The Role of Phospholipase A Activity in Rat Liver Microsomal Lipid Peroxidation

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The involvement of phospholipase(s) A in lipid peroxidation of rat liver microsomes was investigated by: (a) determining the effects of phospholipase A inhibitors (p-bromophenylacyl bromide, chlorpromazine, mepacrine) on the accumulation of thiobarbituric acid reactivity or on levels of oxidized phospholipids in response to selected oxidative stimuli and (b) measurement of phospholipase A activities in response to these agents.

Lipid peroxidation in response to various peroxidation systems was inhibited completely by exposure of microsomes to p-bromophenylacyl bromide (250 μM). The effectiveness of p-bromophenylacyl bromide was dependent on the presence of glutathione (200 μM) in preincubation mixtures. Chlorpromazine (100 μM) and mepacrine (100 μM) also effectively inhibited peroxidation, and their potency was independent of glutathione. The accumulation of oxidized phospholipids in response to the potent peroxidation stimulus alloxan/ferrous ion was similarly inhibited by p-bromophenylacyl bromide, although the level of oxidized phospholipid in response to the initiator ADP/ferrous ion was not affected.

Microsomal phospholipase A₁ activity, assessed using a liposomal substrate, was substantially enhanced by promoters of lipid peroxidation. Phospholipase A₂ activity was not detected using a liposomal substrate but was evident using radiolabeled microsomes as endogenous substrate and was enhanced by oxidative stimuli.

We conclude that phospholipase A activity may play an integral role in the microsomal lipid peroxidation mechanism. Based on this study, we hypothesize a role for phospholipases in facilitating propagation reactions.

An association between lipid peroxidation and enhanced phospholipase A activity has been demonstrated in various membranes (1-4) including rat liver mitochondria (1), hepatic lysosomes (3), and rat hepatic microsomes (2). Furthermore, both site-specific (4) and nonspecific (5-7) phospholipase inhibitors have been demonstrated to inhibit lipid peroxidation. It has been tentatively concluded that fatty acyl hydroperoxides may be preferred substrates for phospholipases, explaining the "increase" in measured phospholipase activity (8, 9) in response to peroxidizing agents.

To date, this association between lipid peroxidation and phospholipase A activity has not been fully explored. In particular, the concept that phospholipase A activation may be necessary for lipid peroxidation has not been directly addressed nor has possible differential effects of phospholipase A activation on initiation and propagation of peroxidation.

Lipid peroxidation consists of distinct phases of initiation (formation of catalytic levels of lipid hydroperoxides) and propagation (extension of lipid hydroperoxide formation upon degradation of initiating species via formation of reactive radicals (10)). Promoters of lipid peroxidation include various forms of chelated iron (11) or of copper (12) which act as either initiators or propagators. Reducing agents such as ascorbic acid (13) or superoxide anion (14) may promote peroxidation by reducing ferric ion or ferric chelates to the ferrous form. A recent report (15) has also suggested that specific ferric to ferrous ion ratios are required for initiation reactions and that oxidants may promote ferrous ion-induced peroxidation. We have recently observed that the diabeticogenic agent alloxan, in the presence of ferrous ion and glutathione, acts as a potent promoter of lipid peroxidation (data described in the Miniprint Section 1), although its precise role in the peroxidation mechanism remains to be clarified. Alloxan is a known source of free radicals and hydrogen peroxide (16) and thus may promote peroxidation reactions indirectly. Ferric ion may substitute with equal effectiveness for ferrous ion, although the peroxidative response to alloxan/ferrous ion is sensitive to inhibition by superoxide dismutase, whereas the alloxan/ferrous ion-induced peroxidation is insensitive to the enzyme, suggesting that superoxide-mediated reduction of ferric ion is required for the alloxan/ferrous ion-induced peroxidative response. The alloxan/ferrous ion-induced peroxidative response was not affected by catalase or hydroxyl radical scavengers, whereas it was prevented by the iron chelator, DETAPAC.

