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INHIBITION OF HEPATIC MICROSOMAL LIPID PEROXIDATION BY DRUG SUBSTRATES WITHOUT DRUG METABOLISM

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Abstract—Experiments were performed to study the mechanism of action of drug substrates on lipid peroxidation in rat hepatic microsomes. Addition of the drug substrates, aniline, β-diethylaminoethyl diphenylpropylacetate (SKF-525A), aminopyrine, benzo[*a*]pyrene or ethylmorphine, to hepatic microsomes causes almost complete inhibition of NADPH-induced (enzymatic) lipid peroxidation. These substrates also produce similar inhibition of ascorbate-induced (non-enzymatic) lipid peroxidation in microsomes in which drug-metabolizing enzymes were inactivated by heat treatment. The substrate concentrations producing half-maximal inhibition ($K_{1/2}$) are also similar for NADPH- and ascorbate-induced lipid peroxidation. Addition of metyrapone, an inhibitor of drug metabolism, has no effect on either the $K_{1/2}$ values or on the maximal substrate inhibition of NADPH-induced lipid peroxidation. All five drug substrates also inhibit Fe^{2+}-stimulated oxidation of linoleic acid. These results demonstrate that inhibition of lipid peroxidation in hepatic microsomes by drug substrates is independent of drug metabolism and is probably due to the antioxidant properties of the substrates.

The potential for interactions between drug metabolism and lipid peroxidation in hepatic microsomes has been recognized for many years because of the involvement of several common factors in the two processes. For example, microsomal metabolism of many xenobiotics requires NADPH as a source of electrons [1, 2], and peroxidation of microsomal lipids is also stimulated by NADPH [3–8]. The flavoprotein, NADPH-cytochrome *c* reductase, is utilized similarly both in metabolism of xenobiotics [1, 2] and in NADPH-induced lipid peroxidation [8, 9]. In addition, an essential role for microsomal phospholipids in cytochrome P-450-dependent drug metabolism has been established [1, 2].

The functional relationship between microsomal drug metabolism and lipid peroxidation has been the subject of many investigations. Increases in lipid peroxidation usually cause a decrease in the capacity for drug metabolism, at least in part by promoting the degradation of cytochrome P-450 which is required for metabolism [10–16]. In addition, various drug substrates have been shown to inhibit lipid peroxidation in hepatic microsomes [16–21]. Orrenius *et al.* [17] proposed that this inhibition is the result of a competition between drug metabolism and lipid peroxidation for reducing equivalents derived from NADPH. However, Pederson and Aust [21] studied the inhibition of both NADPH- and ascorbate-induced lipid peroxidation in hepatic microsomes by benzo[*a*]pyrene and reached a different conclusion. They proposed that the inhibition was caused by the antioxidant properties of a benzo[*a*]pyrene metabolite which is formed in the presence of NADPH. Nevertheless, the hypothesis of Pederson and Aust, like that of Orrenius *et al.*, requires that drug substrates be metabolized if inhibition of lipid peroxidation is to occur.

The objective of this investigation was to study further the relation between drug metabolism and lipid peroxidation in rat hepatic microsomes. The effects of various drug substrates on lipid peroxidation were studied in three different systems: (1) NADPH-induced (enzymatic) lipid peroxidation in normal microsomes; (2) ascorbate-induced (non-enzymatic) lipid peroxidation in microsomes in which drug-metabolizing enzymes had been heat-inactivated; and (3) ferrous iron-stimulated peroxidation of linoleic acid. The results indicate that substrate inhibition of lipid peroxidation does not require substrate metabolism. A preliminary report of these results has appeared previously [22].

MATERIALS AND METHODS

Male Sprague–Dawley rats weighing 200–250 g were obtained from Zivic–Miller Laboratories, Pittsburgh, PA. The animals were decapitated and the livers rapidly removed. The livers were then homogenized in a solution containing 0.154 M KCl and 0.05 M Tris–HCl (pH 7.4), and microsomes were obtained by differential centrifugation. In some experiments microsomal enzymes were inactivated by heating the microsomal pellet at 100° for 4 min. These microsomes are referred to as heat-treated microsomes. For all experiments the microsomes, either normal or heat-treated, were resuspended in 0.1 M phosphate buffer (0.081 M K_2HPO_4 and 0.019 M KH_2PO_4; pH 7.4) at a final concentration of 1–2 mg of microsomal protein/ml.

