EFFECTS OF VARIOUS COMPOUNDS ON LIPID PEROXIDATION MEDIATED BY DETERGENT-SOLUBILIZED RAT LIVER NADPH-CYTOCHROME C REDUCTASE

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Abstract—A reconstituted lipid peroxidation system containing NADPH-cytochrome *c* reductase isolated from detergent-solubilized rat liver microsomes was used to determine the effects of several compounds, including drugs, on the lipid peroxidation activity. EDTA and ferrous ion were essential requirements for reconstitution of the activity. The addition of 1,10-phenanthroline to the system containing both EDTA and ferrous ion further enhanced the activity. Pyrocatechol, thymol, *p*-aminophenol, imipramine, *p*-chloromercuribenzoate (PCMB) and α -tocopherol exhibited strong inhibition, aniline, *N*-monomethylaniline, aminopyrine, benzphetamine, SKF 525-A and NADP exhibited moderate inhibition, and phenol, benzoic acid, acetanilide and nicotinamide exhibited less or no inhibition at the concentrations lower than 1000 μ M. Metal ions such as Hg^+ , Hg^{2+} , Co^{2+} , Cu^{2+} , Mn^{2+} and U^{6+} inhibited lipid peroxidation strongly. In addition, Cd^{2+} , Sr^{2+} and Ca^{2+} exhibited less potent to moderate inhibition, and Ba^{2+} and Mg^{2+} were without effects on the activity. Among sulfhydryl compounds tested, dithiothreitol inhibited lipid peroxidation to a greater extent than did the other three compounds, glutathione, cysteine and mercaptoethanol.

Orrenius *et al.* (1) first reported that aminopyrine and codeine, which are known to be oxidatively metabolized by a NADPH-dependent enzyme system, inhibited microsomal NADPH-dependent lipid peroxidation. Based on this evidence, they demonstrated the possibility that the inhibition of lipid peroxidation by these drugs was due to competition between the two processes for a common NADPH-oxidizing enzyme. Thereafter, Wills (2) proposed that the inhibition of lipid peroxidation did not appear to be caused by the competition between the two processes since a drug which undergoes hydroxylation reaction by liver microsomes did not necessarily inhibit lipid peroxidation. This idea was further supported by the result that aminopyrine inhibited ascorbate-dependent lipid peroxidation. In addition, non-enzymic lipid peroxidation has also been reported to be inhibited by some drugs (3).

Oshino *et al.* (4) found that the activity of cyanide sensitive factor (CSF), which catalyzes desaturation reaction of saturated fatty acids, was supported either by NADH or NADPH.

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From this and other experimental results, they confirmed that electrons from NADPH were transferred to CSF via NADPH-cytochrome *c* reductase and cytochrome b_5 . Further, Oshino and Sato (5) proposed the possibility that some compounds including *p*-cresol, aniline and *p*-aminophenol were oxidatively metabolized by CSF since such were capable of enhancing the reoxidation rate of NADH-reduced cytochrome b_5 . The latter finding might also suggest that electrons transferred from NADPH to cytochrome b_5 via NADPH-cytochrome *c* reductase are utilized rapidly when these compounds are added to microsomes and that the enhanced electron flow from NADPH-cytochrome *c* reductase to cytochrome b_5 should occur, resulting in inhibition of lipid peroxidation, in a manner similar to that proposed by Orrenius *et al* (1). In an apparent accordance with this hypothesis, aniline and *p*-aminophenol are reported to be potent inhibitors of microsomal NADPH-dependent lipid peroxidation (2, 6). Since many phenol and amine compounds have been established as antioxidants, these compounds were expected to inhibit lipid peroxidation without aids of these possibilities as above described. To confirm the direct effects of these compounds on NADPH-dependent lipid peroxidation, it was necessary to use a purified preparation of an enzyme which is active for NADPH-dependent lipid peroxidation and is free from both cytochrome P-450 and cytochrome b_5 .