In the present study, we have explored the role of phospholipase A in microsomal lipid peroxidation by determining the effects of phospholipase A inhibition on peroxidative responses promoted by selected stimuli and by investigating the effects of these agents on microsomal phospholipase A activity. Our assessments of lipid peroxidation were made by both a general assay (thiobarbituric acid reactivity) and the more

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2 Portions of this paper (including part of "Experimental Procedures," part of "Results," Figs. 8, 10, and Table II) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 861M-3308, cite the authors, and include a check or money order for $2.00 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

3 The abbreviations used are: DETAPAC, diethyleneetriaminepentaacetic acid; PBB, p-bromophenylacyl bromide; TPA, 2-thiobarbituric acid; EGTA, [ethylenebis(oxyethylenenitrilo)tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
specific measurement of oxidation products of the phosphatidylcholine fraction.

**EXPERIMENTAL PROCEDURES**

**Materials**—Alloxan, ADP, ascobic acid, chlorpromazine, DETAPAC, 2,4-dinitrophenyhydrizylene, EGTA, ferric chloride, glutathione (reduced form), mecaprine, phosphatidylcholine (egg), PBB, and lysophosphatidylcholine (TLC marker) were obtained from Sigma. 1-Rutanol (spectrophotometric grade) and ferrous sulfate were purchased from EMD Chemicals, Inc., New Jersey. 1-Palmitoyl-2-[3H]arachidonylphosphatidylcholine and 5,6,8,9,11,12,14,15-[3H]harachidonic acid were obtained from New England Nuclear. Thin layer plates and supplies were purchased from Altitech Associates, Inc. (Deerfield, IL).

**Preparation of Microsomes**—Male Sprague-Dawley rats (200–300 g) were obtained from Harlan Sprague-Dawley, Inc. (Indianapolis, IN). Liver microsomes were prepared from 20 g of tissue by preparing a 10% homogenate in 0.15 M KCl, 10 mM Tris buffer, pH 7.4, and performing differential centrifugation. The pellet obtained by centrifuging the 20,000 × g supernatant at 100,000 × g was resuspended in homogenization buffer (5–10 mg of protein/ml) and assayed protein content (17).

**Lipid Peroxidation Assay**—Microsomes (500 µg of protein/ml) were incubated at 37 °C in 100 mM Tris buffer, pH 7.4 (1-ml total volume) in the presence of promoters of lipid peroxidation. GSH (200 µM) was included in reaction mixtures unless otherwise noted. The effects of phosphatidylcholine were investigated by exposing microsomes to PBB (250 µM) for 30 min prior to addition of the initiator or propagation to reactor mixtures. Following incubation, the method of Mihara et al. (18) was used to derivatize TBA-reactive substances (malondialdehyde plus other peroxidative products) which assay spectrophotometrically by the method of Yagi (19).

**Preparation of Radiolabeled Substrate—**3-14C-Labeled and unlabeled phosphatidylcholine were dried under nitrogen and resuspended in water by sonication with a probe sonicator (Model 80201) at setting 3 for 15 sec. Phosphatidylcholine was dissolved such that each 200 µl of the 6–12 ml of substrate preparation contained 100 nmoI of phospholipid containing 10,000 cpm.

**Phospholipase Assay**—Microsomes (500 µg of protein/ml) were incubated for 1 h at 37 °C in the presence of 100 nmoI of liposomal substrate/ml, 100 mM Tris buffer, pH 7.5, and the described agents. Alloxan was dissolved in 10 mM HCl prior to dispensing. Incubations were halted by the addition of MeOH/CHCl3 (2:1) and the lipids were extracted by the Bligh and Dyer method (20). The chloroform layer was evaporated to dryness under nitrogen and redissolved in 50 µl of chloroform prior to application onto a TLC plate. Thin layer plates containing 50–60% of their maximum in chloroform/methanol/acetic acid/water (65:25:3:4) dried, and then developed the entire length of the plate in hexane/diethyl ether/glacial acetic acid (80:20:1). Lipids were visualized by exposure to iodine, the lanes were marked, and the iodine was allowed to sublime overnight. Lysophosphatidylcholine, phosphatidylcholine, and free fatty acid-containing spots were scraped into scintillation vials and counted in a toluene-based scintillant. Phospholipase A1 activity was defined as the percentage of recovered counts obtained in lysophosphatidylcholine, and phospholipase A2 activity was defined by the percentage of counts in free fatty acid.

