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EFFECT OF HYDROGEN PEROXIDE ON THE INITIATION OF MICROSOMAL LIPID PEROXIDATION*

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Abstract—Hydrogen peroxide reacts with reduced transition metals to generate the highly reactive hydroxyl radical (·OH), most often proposed as the predominant species for initiating microsomal lipid peroxidation. To assess the potential involvement of ·OH, generated from hydrogen peroxide, in microsomal lipid peroxidation, we have altered the concentration of microsomal hydrogen peroxide and measured the resulting rates of malondialdehyde production. Hydrogen peroxide concentration in microsomes was changed by adding exogenous catalase, by washing to reduce both endogenous catalase activity and hydrogen peroxide-dependent glutathione oxidase activity, and by inhibiting endogenous catalase activity with azide in either the presence or absence of exogenous hydrogen peroxide. In only one instance was the rate of lipid peroxidation affected; exogenous hydrogen peroxide added to microsomes, previously incubated with azide, inhibited lipid peroxidation, the opposite effect from that predicted if ·OH, generated from hydrogen peroxide, is actually the major initiating species. Neither these results, nor the inability of known ·OH traps to inhibit microsomal lipid peroxidation, support the role of free hydrogen peroxide in the initiation of microsomal lipid peroxidation.

Lai *et al.* [1–3], Fong *et al.* [4], and Koster and Slee [5] have all suggested that the hydroxyl radical (·OH) is the initiating species in NADPH-cytochrome P-450 reductase-dependent peroxidation of microsomal lipids. They propose that, during oxidation of NADPH by microsomes, reduced iron and hydrogen peroxide (H_2O_2), formed by a superoxide-dependent mechanism, react to form ·OH [1–5]. This series of reactions is known as the iron-catalyzed Haber–Weiss reaction:

$$O_2^- + Fe^{3+} \rightarrow Fe^{2+} + O_2$$

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \cdot OH + OH^-.$$

We have questioned whether this mechanism represents the predominant means by which the initiator of microsomal lipid peroxidation is generated [6]. The endogenous catalase activity present in microsomes [7] should scavenge H_2O_2, thereby affecting the amount available to react with reduced iron to generate ·OH. Thurman *et al.* [8] have shown that, in order to quantitate H_2O_2 generation by microsomes, an inhibitor of catalase must be added, thus indicating that the steady-state concentration of H_2O_2 must be very low in microsomes incubated with only NADPH. Furthermore, Cohen and Cederbaum [9] have shown that azide-treated microsomes produce more ·OH than do control microsomes. Therefore, addition of exogenous H_2O_2 and/or the inhibition of endogenous catalase activity should cause an increase both in ·OH production and in subsequent rates of microsomal lipid peroxidation. However, we are unaware of any published reports which thoroughly investigate the effects of H_2O_2, azide, and catalase on microsomal lipid peroxidation.

To address this question, we have studied the effects of adding exogenous catalase to microsomes, washing microsomes to remove endogenous catalase activity, and inhibiting endogenous catalase activity in microsomes with azide in the presence or absence of exogenous H_2O_2. None of these methods affected the observed rates of lipid peroxidation as measured by malondialdehyde (MDA) formation except the addition of both azide and H_2O_2 to microsomes incubated with ADP-chelated iron (ADP-Fe^{3+}) and NADPH. Simultaneous addition of azide and H_2O_2, contrary to what might be predicted from the iron-catalyzed Haber–Weiss reaction, caused decreased rates of lipid peroxidation. The prevalent theory for the initiation of microsomal lipid peroxidation, that of an iron-catalyzed Haber–Weiss reaction generating ·OH, is not supported by these results.

MATERIALS AND METHODS

Chemicals. NADPH, ADP, 2-thiobarbituric acid, butylated hydroxytoluene, glutathione reductase, sodium azide, and Sephadex G-25 were all purchased from the Sigma Chemical Co. (St. Louis, MO). Malondialdehyde bis(dimethyl acetal) was obtained from the Aldrich Chemical Co. (Milwaukee, WI). Catalase was purchased from Sigma (30,000 units/mg), Boehringer-Mannheim (Indianapolis, IN) (65,000 units/mg), and Millipore (Freehold, NJ) (three different preparations: 86,902 units/mg, 44,087 units/mg, and 11,054 units/mg, furnished

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lyophilized). Other chemicals used were of reagent grade or better and used without further purification.