Pederson and Aust (7) and Pederson *et al.* (8) confirmed that the NADPH-dependent lipid peroxidation was catalyzed by NADPH-cytochrome *c* reductase since the lipid peroxidation activity could be reconstituted using NADPH-cytochrome *c* reductase isolated from bromelain-treated rat liver microsomes. The proteolytic digestion of microsomal membranes cleaves hydrophobic peptides of the membrane bound enzymes, and the hydrophobic nature of membrane bound enzymes is successfully retained when microsomes are solubilized with detergents (9-12). Therefore, to establish the effects of some compounds on the NADPH-cytochrome *c* reductase mediated lipid peroxidation, we partially purified NADPH-cytochrome *c* reductase from detergent solubilized microsomes.

Metal ions including Mn^{2+} are also known to inhibit microsomal NADPH-dependent lipid peroxidation (13, 14). Recent studies by Fong *et al.* (15) have demonstrated that Mn^{2+} inhibits lipid peroxidation functioning as an efficient superoxide anion scavenger. In this paper, we also report the ability of metal ions to inhibit the NADPH-cytochrome *c* reductase mediated lipid peroxidation.

MATERIALS AND METHODS

Male rats of Crl:CD(SD) strain weighing 100 to 150 g were used throughout this study. In some experiments, the animals were given 0.1% sodium phenobarbital in the drinking water for three days. All animals were fasted for 48 hr prior to sacrifice. Liver microsomes were prepared as described previously (16). The pooled microsomes were washed once with 1.15% KCl. The washed microsomes were suspended in redistilled water at a concentration of approx. 30 mg protein per ml and stored under an atmosphere of nitrogen at -70° until use. Protein was determined by the method of Lowry *et al* (17). Values represented in the figures and tables are the means of duplicate or triplicate determinations.

Preparation of substrate lipids

Lipids used as a substrate of lipid peroxidation were extracted from intact liver microsomes by the method of Folch *et al* (18). The extracted lipids were dissolved in chloroform at a concentration of about 100 mg lipid phosphorus per ml and were stored under nitrogen at -70° . For the assay of lipid peroxidation, the lipid preparation was suspended in redistilled water by sonication three times at 10 second intervals. Lipid phosphorus was determined by the method of Bartlett (19).

*Partial purification of NADPH-cytochrome *c* reductase from rat liver microsomes*

The pooled microsomes from phenobarbital-treated rat livers were treated once with 0.1 M pyrophosphate (pH 7.4) and were used for purification of NADPH-cytochrome *c* reductase. The pyrophosphate-extracted microsomes were solubilized with Emulgen 913. NADPH-cytochrome *c* reductase was purified from the solubilized microsomes by means of DEAE-Sephadex (A-50) column, ω -amino-*n*-octyl Sepharose 4B column and hydroxylapatite column as reported previously (20) except that 150 mM rather than 125 mM KCl was added to the solubilized microsomes and to the buffer for equilibrating DEAE-Sephadex column. Sizes of DEAE-Sephadex, ω -amino-*n*-octyl Sepharose 4B and hydroxylapatite columns were 5.0×10.0 cm, 1.5×7.5 cm, and 3.0×10.0 cm, respectively. Further, the eluate from the column of ω -amino-*n*-octyl Sepharose 4B was concentrated with polyethyleneglycol (6,000), followed by dialyzation against 20 mM K-phosphate (pH 7.25) containing 20% glycerol for 24 hr. The specific content of the purified NADPH-cytochrome *c* reductase was 21.05 unit/mg protein and the recovery of the activity was 24.4%. This NADPH-cytochrome *c* reductase preparation was free from cytochrome P-450 and cytochrome *b*₅. The NADPH-cytochrome *c* reductase activity was measured by the method of Phillips and Langdon (21). A unit of NADPH-cytochrome *c* reductase activity is defined as one μ mole cytochrome *c* reduced per min.

*Assay of NADPH-cytochrome *c* reductase mediated lipid peroxidation activity*

A typical incubation mixture for the assay of NADPH-cytochrome *c* reductase mediated lipid peroxidation consisted of 0.25 M Tris-HCl (pH 6.8), 0.25 M NaCl, 0.5 μ M FMN, 25 μ M EDTA, 50 μ M $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$, 3 mM extracted lipids (as lipid phosphorus) and 0.01 unit of NADPH-cytochrome *c* reductase. The reaction was started by the addition of 0.2 mM NADPH. The lipid peroxidation activity was estimated by determining malondialdehyde formed by the thiobarbituric acid method as described previously (20). Incubations were carried out in the presence and absence of NADPH-cytochrome *c* reductase. The values for NADPH-cytochrome *c* reductase mediated lipid peroxidation were thus obtained by subtracting the optical density at 532 nm of the control ($-$ NADPH-cytochrome *c* reductase) from the experimental incubation ($+$ NADPH-cytochrome *c* reductase). A mmolar extinction coefficient of $156 \text{ mM}^{-1} \text{ cm}^{-1}$ (13) for malondialdehyde-thiobarbituric acid complex was used for calculating the lipid peroxidation activity.