**Endogenous Substrate Phospholipase Assay**—As an alternative to the use of a liposomal substrate, microsomes were radiolabeled by exposure to [3H]harachidonic acid (0.1 µCi, 83.6 Ci/nmol) for 30 min at 37 °C in 100 mM Tris buffer, pH 7.4, in the presence of 80 µM coenzyme A, 1 mM MgCl2, and 1.6 mM ATP. Incubations labeled the microsomal phospholipids with the phosphatidylcholine fraction containing the majority of the radioactivity. Incubations were continued in the same buffer system in the presence of oxidants using the concentrations described in the text. Reaction mixtures were processed as described for the liposomal assay except that radioactivity was measured for all phospholipid and neutral lipid fractions.

**Assay of Phospholipid Oxidation Products**—The procedure of Poli et al. (21) was used to prepare 2,4-dinitrophenyhydrizylene derivatives of carbonyl compounds of microsomal reaction mixtures. Microsomal reaction mixtures (1 ml) were mixed with 1 ml of dinitrophenyhydrizylene reagent (0.34 mg/ml in 1 M HCl) and allowed to react at room temperature in the dark for 2 h. The total lipid fraction was then extracted (20), and extracts were evaporated to dryness under nitrogen redissolved in methanol and applied to silicic acid TLC plates. The plates were developed initially in methylene chloride which separated unreacuted reagent and neutral lipids from the phospholipid fraction (which remained at the origin). The plates were developed until at least one-half of their length was free of reagent or neutral lipid-associated dinitrophenyhydrizylene. Phospholipids were then separated on this section of the plate by subsequent development using the first solvent system described above for the phospholipase assay. The phosphatidylcholine band (identified using a standard) was scraped off of the plates, eluted with methanol, and measured spectrophotometrically (22). Carbonyl-containing phospholipids were originally described by Tam and McCay (23) who determined that following derivatization with dinitrophenyhydrizylene, the resulting phenylhydrizone functional groups were localized on the β-position polysaturated fatty acids of the phospholipids.

**RESULTS**

Exposure of rat liver microsomes in the initiator (11) ADP/ferrous ion (10 µM) resulted in an early (first 4–5 min) phase of rapid induction of oxidant activity (as measured by TBA activity), followed by a slower accumulation of TBA-reactive products. Pre-exposure of microsomes to PBB (250 µM) completely blocked lipid peroxidation (Fig. 1). These reaction mixtures contained glutathione (200 µM) (a necessary factor for alloxan-induced propagation reactions (see Fig. 9)). In the absence of glutathione, the peroxidative response to ADP/ferrous ion is not affected by PBB. The effectiveness of PBB (250 µM) as an inhibitor of microsomal phospholipase A activity in the presence/absence of glutathione paralleled its effectiveness as an inhibitor of lipid peroxidation (Fig. 2). PBB (with or without GSH), incubated for periods up to 30 min with microsomal reaction mixtures in which peroxidation was complete (in response to alloxan/iron), did not affect subsequent measurement of TBA reactivity (data not shown). Chlorpromazine (100 µM) inhibited ADP/ferrous ion-induced lipid peroxidation in the absence of GSH (95% inhibition of relative intensity, p < 0.001), as did mepacrine (100 µM) (83% inhibition of relative intensity, p < 0.001).

Initiation of lipid peroxidation by ascorbic acid and ADP/ferrous ion in the presence of glutathione exhibited a "lag" period during which lipid peroxidation proceeds at a slow rate, followed by a period of rapid lipid peroxidation, as described by others (24). Pre-exposure of microsomes to PBB (250 µM) completely blocked lipid peroxidation (Fig. 3).

We have observed that exposure of rat liver microsomes to

![Fig. 1. Inhibition of ADP/ferrous ion-initiated lipid peroxidation of rat liver microsomes by PBB. Microsomes (500 µg of protein/ml) were incubated for 30 min at 37 °C in the presence (●) or absence (○) of the phospholipase A inhibitor PBB (250 µM). Microsomes (control and PBB-treated) were then exposed to ADP (1 mM final concentration) and ferrous sulfate (10 µM) for the given duration. Lipid peroxidation was assessed by measurement of TBA reactivity as described under "Experimental Procedures."](image-url)
following exposure to ADP/ferrous ion prevented peroxidative responses attributed to alloxan (Fig. 4). Similar peroxidative responses to copper (a reported propagator (12)) were also inhibited by PBB (Fig. 4). Alloxan/ferrous ion-induced lipid peroxidation was also inhibited by chlorpromazine (85% inhibition by 100 pM, p < 0.001) and mepacrine (78% by 100 pM, p < 0.001).