Preparations of microsomes. Male Sprague–Dawley rats (250–275 g) were obtained from Spartan Research Animals, Haslett, MI. Liver microsomes were prepared by the procedure of Pederson and Aust [10]. Isolated microsomes were washed by homogenization in 4 vol. of ice-cold, argon-purged 1.15% KCl, 0.2% nicotinamide and centrifugation at 105,000 *g* for 90 min.

Lipid peroxidation assays. Stock solutions of microsomes, ADP-Fe^{3+}, and NADPH, prepared in 30 mM NaCl (pH 7.0) were combined to obtain complete lipid peroxidation incubation mixtures: 0.5 mg microsomal protein/ml, ADP-Fe^{3+} (1.7 mM ADP, 0.1 mM $FeCl_3$, pH 7.0), and NADPH (0.1 mM). Peroxidation was initiated by the addition of NADPH. Additions to these complete incubation mixtures are described in the figure legends. When both azide and H_2O_2 were added to microsomes, azide was always added prior to H_2O_2 to inhibit the endogenous catalase activity present in microsomes. When only H_2O_2 was added to microsomes, it was done just prior to the initiation of peroxidation by NADPH. All solutions used in these incubations were prepared from distilled-deionized H_2O passed through a Chelex chelating resin (Bio-Rad Laboratories, Richmond, CA) column to remove contaminating metals. MDA and/or lipid hydroperoxide were assayed by the procedures of Buege and Aust [11]. Rates of MDA formation in each incubation mixture, calculated from the amount of MDA formed during the first 3 min following the addition of NADPH, were compared to simultaneously run control incubations (complete systems) and expressed as a percentage of the control rate. Catalase activity was assayed by the procedure of Beers and Sizer [12]. The procedure of Paglia and Valentine [13] was used for determination of hydrogen peroxide-dependent glutathione oxidase activity. Microsomal protein content was determined by the method of Lowry *et al.* [14].

RESULTS

Figure 1 shows the results of an experiment designed to demonstrate the validity of MDA [as measured by the thiobarbituric acid (TBA) assay] as a reliable index of microsomal lipid peroxidation. The time course of MDA formation from microsomes parallels that of lipid hydroperoxide (LOOH) formation over the first 10 min of the incubation. Furthermore, microsomal lipid peroxidation is both an NADPH and ADP-Fe^{3+}-dependent process, because in the absence of either NADPH or ADP-Fe^{3+}, rates of lipid hydroperoxide or MDA formation from microsomal lipid peroxidation incubation mixtures were not significantly different from control incubations (Fig. 1).

Figure 2 is representative of the time course of microsomal NADPH-dependent lipid peroxidation and shows the effect of H_2O_2 added either without or with azide to inhibit endogenous catalase. The addition of either azide (0.4 mM) or H_2O_2 (0.1 mM) to microsomal incubation mixtures containing both NADPH and ADP-Fe^{3+} had no effect on the rate of lipid peroxidation. However, upon the addition of both H_2O_2 and azide, the initial rate of lipid peroxidation was decreased markedly. The simultaneous addition of H_2O_2 (0.1 mM), NADPH (0.1 mM) and azide (0.4 mM) to microsomes in the absence of ADP-Fe^{3+} did not result in lipid peroxidation (Fig. 2). This result indicates that neither endogenous heme iron, such as cytochrome P-450 or cytochrome b_5, nor non-heme iron was able to effectively react with H_2O_2 to initiate lipid peroxidation.

The ability of azide (0.4 mM) to inhibit the endogenous catalase activity of microsomes is dem-