Materials

Emulgen 913, a non-ionic detergent, was kindly provided by Kao-Atlas Co., Japan,

and α -tocopherol was a gift from Eisai Co., Japan. NADP and NADPH were purchased from Boehringer Mannheim, FMN from Wako Pure Chemicals, Japan. Sepharose 4B and DEAE-Sephadex (A-50) were purchased from Pharmacia Fine Chemicals Co., hydroxylapatite (Bio Gel HT) from Bio-Rad. The ω -amino-*n*-octyl derivative of Sepharose 4B was prepared from cyanogen bromide-activated Sepharose 4B and 1,8-diamino-octane by a method described by Cuatrecasas (22). Commercial aniline was redistilled under vacuum and the distillate was stored under nitrogen at -20° . Other chemical reagents of analytical grade were purchased from commercial sources and were used without further purification.

RESULTS

The requirements for reconstitution of the NADPH-cytochrome *c* reductase mediated lipid peroxidation activity were examined (Table 1). As was demonstrated by Pederson and Aust (7) and Pederson *et al.* (8), EDTA as well as ferrous ion was an essential requirement for the activity. In addition, NaCl was also required for the maximal activity. FMN exhibited a slight enhancement of the activity. To determine the concentration of EDTA for the maximal activity, the effect of varying concentrations of EDTA on the lipid peroxidation activity was examined in the presence of 50 μ M ferrous ion (Fig. 1). A 25 μ M EDTA concentration was found to produce a maximal enhancement of the activity. In a preceding paper (20), we reported that 1,10-phenanthroline and 2,2'-bipyridine were also capable of supporting lipid peroxidation activity but to extents lesser than EDTA when such were added in place of EDTA. Fig. 2 shows the effects of 1,10-phenanthroline and 2,2'-bipyridine on lipid peroxidation activity. In this experiment, either of these chelating reagents was added to the incubation mixture containing EDTA, ferrous ion and other necessary components. As can be seen, 1,10-phenanthroline exhibited the enhancement of lipid peroxidation at a concentration near 25 μ M. 2,2'-Bipyridine also enhanced the activity but the enhancement increased gradually with increase in the concentrations.

Wills (2) reported that phenols and aromatic amines are potent inhibitors of NADPH-

TABLE 1. Requirements for lipid peroxidation activity in the reconstituted system

Incubation conditions	Lipid peroxidation activity ^a
Complete system ^b	1.03
-Fe ²⁺	0.02
-EDTA	0.00
-FMN	0.98
-NaCl	0.56
-EDTA, Fe ²⁺	0.00
-FMN, NaCl	0.49

a; μ mole malondialdehyde/unit NADPH-cytochrome *c* reductase /10 min

b; Complete system for lipid peroxidation activity contained all necessary components described in *MATERIALS AND METHODS*.

FIG. 1. Enhancement of NADPH-cytochrome *c* reductase mediated lipid peroxidation by EDTA. The incubation system contained all necessary components as described in MATERIALS AND METHODS except that varying concentrations of EDTA were used. MDA; malondialdehyde.