Microsomal phospholipase A1 activity (assessed using a liposomal phosphatidylcholine substrate) was enhanced by both initiators and propagators of lipid peroxidation (Table I). ADP/ferrous ion elicited a higher level of phospholipase A1 activity than did ADP/ferric ion (272 versus 162% of control), whereas the effect of ascorbic acid/ADP/ferric ion on phospholipase A1 activity was nearly equal to that of ADP/ferrous ion.

**Fig. 2.** Glutathione requirement for PBB-induced inhibition of microsomal lipid peroxidation and phospholipase A1 activity. Microsomes (500 ng of protein/ml) were incubated for 30 min at 37 °C in the presence/absence of glutathione (200 mM) and in the presence/absence of PBB (250 pM) (with or without glutathione). Reaction mixtures were then exposed to ADP (1 mM)/ferrous sulfate (10 pM) or control incubation for the given duration. Lipid peroxidation (A) was assessed (TBA reactivity), and phospholipase A1 activity (B) was measured as described under "Experimental Procedures." Open bars, control incubations; solid bars, ADP/ferrous ion-exposed microsomes; cross-hatched bars, PBB-treated microsomes exposed to ADP/ferrous ion.

**Fig. 3.** Inhibition of ascorbic acid/ADP/ferric ion-promoted lipid peroxidation by PBB. Microsomes (500 ng of protein/ml) were incubated for 30 min at 37 °C in the presence (1) or absence (2) of the phospholipase A inhibitor PBB (250 pM). Reaction mixtures were then exposed to ascorbic acid (500 pM final concentration), ADP (1 mM), and ferrous sulfate (10 pM) for the given duration. Lipid peroxidation was assessed by measurement of TBA reactivity as described under "Experimental Procedures."

**Fig. 4.** Inhibition of alloxan- or copper-induced propagation of lipid peroxidation of rat liver microsomes by PBB. Microsomes (500 ng/ml) were exposed to the peroxidation initiator ADP (1 mM)/ferrous ion (10 pM) for 5 min. Incubation was then continued in the presence or absence of PBB for 30 min, followed by an exposure for 5 min to either alloxan (100 pM) (2) or copper (10 pM) (3) or no additional agent (1) without PBB (---) or with PBB (----). Lipid peroxidation was then assessed as described under "Experimental Procedures." Peroxidation was assessed at various intermediate stages as well as final levels stopping reaction at appropriate times.

**Table I**

Effects of initiators and propagators of lipid peroxidation on phospholipase A1 activity of rat liver microsomes at pH 7.5

| Agents                  | Phospholipase A1 activity (nmol/h/mg protein) | % of control |
|-------------------------|-----------------------------------------------|-------------|
| Control (GSH, 200 mM)   | 10.2 ± 1.4                                   | 100         |
| Control without GSH     | 10.0 ± 0.8                                   | 98          |
| ADP (1 mM)/ferrous ion  | 27.8 ± 1.6                                   | 272         |
| ADP (1 mM)/ferric ion   | 16.5 ± 2.0                                   | 162         |
| Ascorbic acid (500 mM)/ADP (1 mM) | 26.7 ± 3.1 | 262         |
| Ferric ion (10 pM)      | 30.0 ± 3.3                                   | 294         |
| Alloxan (100 pM)/ferrous ion (10 pM) | 54.2 ± 4.0 | 531         |
| Copper (10 pM)          | 14.1 ± 1.2                                   | 117         |
| Copper (10 pM)/ferrous ion (10 pM) | 35.2 ± 4.6 | 345         |
| Copper (100 pM)         | 27.4 ± 2.6                                   | 268         |
| Copper (100 pM)/ferrous ion (10 pM) | 47.0 ± 5.1 | 461         |

* Values denote the mean ± S.E. of four observations.
* p ≤ 0.001.
* p ≤ 0.05.
Alloxan (100 μM) and copper (10 and 100 μM) each augmented microsomal phospholipase A₁ activity: alloxan, 294% of control; copper (10 μM), 117% of control; copper (100 μM), 268% of control. Combinations of alloxan and ferrous ion and of copper and ferrous ion were more potent inducers of phospholipase A₁ activity: alloxan/ferrous ion, 531% of control; copper (100 μM)/ferrous ion, 461% of control.