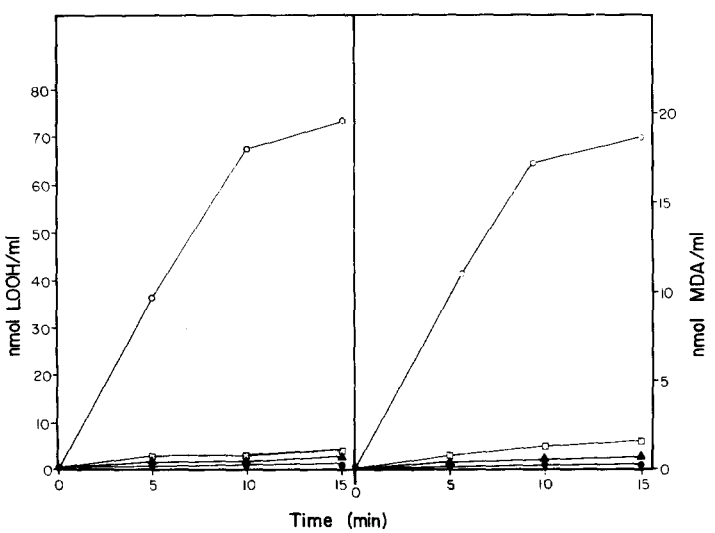

Fig. 1. Time course of lipid hydroperoxide and MDA formation during microsomal lipid peroxidation. Each incubation mixture contained 0.5 mg microsomal protein/ml in 30 mM NaCl (pH 7.0) with the following additions: (●) none; (▲) NADPH (0.1 mM); (□) ADP-Fe^{3+} (1.7 mM ADP, 0.1 mM $FeCl_3$); and (○) NADPH and ADP-Fe^{3+} (complete system). Assay conditions are described in Materials and Methods.

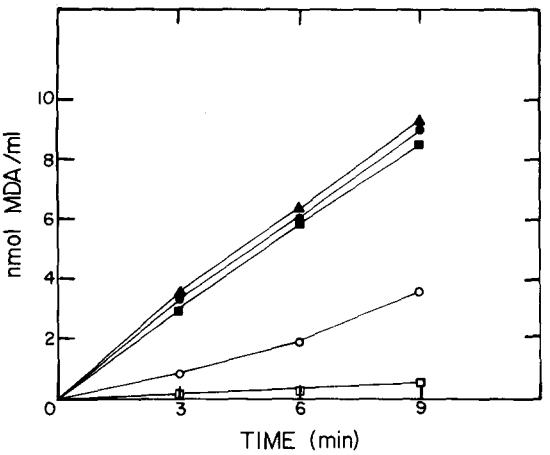

Fig. 2. Effect of H_2O_2, azide, or H_2O_2 plus azide on microsomal lipid perodixation. Each incubation contained 0.5 mg microsomal protein/ml in 30 mM NaCl (pH 7.0) with the following additions at the indicated final concentrations: (▲) ADP-Fe^{3+} (1.7 mM ADP, 0.1 mM $FeCl_3$) and NADPH (0.1 mM) (complete system); (■) complete system plus H_2O_2 (0.1 mM); (●) complete system plus azide (0.4 mM); (○) complete system plus azide (0.4 mM) and H_2O_2 (0.1 mM); and (□) NADPH (0.1 mM), H_2O_2 (0.1 mM), and azide (0.4 mM). Assay conditions are described in Materials and Methods.

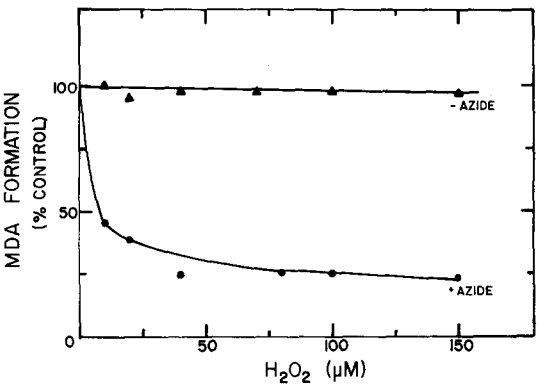

Fig. 4. Effect on H_2O_2 on microsomal lipid peroxidation. All incubation mixtures contained the complete system (Fig. 1) in the absence (▲) or presence (●) of azide to which were added various concentrations of H_2O_2. Rates of MDA formation are expressed as percentages of that observed in the complete system.

onstrated by the results shown in Fig. 3. In the absence of azide, the catalase activity of microsomes is approximately 143 I.U./mg protein. Fifty percent inhibition of this activity was obtained by the addition of 0.025 mM azide (Fig. 3, inset), while 0.4 mM azide caused greater than 97% inhibition of the microsomal catalase activity. Although inhibition of catalase activity by azide should increase the H_2O_2 concentration in microsomes, NADPH and ADP-Fe^{3+}-dependent lipid peroxidation was clearly not affected by azide concentrations up to 1 mM (Fig. 3).

The effect of H_2O_2 on microsomal lipid peroxidation was also studied by adding exogenous H_2O_2 to microsomes (Fig. 4). In the absence of azide, H_2O_2 concentrations up to 150 μM were without effect on the rate of MDA formation. However, in agreement with the results shown in Fig. 2, the addition of H_2O_2 in the presence of azide (0.4 mM) caused a significant decrease in the rate of lipid peroxidation. Fifty percent inhibition occurred in the presence of approximately 20 μM H_2O_2. Control experiments indicated that neither H_2O_2 nor azide had an effect on the TBA assay for MDA.

