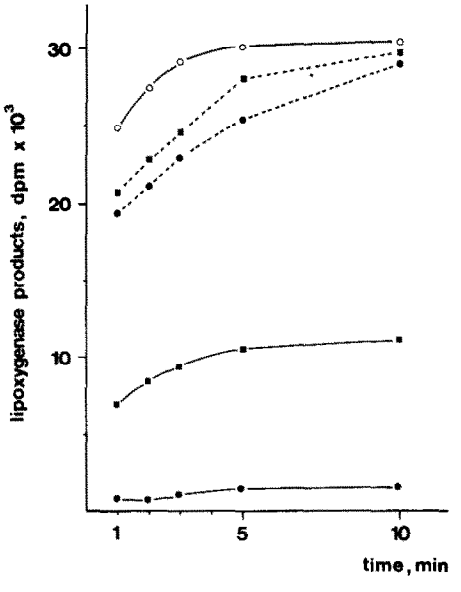

Fig. 7. Influence of $HgCl_2$ and glutathione on the time-course of arachidonic acid peroxidation by soybean lipoxygenase. ○——○ Control: $HgCl_2$ and GSH were omitted. ●——● 1 mM $HgCl_2$ (pre-incubation time 10 min). ■——■ 0.1 mM $HgCl_2$ (pre-incubation time 10 min). ●- - -● 1 mM $HgCl_2$ (pre-incubation time 10 min) followed by the addition of 1.3 mM GSH 5 min before lipoxygenation was started. ■- - -■ 0.1 mM $HgCl_2$ (pre-incubation time 10 min) followed by the addition of 1.3 mM GSH 5 min before lipoxygenation was started. Each point is the mean of four observations.

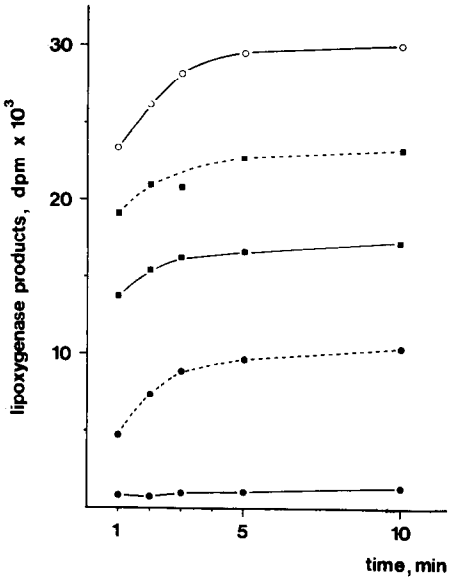

Fig. 8. Influence of phenidone and glutathione on the time-course of arachidonic acid peroxidation by soybean lipoxygenase. O——O Control: Phenidone and GSH were omitted. ●——● Pre-incubation (10 min) of lipoxygenase with 1 mM phenidone before lipoxygenation was started. ■——■ Pre-incubation (10 min) of lipoxygenase with 0.1 mM phenidone before lipoxygenation was started. ●- - -● Pre-incubation (5 min) of lipoxygenase with 1.3 mM GSH before 1 mM phenidone was added to the mixture. Lipoxygenation was started 10 min later. ■- - -■ Pre-incubation (5 min) of lipoxygenase with 1.3 mM GSH before 0.1 mM phenidone was added to the mixture. Lipoxygenation was started 10 min later. Each point is the mean of four observations.