FIG. 2. Effects of 1,10-phenanthroline and 2,2'-bipyridine on NADPH-cytochrome *c* reductase mediated lipid peroxidation. The incubation system contained all necessary components described in MATERIALS AND METHODS except that various concentrations of 1,10-phenanthroline or 2,2'-bipyridine were added. MDA; malondialdehyde.

dependent lipid peroxidation in rat liver microsomes. The effects of various compounds on the NADPH-cytochrome *c* reductase mediated lipid peroxidation are shown in Table 2. Phenol compounds such as pyrocatechol and thymol and some amine compounds such as *p*-aminophenol and imipramine strongly inhibited the NADPH-cytochrome *c* reductase mediated lipid peroxidation. Other amine compounds, aniline, N-monomethylaniline, aminopyrine, benzphetamine and SKF 525-A also inhibited the activity but to lesser extents than *p*-aminophenol and imipramine. Acetanilide and ethylmorphine did not inhibit lipid peroxidation at concentrations lower than 1000 μ M. Wills (2) also reported that about 91% inhibition of microsomal lipid peroxidation was attainable by addition of 1000 μ M PCMB. Our experiments have shown that 0.1 μ M PCMB inhibited about 51% of the NADPH-cytochrome *c* reductase mediated lipid peroxidation activity, and 10 μ M PCMB completely inhibited the activity. α -Tocopherol is a widely used antioxidant drug. As was expected, α -tocopherol at concentrations below 5 μ M inhibited lipid peroxidation completely. Nicotinamide did not inhibit lipid peroxidation at concentrations below 1000 μ M. NADP is known to be a competitive inhibitor of NADPH-cytochrome *c* reductase (23). As shown in Table 2, this compound inhibited lipid peroxidation about 20% and 29% at 500 μ M and 1000 μ M concentrations, respectively.

The effects of metal ions on the NADPH-cytochrome *c* reductase mediated lipid peroxidation were also examined (Table 3). Known inhibitors of microsomal lipid peroxidation, such as Co^{2+} and Mn^{2+} also inhibited the NADPH-cytochrome *c* reductase mediated lipid peroxidation. Other metal ions such as Hg^+ , Hg^{2+} , Cd^{2+} , Cu^{2+} and U^{6+} also inhibited lipid peroxidation.

Previous reports from our own (24) and other laboratories (2, 25) indicated that microsomal lipid peroxidation is inhibited by glutathione. Our results (24) further showed that cysteine and dithiothreitol also inhibited lipid peroxidation but to extents lesser than gluta-

TABLE 2. Effects of some compounds on NADPH-cytochrome *c* reductase mediated lipid peroxidation

Addition	Concn. (μ M)	Lipid peroxidation (%)
None (control)	—	100
Phenol	1000	101.0
Pyrocatecol	250	0.0
Thymol	250	30.3
	500	1.1
Benzoic acid	1000	96.5
Aniline	250	94.5
	1000	87.4
<i>p</i> -Aminophenol	50	34.6
	100	6.7
N-Monomethylaniline	750	92.5
	1000	80.3
Acetanilide	1000	103.5
Aminopyrine	250	91.9
	1000	73.8
Imipramine	500	21.7
	1000	13.1
Ethylmorphine	1000	101.0
Benzphetamine	500	71.9
	1000	57.9
SKF 525-A	500	73.8
	1000	39.3
PCMB	0.1	49.0
	10	0.0
α -Tocopherol	1.0	75.3
	5.0	0.0
NADP	500	79.8
	1000	71.1
Nicotinamide	1000	102.8

Control activity was 1.51 μ mole MDA/unit/10 min.TABLE 3. Effects of metal ions on NADPH-cytochrome *c* reductase mediated lipid peroxidation

Addition	Concn. (μ M)	Lipid peroxidation (%)
None (control)	—	100
HgCl	250	28.0
	750	0.0
HgCl ₂	250	0.0
CdCl ₂	500	51.7
	1000	25.8
BaCl ₂	1000	101.3
CoCl ₂	250	2.8
CaCl ₂	1000	88.8
SiSO ₄	1000	89.9
CuCl ₂	500	9.0
MgCl ₂	1000	103.9
MnCl ₂	250	0.0
UO ₂ (NO ₃) ₂	250	30.3
	500	15.2

Control activity was 1.51 μ mole MDA/unit/10 min.

FIG. 3. Inhibition of NADPH-cytochrome *c* reductase mediated lipid peroxidation by sulfhydryl compounds. MDA; malondialdehyde.

thione. Further, mercaptoethanol did not exhibit significant inhibition. The inhibition of the NADPH-cytochrome *c* reductase mediated lipid peroxidation by these sulfhydryl compounds is shown in Fig. 3. Our results indicate that dithiothreitol rather than glutathione inhibited lipid peroxidation to a greater extent than did the other sulfhydryl compounds.