Oxygen (100%) was bubbled through the suspension of microsomes for 1 min prior to the start of each experiment. The incubation medium consisted of 2.5 ml of the microsomal suspension, the appropriate concentration of drug substrate, and NADPH

(0.4 mM; Type I; Sigma Chemical Co., St. Louis, MO) or ascorbate (0.4 mM) to initiate lipid peroxidation. The drug substrates used were aniline (Aldrich Chemicals, Milwaukee, WI), β-diethylaminoethyl diphenylpropylacetate (SKF-525A) (Smith, Kline & French, Philadelphia, PA), aminopyrine (Aldrich Chemicals), benzo[*a*]pyrene (Eastman Organic Chemicals, Rochester, NY) and ethylmorphine (Merck & Co., Rahway, NJ). The samples were incubated at 37° for various lengths of time. Lipid peroxidation was determined by measuring the amount of malonaldehyde formed by hepatic microsomes with the thiobarbituric acid test (TBA test) by using the method of Ottolenghi [23]. After the incubation period, the samples were placed on ice and 0.625 ml of 40% trichloroacetic acid (TCA) was added to each sample. Thiobarbituric acid (2.5 ml of a 0.67% solution) was then added and each sample was incubated at 90° for 20 min. After this incubation, the samples were centrifuged at 30,000 *g* in a Sorvall model SS-3 centrifuge (Ivan Sorvall Co., Norwalk, CT) for 5 min. The samples were then diluted with 9 vol. of water and the amount of malonaldehyde was determined by measuring the optical density of each sample at 535 nm with a Gilford model 300-N spectrophotometer (Gilford Instrument Co., Oberlin, OH) and by using a molar extinction coefficient of $1.56 \times 10^5 M^{-1} cm^{-1}$ [24]. In some experiments microsomal metabolism of aniline, benzo[*a*]pyrene or ethylmorphine was measured as described previously [25].

The effects of drug substrates on ferrous iron (Fe^{2+})-stimulated oxidation of linoleic acid were also studied. The methods used were similar to those used by Matsushita *et al.* [26] to study oxidation of linoleic acid by air. Linoleic acid (0.09 g, Grade III, 99% pure, Sigma Chemical Co.) was dissolved in 0.7 ml of 1 N NaOH and then diluted to 50 ml with 0.2 M phosphate buffer (0.162 M K_2HPO_4 and 0.038 M KH_2PO_4; pH 7.4). Oxygen (100%) was bubbled through this solution. Then 2 ml aliquots of the linoleic acid solution were incubated for 2 hr at 37° with 2.5 mM Fe^{2+} (added as $FeSO_4$) and, in some cases, appropriate concentrations of drug substrates. The amount of lipid peroxidation which occurred during this time was measured with the TBA test. After the incubation period, 0.5 ml TCA (40%) and 0.5 ml TBA (2%) were added to each sample and the samples were incubated at 90° for 20 min. Then 1 ml of glacial acetic acid and 2 ml of chloroform were added to each sample. The samples were stirred and centrifuged at 30,000 *g* for 5 min. The amount of TBA-reactive material in each sample was determined by reading the optical density of the aqueous layer at 535 nm.

It has been reported that linoleic acid does not form malonaldehyde when it is exposed to peroxidizing conditions [27]. However, a number of investigators have obtained a positive TBA test upon peroxidation of pure linoleic acid [26, 28, 29]. The identity of the product which forms the red pigment is unknown, although Asakawa and Matsushita [30] have suggested that 2, 4-alkadienal, a product of the peroxidation of linoleic acid, may be oxidized to malonaldehyde which then reacts with the TBA. In any case, the identity of the TBA-reactive substance is not important in our study. In the present study, a positive TBA test was obtained and used to study the effects of drug substrates on the Fe^{2+}-stimulated oxidation of linoleic acid.