The involvement of H_2O_2 in microsomal lipid peroxidation has been studied previously by adding exogenous catalase. However, the results of these studies are contradictory [5, 15, 16]. Accordingly, five different commercial catalase preparations were tested for their effects on this process. The results are shown in Fig. 5. Three of the five catalase prep-

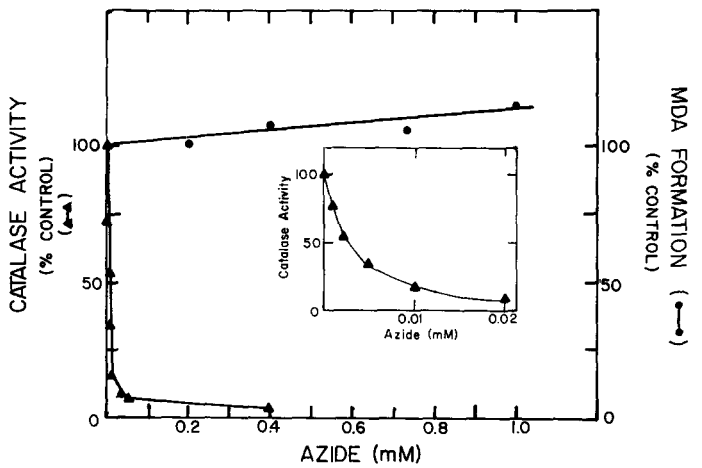

Fig. 3. Effect of azide on endogenous catalase activity in microsomes and microsomal lipid peroxidation. Endogenous catalase activity in microsomes was measured in a 1 ml cuvette containing 0.1 mg microsomal protein/ml, 20 mM H_2O_2 in 0.05 M KH_2PO_4 (pH 7.5) and various concentrations of sodium azide. Microsomal lipid peroxidation incubations contained the complete system (described in Fig. 1) to which were added various concentrations of sodium azide. Rates of MDA formation are expressed as percentages of that observed in the complete system.

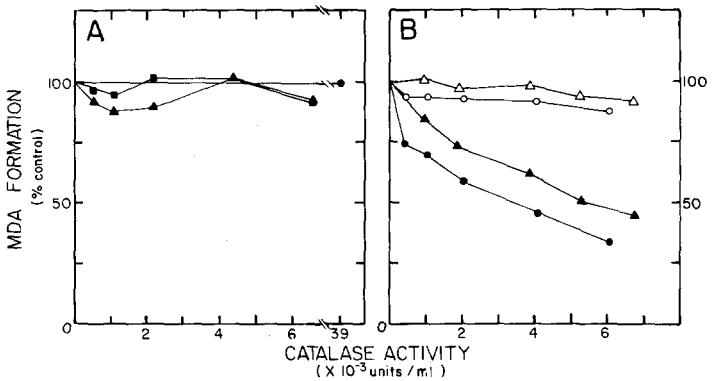

Fig. 5. Effect of different commerical catalase preparations on microsomal lipid peroxidation. All incubations contained the complete system (Fig. 1) to which were added various amounts of the following catalase preparations: Panel A: (●) Boehringer-Mannheim (65,000 units/mg); (■) Millipore (86,902 units/mg); and (▲) Millipore (11,054 units/mg, furnished lyophilized). Panel B: (▲) Sigma (30,000 units/mg); and (●) Millipore (44,087 units/mg). The catalase preparations in Panel B were also first chromatographed on Sephadex G-25, assayed for activity, and then added to the complete system. The effect of these two chromatographed catalase preparations on MDA formation is also shown in Panel B: (△) chromatographed Sigma (30,000 units/mg); and (○) chromatographed Millipore (44,087 units/mg). Rates of MDA formation in both panels A and B are expressed as percentages of that observed in the complete system.

arations had no effect on microsomal lipid peroxidation (Fig. 5A). However, two catalase preparations significantly inhibited the rate of MDA formation (Fig. 5B). Pederson and Aust [16] suggested that the conflicting reports of the effect of exogenous catalase on microsomal lipid peroxidation could be due to antioxidants present in several commercial enzyme preparations. When the catalase preparations which inhibited lipid peroxidation [Sigma (30,000 units/mg) and Millipore (44,087 units/mg)] were chromatographed on Sephadex G-25 to remove low molecular weight contaminants, no inhibition of MDA formation could be observed upon their addition to the complete system (Fig. 5B).