Alloxan enhanced microsomal phospholipase A₁ activity in a manner which strictly paralleled its effect on lipid peroxidation. That is, alloxan-induced phospholipase A₁ activity required glutathione (data not shown), was potentiated by iron (Table I), and was inhibited completely by DETAPAC (data not shown). Furthermore, the alloxan dose response for phospholipase A₁ exhibited a biphasic dose-response pattern (Fig. 5) which closely resembled the peroxidation response (Fig. 6).

Phospholipase A₂ activity was not detected in response to any of the peroxidation stimuli using the liposomal assay system. Using an assay in which microsomes were preradio-labeled with [³H]arachidonic acid (80% incorporation into phosphatidylcholine) and subsequently exposed to alloxan/GSH/ferrous ion, we measured a rapid accumulation of radio-labeled lysophosphatidylcholine (indicative of phospholipase A₂ activity) and of radio-labeled free fatty acids (representative of phospholipase A₃) with a parallel loss of radioactivity in phosphatidylcholine (Fig. 6, A and B). The enhanced accumulation of radioactivity of free fatty acids was transient, however, suggesting that significant reacylation may have occurred. Indeed, accumulation of radioactivity in the phospholipid precursor phosphatidic acid was prominent in response to alloxan (Fig. 6C). The accumulation of radioactivity into lysophospholipids, free fatty acids, and phosphatidic acid as well as the loss of radioactivity in phosphatidylcholine was completely blocked by 250 μM PBB (data not shown).

Alloxan/ferrous ion and ADP/ferrous ion each promoted the formation of oxidized phospholipid molecules containing carbonyl groups reactive with 2,4-dinitrophenylhydrazine (18.0 ± 0.6 μM for alloxan/ferrous ion; 5.3 ± 0.50 μM for ADP/ferrous ion). We have demonstrated a positive relationship between the process of microsomal lipid peroxidation and phospholipase

**Discussion**

We have demonstrated a positive relationship between the process of microsomal lipid peroxidation and phospholipase
A activity. Inhibition of phospholipase A activity by PBB (at a level verified effective) prevented peroxidative responses to each of the peroxidative stimuli utilized. PBB acts as a site-specific phospholipase A inhibitor which binds a histidine residue at the active site of phospholipase A enzyme (35, 26). PBB is not specific for phospholipase A (27), however; and its use must be viewed with caution. Therefore, we have verified that other inhibitors of phospholipase(s) A, chlorpromazine and mecaprine, also inhibited lipid peroxidation in response to both ADP/ferrous iron and to alloxan/ferrous ion.

The effectiveness of PBB as an inhibitor of iron-promoted initiation reactions was dependent on the presence of glutathione during the PBB preincubation with microsomes. Similarly, inhibition of microsomal phospholipase A activity by PBB required glutathione, suggesting that PBB-induced inhibition of lipid peroxidation is indeed related to phospholipase A inhibition. In view of the reported reactivity of PBB with thiols (27), we investigated the possibility that PBB might react with GSH to in some manner inhibit the TBA assay. PBB (with or without glutathione) does not inhibit the TBA reaction, and preincubation of PBB with GSH does not render the PBB into an inhibitor of the TBA reaction. Phospholipase assays are similarly not affected by addition of PBB and GSH to reaction mixtures immediately prior to extraction.

The phospholipase activity of rat liver microsomes was measured in the presence of various promoters of lipid peroxidation. The liposomal assay detected only phospholipase A activity (the predominant phospholipase of microsomes (28) as phospholipase A activity was minimal and not influenced by our interventions (including the addition of calcium (5 mM)). The membrane-embedded nature of phospholipase A (29) or reacylation of hydrolyzed fatty acids (see below) may explain its apparent lack of activity against liposomal substrate. Phospholipase A activity in response to oxidative stimuli was evident using the assay based on preradiolabeled microsomes. The enhanced accumulation of radioactivity in free fatty acids was transient, however, and would be missed using only long-term incubations. Reacylation of fatty acids into phospholipids was indicated by the accumulation of radioactivity in phosphatidic acid.