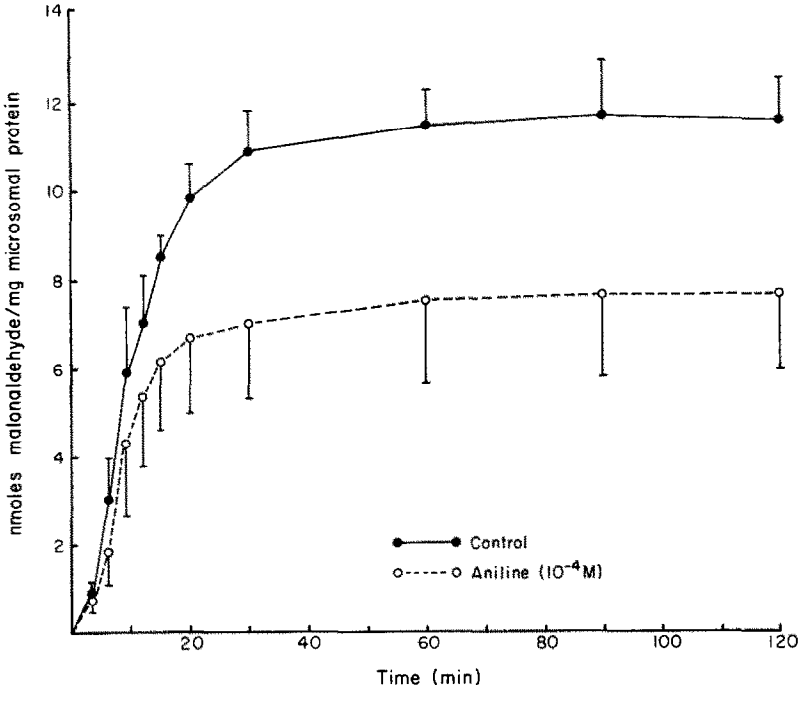

Fig. 1. Time course of NADPH-induced lipid peroxidation in normal microsomes in the presence and the absence of 10^{-4}M aniline. Each point is the mean value for five experiments and the bars are the standard errors of the means.

Table 1. Inhibition of NADPH-induced lipid peroxidation by drug substrates*

Substrate (concn)	Maximal inhibition (%)	$K_{1/2}$ (M)
Aniline (5 mM)	96 (±1)	$2.2\ (\pm0.2) \times 10^{-4}$
SKF-525A (1 mM)	95 (±1)	$7.7\ (\pm0.2) \times 10^{-5}$
Aminopyrine (10 mM)	95 (±1)	$3.5\ (\pm1.6) \times 10^{-3}$
Benzo[*a*]pyrene (0.05 mM)	95 (±1)	$6.4\ (\pm0.4) \times 10^{-6}$
Ethylmorphine (10 mM)	76 (±3)	$1.1\ (\pm0.2) \times 10^{-3}$

* The values shown represent the maximal per cent inhibition of lipid peroxidation and the $K_{1/2}$ values for each of the drug substrates. The substrate concentrations shown are those which produce maximal inhibition. Each value is the mean for four to six experiments and the numbers in parentheses are the standard errors of the means. The value for NADPH-induced lipid peroxidation in the absence of substrates is 16.2 (±0.1) nmoles malonaldehyde/mg of microsomal protein.

RESULTS

The time course of lipid peroxidation induced by NADPH in normal microsomes is shown in Fig. 1. Malonaldehyde formation increased rapidly during the first 15–20 min of incubation and reached a maximum at approximately 30 min. The amount of malonaldehyde present remained constant over the next 100 min. As other investigators have demonstrated previously, NADPH-induced lipid peroxidation is inhibited by a variety of drug substrates. The effect of an intermediate dose (10^{-4} M) of aniline on NADPH-induced lipid peroxidation is also shown in Fig. 1. After 3 min of incubation, the per cent inhibition caused by aniline (30–40 per cent) was relatively constant over the entire incubation period. Higher concentrations of aniline produced greater decreases in lipid peroxidation which are clearly independent of incubation time. Similar results were obtained with other drug substrates, i.e. the effects of the substrates on lipid peroxidation were, for the most part, independent of incubation time.

The maximal inhibition produced by five different drugs is shown in Table 1. Because of the extremely small amounts of malonaldehyde produced in the presence of high concentrations of the drugs, only the extent (90 min incubation values), and not the rate, of lipid peroxidation was evaluated. However, the degree of inhibition clearly indicates that the rates are diminished considerably. Addition of aniline, SKF-525A, benzo[*a*]pyrene or aminopyrine to microsomal preparations resulted in at least 95 per cent inhibition of lipid peroxidation, but ethylmorphine, in concentrations as high as 10 mM, decreased malonaldehyde production by only 76 per cent. The concentration of substrate which produces one-half of the maximal inhibition, $K_{1/2}$, was also calculated for each drug substrate. This was done by measuring the inhibition produced at various substrate concentrations and then constructing double-reciprocal plots. The $K_{1/2}$ values obtained from these plots are also shown in Table 1.