Previously, it was demonstrated that endogenous catalase activity could be significantly removed from microsomes by washing [17]. In this study, washed microsomes were found to contain both lower catalase activity and lower H_2O_2-dependent, glutathione oxidase activity than was found in unwashed microsomes, yet rates of microsomal lipid peroxidation were essentially unaffected (Table 1). Washed microsomes to which exogenous catalase was added to an activity equal to that of unwashed microsomes also showed rates of lipid peroxidation similar to those seen with unwashed control microsomes (Table 1).

DISCUSSION

In this paper, we have examined the potential role of microsomal H_2O_2 in the promotion of lipid peroxidation. We have postulated that by affecting microsomal H_2O_2 metabolism we should observe, in the presence of reduced iron, corresponding changes in lipid peroxidation if it is initiated by an iron-catalyzed Haber–Weiss reaction.

The concentration of H_2O_2, generated *in situ*, was altered by the addition of azide (0.4 mM) to microsomes. This treatment, which caused 97% inhibition of microsomal catalase activity, did not affect the rate of lipid peroxidation. Correspondingly, the rate of microsomal lipid peroxidation was also not affected by washing microsomes to remove both endogenous catalase activity and H_2O_2-dependent glutathione oxidase activity. Furthermore, the addition of exogenous catalase (previously chromatographed to remove contaminating antioxidants) to microsomes had no effect on lipid peroxidation. Using even extremely high concentrations of catalase

Table 1. Effect of washing and the addition of catalase on microsomal lipid peroxidation*

Microsomes	Catalase activity (units/mg)	H_2O_2-dependent glutathione oxidation (μmoles NADPH·mg^{-1}·min^{-1})	MDA formation (% control)	
			No catalase added	Catalase added
Unwashed	143	0.119	100	
Washed once	33	0.060	113	97
Washed twice	23	0.059	100	100

* Microsomes were washed as outlined in Materials and Methods. Catalase activity in microsomes was measured in phosphate buffer (pH 7.5) containing 20 mM H_2O_2. Hydrogen peroxide dependent glutathione oxidase activity was assayed as described in Materials and Methods. Lipid peroxidation incubations consisted of the complete system to which was added exogenous catalase to a specific activity equal to that of unwashed microsomes. Rates of MDA formation are expressed as percentages of that observed in the complete system with unwashed microsomes.

(0.6 mg protein/ml), we could not observe the inhibition of MDA formation which was reported by Koster and Slee [5]. These results clearly indicate that the endogenous catalase activity in microsomes is neither critical for, nor inhibitory toward, the formation of the initiating species of microsomal lipid peroxidation.

The addition of organic hydroperoxides to microsomes has been shown to promote lipid peroxidation by a mechanism dependent upon the peroxidase activity of cytochrome P-450 [18]. From our results, it appears that exogenous H_2O_2 does not interact with cytochrome P-450 in a manner similar to that of organic hydroperoxides. The addition of only H_2O_2 to azide-treated microsomes did not initiate lipid peroxidation. However, when H_2O_2 was added to microsomes incubated with azide, ADP-Fe^{3+}, and NADPH, decreased rates of MDA formation were observed, apparently in agreement with results reported by Kornbrust and Mavis [15]. This is the opposite effect from that which one would expect if the initiation of microsomal lipid peroxidation occurred via an iron-catalyzed Haber–Weiss reaction. In the absence of azide, exogenous H_2O_2 did not affect microsomal NADPH and ADP-Fe^{3+}-dependent lipid peroxidation, presumably because the H_2O_2 would be efficiently decomposed by endogenous catalase.

Small increases in the H_2O_2 concentration in azide-treated microsomes have been reported to cause increased ·OH production [9] and should cause corresponding increases in MDA formation. However, our results suggest that the formation of an initiator of microsomal lipid peroxidation is more complex than the exclusive generation of free ·OH by an iron-catalyzed Haber–Weiss mechanism. When one also considers previously published reports which demonstrated that ·OH traps could not inhibit ADP-Fe^{3+}-catalyzed microsomal lipid peroxidation [15, 19], a strong argument can be made against the involvement of free ·OH in the initiation of microsomal lipid peroxidation.

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