Agents which promote lipid peroxidation substantially enhance microsomal phospholipase A activities. Others have reported that phospholipid epoxydes are readily hydrolyzed by phospholipases and suggested that phospholipid oxidation products are preferred substrates for phospholipase enzymes (8, 9). Indeed, we observed a strong correlation between the biphasic dose-response relationships of alloxan with respect to lipid peroxidation and phospholipase A activity. Besides the possibility that the oxidized phospholipids are preferred substrates, it is possible that free radicals or other oxidants directly activate phospholipases.

Regardless of the mechanism for apparent phospholipase activation, an important consideration is the role that this activity plays in the lipid peroxidation mechanism. One possibility is that the inhibition of peroxidation by phospholipase A inhibitors is simply a result of confining peroxidation products to a form that was not TBA-reactive, i.e. as intact phospholipids. However, the formation of phospholipid oxidation products in response to alloxan/ferrous ion was also inhibited by PBB, indicating that the peroxidative response to phospholipase inhibitors cannot be explained in this manner. In contrast to its substantial inhibition of alloxan-induced phospholipid oxidation products (reactive with 2,4-dinitrophenylhydrazine), PBB did not affect the level of these products in response to the initiator, ADP/ferrous ion. We hypothesize that the phospholipid oxidation products measured in response to ADP/ferrous iron are remnants of phospholipid hydroperoxides derived from primary initiation reactions. We further hypothesize that the oxidized phospholipids induced by alloxan/ferrous ion represent an extension of primary initiation reactions (secondary initiation or propagation) which is phospholipase A-dependent. Unesterified fatty acids released by phospholipases may be more effective in interacting with initiators and may be involved in a "second wave" of initiation. Alternatively, unesterified fatty acid radicals may be better able to reinitiate peroxidative reactions (i.e. propagate peroxidation) than are intact phospholipid radicals or radicals of scission products released nonenzymatically from them. The latter suggestion is analogous to one developed by Parthasarathy et al. (4) in a related study. The authors demonstrated that PBB inhibits the lipid peroxidation associated with endothelial cell modification of low density lipoprotein. They interpreted their data to indicate that free fatty acids might more readily propagate lipid peroxidation due to facilitated interaction with low density lipoprotein molecules and/or endothelial cells. Our results obtained using the rat liver microsomal lipid peroxidation model system indicate that enhanced lipolytic activity may prove to be an integral component of the mechanism of lipid peroxidation.

Although phospholipase activity appears to be directly involved in the lipid peroxidation mechanism, it also may play a role in protecting membranes against peroxidative injury. Glutathione peroxidase reduces fatty acyl hydroperoxides as substrates (30, 31) and thus requires phospholipase activity to provide the substrate with which it can react. Conditions favoring the accumulation of initiation products may thus be alleviated by phospholipase activity.

In summary, we have demonstrated a dependence of rat liver microsomal lipid peroxidation on phospholipase A activities and have shown that a variety of promoters of lipid peroxidation act directly or indirectly as stimuli to increase phospholipase A activity. The data presented suggest that phospholipase activity is involved at a stage of the lipid peroxidation mechanism beyond primary initiation.

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Supplementary Material to
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EXPERIMENTAL PROCEDURES

Chemicals. Alixens, DETAPAC, ferric chloride, ferrous sulfate, and mammary were obtained from Sigma Chemical Company, St. Louis, MO. Defatted (arachis oil) grade) and ferrous sulfate were purchased from Aldrich Chemical, Milwaukee, WI.

Enzymes. Bovine erythrocytes superoxide dismutase (EC 1.15.1.1) was obtained from Miles Laboratories, Elkhart, Indiana and bovine liver catalase (EC 1.1.1.12) and purchased from Sigma, St. Louis, MO.