In order to determine substrate effects on lipid peroxidation in the absence of substrate metabolism,

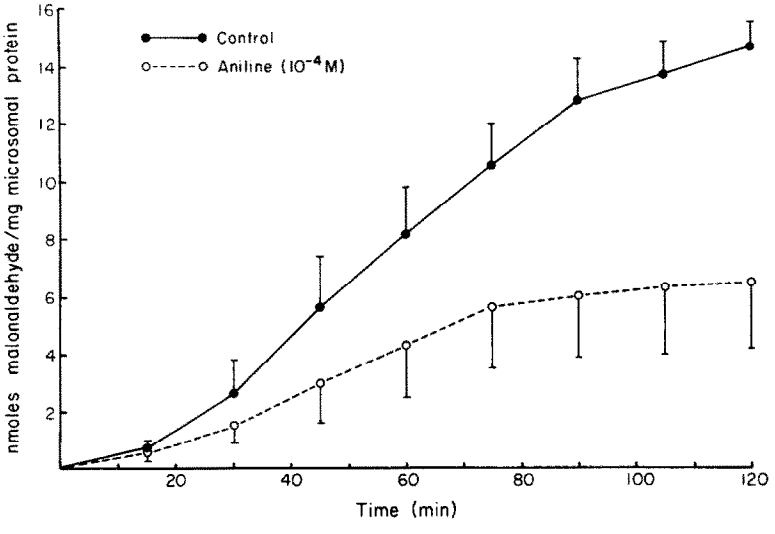

Fig. 2. Time course of ascorbate-induced lipid peroxidation in heat-treated microsomes in the presence and the absence of 10^{-4} M aniline. Each point is the mean value for five experiments and the bars are the standard errors of the means.

Table 2. Inhibition of ascorbate-induced lipid peroxidation by drug substrates in heat-treated microsomes*

Substrate (concn)	Maximal inhibition (%)	$K_{½}$(M)
Aniline (5 mM)	97 (±1)	$2.0\ (\pm0.1) \times 10^{-4}$
SKF-525A (1 mM)	97 (±1)	$7.7\ (\pm0.6) \times 10^{-5}$
Aminopyrine (10 mM)	96 (±1)	$2.1\ (\pm0.4) \times 10^{-4}$
Benzo[*a*]pyrene (0.05 mM)	95 (±1)	$10.0\ (\pm1.3) \times 10^{-6}$
Ethylmorphine (10 mM)	73 (±2)	$2.4\ (\pm0.7) \times 10^{-3}$

* The values shown represent the maximal per cent inhibition of lipid peroxidation and the $K_{½}$ values for each of the drug substrates. The substrate concentrations shown are those which produce maximal inhibition. The experiments were performed with heat-treated microsomes. Each value is the mean for five to six experiments and the numbers in parentheses are the standard errors of the means. The value for ascorbate-induced lipid peroxidation in the absence of substrates is 14.8 (±0.5) nmoles malonaldehyde/mg of microsomal protein.

lipid peroxidation was induced non-enzymatically by ascorbate in microsomal preparations which had been heat-treated (100° for 4 min) to inactivate drug-metabolizing enzymes (heat-treated microsomes). No NADPH-induced lipid peroxidation and no benzo[*a*]pyrene or ethylmorphine metabolism could be demonstrated in heat-treated microsomes. The time course of ascorbate-induced lipid peroxidation in heat-treated microsomes is shown in Fig. 2. Malonaldehyde formation in response to ascorbate occurred more slowly than NADPH-induced lipid peroxidation in normal microsomes and does not appear to reach maximal levels even after 120 min of incubation.

Drug substrates have the same effects on non-enzymatic, ascorbate-induced lipid peroxidation as on the enzymatic, NADPH-induced response. The effect of aniline on ascorbate-induced lipid peroxidation in heat-treated microsomes is shown in Fig. 2. After 15 min of incubation, the per cent inhibition caused by aniline was relatively constant over the entire incubation period. Similar results were obtained with the other substrates. For the ascorbate-induced lipid peroxidation experiments, all incubations were carried out for 90 min. The effects of drug substrates on ascorbate-induced LP are summarized in Table 2. Aniline, SKF-525A, benzo[*a*]pyrene and aminopyrine produced maximal inhibitions of at least 95 per cent. However, ethylmorphine (10 mM) produced only 73 per cent inhibition. These values are similar to those obtained when NADPH was used to induce lipid peroxidation in normal microsomes (Table 1). The $K_{½}$ values for substrate inhibition of ascorbate-induced lipid peroxidation in heat-treated microsomes are also shown in Table 2. In each case, the $K_{½}$ values for substrate inhibition of NADPH- and ascorbate-induced lipid peroxidation are similar. These results indicate that inhibition of lipid peroxidation by drug substrates is not dependent upon the presence of microsomal drug-metabolizing activity.