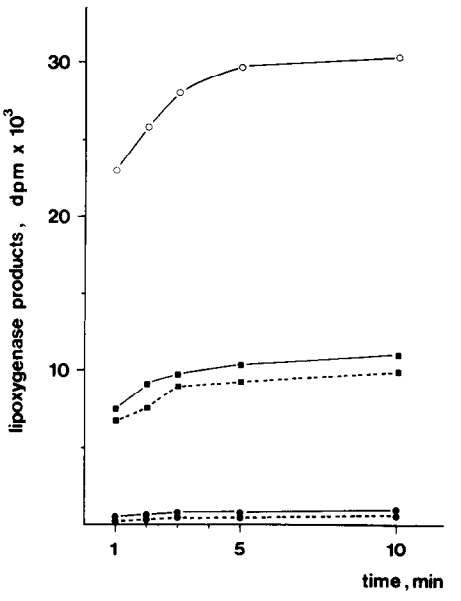

Fig. 9. Influence of acetone phenylhydrazone (AcPh) and glutathione on the time-course of arachidonic acid peroxidation by soybean lipoxygenase. O——O Control: AcPh and GSH were omitted. ●——● Pre-incubation (10 min) of lipoxygenase with 0.1 mM AcPh before lipoxygenation was started. ■——■ Pre-incubation (10 min) of lipoxygenase with 0.001 mM AcPh before lipoxygenation was started. ●- - -● Pre-incubation (5 min) of lipoxygenase with 1.3 mM GSH before 0.1 mM AcPh was added to the mixture. Lipoxygenation was started 10 min later. ■- - -■ Pre-incubation (5 min) of lipoxygenase with 1.3 mM GSH before 0.001 mM AcPh was added to the mixture. Lipoxygenation was started 10 min later. Each point is the mean of four observations.

interactions of glutathione with the lipoxygenase inhibitors $HgCl_2$, phenidone and acetone phenylhydrazone as stated before. Reviewing these time-dependent inhibition studies the inhibitory patterns of the compounds tested cannot be explained by direct interference with the self-inactivation processes to which lipoxygenases are subjected during catalysis and which still remain open for speculation.

DISCUSSION

According to our experimental data, a merely chemical interaction between phenidone and glutathione seems to be improbable. Optimal concentrations of 1–2 mM glutathione exhibit a 35% reactivation of the phenidone-modified lipoxygenase enzyme. Reviewing the effects of reduced and *S*-methylated glutathione, free sulfhydryl groups seem to be essential for the inhibitor effect of glutathione against lipoxygenase inhibition by phenidone or mercuric chloride. When lipoxygenase was pre-incubated first with phenidone a possible reactivation of the enzyme activity with glutathione or other thiol compounds did not occur, suggesting that phenidone is bound more closely to the enzyme than mercuric chloride.

Reviewing the effects of thiol compounds like cysteine, sodium hydrogensulfide, 2-mercaptoethanol, dithioerythritol and especially reduced glutathione on the lipoxygenase inhibitors phenidone, mercuric chloride, 1, 5-DHN, NDGA, and acetone phenylhydrazone, we found three different modes of action.

(1) Inhibition of lipoxygenase activity by mercuric chloride can be totally abolished by gluthathione and other thiol compounds, no matter whether inhibitor or thiol substance were pre-incubated first with the enzyme.

(2) Pre-incubation of lipoxygenase-1 with thiol compounds reduces the inhibitory efficacy of phenidone remarkably, if the inhibitor is added afterwards but glutathione does not restore the activity of the phenidone-modified enzyme.

(3) The efficacy of the other inhibitors of the lipoxygenase pathway tested (1,5-DHN, NDGA, acetone phenylhydrazone) is not significantly influenced by glutathione or other thiol compounds.

Whereas mercurials seem to act via direct interaction with free sulfhydryl groups of lipoxygenase and thiol compounds, the effect of phenidone is probably inhibited in a competitive manner by glutathione. Comparing the interaction of phenidone and glutathione, the effect of competition is a reduction of the apparent affinity of phenidone for the enzyme receptor in the presence of glutathione as predicted by the law of mass action. In this case the magnitude of response is not altered, since a high enough ratio of phenidone: glutathione seems to force complete occupancy of receptors by phenidone

despite the presence of glutathione. These experimental results, especially the double reciprocal plot according to Lineweaver-Burk, suggest a competitive antagonism for the interaction of glutathione and phenidone. The other inhibitors not influenced by thiol compounds therefore may have a site of action different from that of phenidone.

Concerning the combined action of the inhibitors phenidone and 1, 5-DHN, further investigations indicated that a more than additive interaction of both inhibitors seems to be improbable, although thiol compounds decrease the inhibitory efficacy of phenidone but not of 1, 5-DHN. Therefore, these two inhibitors are not supposed to act via completely different sites of action.

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