DISCUSSION

Effects of drugs, metal ions and sulfhydryl compounds on the NADPH-cytochrome *c* reductase mediated lipid peroxidation were determined herein. Since the purified preparation of NADPH-cytochrome *c* reductase did not contain cytochrome P-450 and cytochrome *b*₅, the inhibition of lipid peroxidation by these compounds can be free from the effects of these electron transfer enzymes. As was shown in this paper, known inhibitors of microsomal lipid peroxidation also inhibited the NADPH-cytochrome *c* reductase mediated lipid peroxidation. This finding suggests that inhibition by these compounds is the result of direct effects on lipid peroxidation rather than a competition of the common NADPH-oxidizing enzyme.

It was found that the addition of 1,10-phenanthroline enhanced the NADPH-cytochrome *c* reductase mediated lipid peroxidation even in the presence of adequate amounts of EDTA and ferrous ion. This result probably suggests that EDTA and 1,10-phenanthroline enhance lipid peroxidation by different mechanism(s).

Based on the fact that microsomal NADPH-cytochrome *c* reductase activity was inhibited by some mercurial compounds, it was confirmed that NADPH-cytochrome *c* reductase contained sulfhydryl groups (21, 23, 26). In accordance with the previous view, Wills (2) reported that PCMB inhibited microsomal lipid peroxidation and the inhibition of lipid peroxidation was approx. 91 % by 1 mM PCMB. In the present study, we found that the NADPH-cytochrome *c* reductase mediated lipid peroxidation was inhibited completely by addition of 10 μM PCMB. The difference between these studies in the ability of PCMB to inhibit lipid peroxidation is probably due to the difference of the total sulfhydryl groups present in these incubation mixtures since we used a small amount of purified NADPH-cytochrome *c* reductase and Wills used microsomes. The organic mercurial PCMB inhibited

lipid peroxidation activity to a greater extent than did inorganic mercurial compounds, HgCl and HgCl_2 . NADPH-cytochrome *c* reductase, which we purified from detergent-solubilized microsomes, appears to retain a hydrophobic nature. Therefore, the more potent inhibition of lipid peroxidation by PCMB might be caused by the hydrophobic nature of PCMB as compared to the inorganic mercurials.

Although data are not shown, the inhibitory effects of NADP on lipid peroxidation activity and on NADPH-cytochrome *c* reductase activity were compared since it was expected that the inhibition by NADP of NADPH-cytochrome *c* reductase paralleled inhibition of the lipid peroxidation activity. The activity of NADPH-cytochrome *c* reductase as measured by cytochrome *c* reduction rate was inhibited by 73% and 84% by the presence of 500 μM and 1000 μM NADP, respectively. These results indicate that NADP inhibited cytochrome *c* reduction rate by NADPH to a greater extent than NADPH-cytochrome *c* reductase mediated lipid peroxidation activity. This seems to be rather anomalous and at present unexplainable. However, since a unit of NADPH-cytochrome *c* reductase (1000 nmol cytochrome *c* reduced/min) mediated only one-tenth the capacity in lipid peroxidation (100 nmol malondialdehyde formed/unit NADPH-cytochrome *c* reductase/min), it appears that a rapid flow of electrons to cytochrome *c* might be one of the causes of the potent inhibition by NADP.

In a previous report from this laboratory (24), we demonstrated that glutathione inhibits microsomal lipid peroxidation to a greater extent than cysteine, dithiothreitol and mercaptoethanol. In this study we observed that dithiothreitol rather than glutathione inhibited the NADPH-cytochrome *c* reductase mediated lipid peroxidation to a greater extent than the other sulfhydryl compounds. The potent inhibition of microsomal lipid peroxidation by glutathione therefore appeared to be due to the glutathione peroxidase which remained as a contaminant in the washed liver microsomes. In accordance with this assumption, our preliminary experiment indicated that when the microsomes were centrifuged once, a complete removal of glutathione peroxidase activity did not take place. Therefore, the potent inhibition by glutathione seen on liver microsomes seemed to be partly accounted for by the presence of glutathione peroxidase in liver microsomal fraction.

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