To further examine the relationship between drug metabolism and lipid peroxidation, the effect of metyrapone, an inhibitor of cytochrome P-450-dependent metabolism [31, 32], on substrate inhibition of NADPH-induced lipid peroxidation was evaluated. The results of experiments in which aniline was used as the substrate are shown in Fig. 3. At the concentration of metyrapone employed (5×10^{-4} M) the rate of aniline metabolism was decreased by approximately 50 per cent, but metyrapone had no effect on aniline inhibition of lipid peroxidation. The effects of metyrapone on the $K_{½}$ values for aniline and benzo[*a*]pyrene are presented in Table 3. Although substrate metabolism was inhibited 40–50 per cent by metyrapone, the values of $K_{½}$ for both aniline and benzo[*a*]pyrene inhibition of NADPH-induced lipid peroxidation are similar both in the presence and in the absence of metyrapone. Also, metyrapone has no effect on the maximal inhibition of lipid peroxidation produced by either

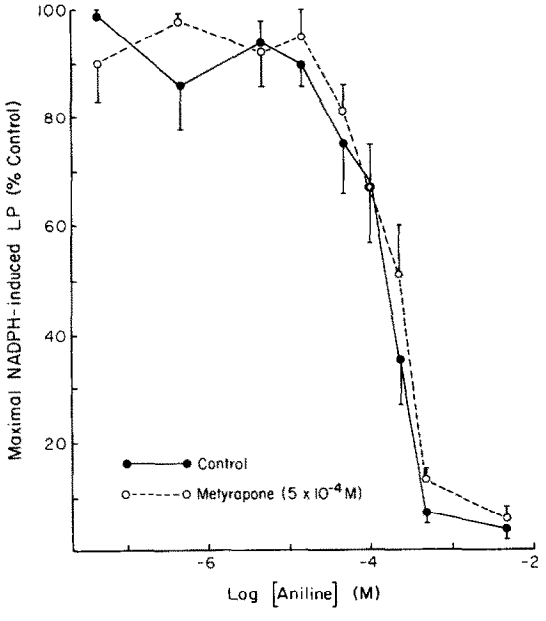

Fig. 3. Effect of metyrapone on aniline inhibition of NADPH-induced lipid peroxidation in normal microsomes. In these experiments aniline metabolism was inhibited by approximately 50 per cent in the presence of metyrapone. Each point is the mean value for three experiments and the bars are the standard errors of the means. The value for lipid peroxidation in the absence of aniline and metyrapone was 9.9(±0.8) nmoles malonaldehyde/mg of microsomal protein.

Table 3. Effects of metyrapone on aniline and benzo[*a*]pyrene inhibition of NADPH-induced lipid peroxidation*

Substrate	Maximal inhibition (%)	K_i (M)
Aniline		
control	96 (±2)†	$2.3\ (\pm0.2) \times 10^{-4}$
metyrapone	93 (±3)	$1.9\ (\pm0.4) \times 10^{-4}$
Benzo[*a*]pyrene		
control	96 (±1)‡	$1.9\ (\pm0.7) \times 10^{-6}$
metyrapone	94 (±1)	$3.0\ (\pm1.1) \times 10^{-6}$

* Each value represents the mean for three experiments and the numbers in parentheses are the standard errors of the means. In these experiments, metyrapone (5×10^{-4} M) caused inhibition of aniline metabolism by approximately 50 per cent and benzo[*a*]pyrene metabolism was inhibited by about 40 per cent.

† The value for lipid peroxidation in the absence of substrate was 9.9 (±0.5) nmoles malonaldehyde/mg of microsomal protein.

‡ The value for lipid peroxidation in the absence of substrate was 12.0 (±2.5) nmoles malonaldehyde/mg of microsomal protein.

substrate. Thus, these results also indicate that substrate inhibition of lipid peroxidation is independent of drug metabolism.