Experimental Design. Rat liver microsomes (90 mg protein/ml) were incubated for 3 min at 37°C in 180 ml buffer, pH 7.4 (0.5 ml total volume) in the presence of 0.1 mM glutathione (GS) or 1.5 μM glutathione disulfide (GSSG). Samples were collected for analysis in the primary Experimental Procedures section. In subsequent experiments tests were performed on the effects of: (1) GS concentration; (2) ferrous sulfate (10 μM); (3) ferric chloride (10 μM); (4) superoxide dismutase (180 U/ml); (5) catalase (560 U/ml); (6) selected redox scavengers, and (7) DETAPAC (1 μM).

RESULTS

Exposure of rat liver microsomes to alloxan in the presence of 100 μM glutathione resulted in activated rates of TBA-reactive substances in the absence of lipid peroxidation. A peak response was observed with a peak effect observed with 100 μM alloxan (Figure 2). Alloxan-induced lipid peroxidation was quantitatively less in the absence of glutathione (Figure 2 and 3). The presence of ferrous ion potentiates the effect of alloxan in the presence of GS (Figure 2 and 3). Ferric ion pretreated alloxan-induced peroxidation equally well as ferrous ion/ADP (Figure 3) although ferric ion/ADP did not affect basal levels of TBA-reactive substances. The effect of alloxan was enhanced by the inhibition of superoxide dismutase, unlike ferrous ion/ADP pretreatment (Figure 4). Suggesting that superoxide-mediated reduction of ferrous ion was required for peroxidation. Pre-exposure of microsomes to ferrous ion/ADP enhanced the initial rate of peroxidation in response to alloxan (Figure 5).

Figure 1. Production of lipid peroxidation of rat liver microsomes by alloxan. Microsomes (100 μg protein/ml) were exposed to alloxan for 15 minutes at 37°C. (-), Buffer pH 7.4 in the presence of 0.1 mM glutathione disulfide (GSSG); (---), Buffer pH 7.4 in the presence of 0.1 mM glutathione (GSH); (---), DMSO control.

Table 1. Effects of Scavenging Agents on Lipid Peroxidation of Rat Liver Microsomes Catalyzed by Alloxan/Ferrous Ion

| Scavenging Agent | TBA Reactivity | % of Control |
|------------------|---------------|-------------|
| None             | 185 ± 18a     | 100         |
| Alloxan (100 μM) | 195 ± 18      | 106         |
| + Catalase (184 U/ml) | 195 ± 18 | 100       |
| + DMSO (2.5 mg/ml) | 195 ± 18 | 100       |
| + NAD (9.6 mM) | 195 ± 18     | 100         |
| + SS (15 μM) | 196 ± 18      | 100         |

Values denote the mean ± SEM of four observations.

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Figure 2. Effect of glutathione on basal and alloxan (180 μM)-induced lipid peroxidation in rat liver microsomes. Exposure of microsomes to the designated concentrations of glutathione were performed in the absence (•) or presence (△) of 100 μM alloxan. Experiments and assessments of lipid peroxidation (TBA-reactive substances) were performed as described in Figure 1. Brackets denote one standard error unit for four observations.

Figure 3. Effect of glutathione on basal and alloxan (180 μM)-induced lipid peroxidation in rat liver microsomes. Exposure of microsomes to the designated concentrations of glutathione were performed in the absence (•) or presence (△) of 100 μM alloxan. Experiments and assessments of lipid peroxidation (TBA-reactive substances) were performed as described in Figure 1. Brackets denote one standard error unit for four observations.

Figure 4. Production of lipid peroxidation of rat liver microsomes by alloxan. Microsomes (100 μg protein/ml) were exposed to alloxan for 15 minutes at 37°C. ( ), Buffer pH 7.4 in the presence of 0.1 mM glutathione disulfide (GSSG); (---), Buffer pH 7.4 in the presence of 0.1 mM glutathione (GSH); (---), DMSO control.

Figure 5. Production of lipid peroxidation of rat liver microsomes by alloxan. Microsomes (100 μg protein/ml) were exposed to alloxan for 15 minutes at 37°C. ( ), Buffer pH 7.4 in the presence of 0.1 mM glutathione disulfide (GSSG); (---), Buffer pH 7.4 in the presence of 0.1 mM glutathione (GSH); (---), DMSO control.