One possible explanation for these results is that antioxidant properties of the substrates are responsible for substrate inhibition of hepatic microsomal lipid peroxidation. In order to determine the ability of substrates to act as antioxidants, the effects of the drugs on Fe^{2+}-stimulated oxidation of linoleic acid were measured. The results of these experiments are shown in Table 4. All substrates inhibited lipid peroxidation in this nonbiological system. No attempt was made to find the concentration of substrate which produces maximal inhibition. It is interesting to note that greater concentrations of benzo[*a*]pyrene and SKF-525A are required to produce inhibition in the linoleic acid system than are required in the microsomal system. Nevertheless, the effects of all drugs on the nonbiological system are similar to those obtained in the microsomal system. Therefore, the results of these experiments indicate that drug substrate inhibition of lipid peroxidation in hepatic microsomes is due to the antioxidant properties of the substrate molecules.

DISCUSSION

The results of these experiments indicate that inhibition of hepatic microsomal lipid peroxidation by drug substrates is not dependent upon substrate metabolism. Three lines of evidence are presented in support of this conclusion: (1) substrate inhibition of ascorbate-induced lipid peroxidation following heat inactivation of microsomal enzyme systems is quantitatively similar to drug inhibition of NADPH-induced lipid peroxidation in normal microsomes; (2) partial inhibition of drug metabolism by metyrapone, a mixed function oxidase inhibitor, has no effect on substrate inhibition of NADPH-induced lipid peroxidation; and (3) drug substrates inhibit Fe^{2+}-stimulated oxidation of linoleic acid which indicates that the substrates are antioxidants. Thus, inhibition of lipid peroxidation by various substrates does not appear to be affected by either the level of activity of microsomal enzymes or by the mode of initiation of lipid peroxidation, i.e. NADPH, ascorbate or ferrous iron.

Our observations differ somewhat from those made previously by other investigators, but the apparent discrepancies may be attributable, at least in part, to differences in experimental technique. Orrenius *et al.* [17] found that aminopyrine almost completely inhibited NADPH-induced lipid peroxidation but had little effect on ascorbate-promoted lipid peroxidation. There are several differences in the incubation conditions employed by Orrenius *et al.* and by us. ADP-chelated Fe^{2+} was added to the incubation mixture in their experiments. In our experiments we relied on the Fe^{2+} which is present as a contaminant in the phosphate buffer. However, we found that the results were no different if we included additional Fe^{2+} or ADP-chelated Fe^{2+} in the incubation mixture, i.e. the drug substrates still inhibited both NADPH- and ascorbate-induced LP in a quantitatively similar manner (data not reported). In addition, Orrenius *et al.* used a concentration of aminopyrine which is only half of the level we determined to be necessary for maximal inhibition, and they made their measurements after only 6 min of incubation. It is possible that at lower substrate concentrations, i.e. concentrations which are less than that required for maximal inhibition, more than 6 min of incubation time is required to demonstrate inhibition of ascorbate-induced LP (see Fig. 2). In fact, Wills [18] used longer incubation times of 15 min and found, as we have reported, that aminopyrine did inhibit ascorbate-induced LP.

The results of Pederson and Aust [21] are also in apparent conflict with some of our findings. These investigators reported that benzo[*a*]pyrene inhibited NADPH-induced lipid peroxidation to a far greater extent than ascorbate-promoted lipid peroxidation. They also found that metyrapone diminished the effect of benzo[*a*]pyrene on NADPH-induced lipid peroxidation. The use of phenobarbital-treated rats by Pederson and Aust may account for some of the differences between their results and those obtained in the present study since untreated animals were used in our experiments. Nevertheless, the results

Table 4. Inhibition of Fe^{2+}-stimulated oxidation of linoleic acid by drug substrates*

Substrate (concn)	Inhibition (%)
Aniline (5 mM)	91 (±1)
SKF-525A (10 mM)	94 (±1)
Aminopyrine (10 mM)	97 (±1)
Benzo[*a*]pyrene (1 mM)	67 (±2)
Ethylmorphine (10 mM)	78 (±1)

* The values shown represent the per cent inhibition of Fe^{2+}-stimulated oxidation of linoleic acid caused by each of the drug substrates. Each value is the mean for four to five experiments and the numbers in parentheses are the standard errors of the means.

of Pederson and Aust, as well as our own, support the conclusion that drug substrates do not inhibit NADPH-induced lipid peroxidation by competing for reducing equivalents from NADPH. On the basis of their observations, Pederson and Aust concluded that a metabolite of benzo[*a*]pyrene was responsible for the inhibition. However, our data indicate that various substrates, including benzo[*a*]pyrene, inhibit lipid peroxidation in the absence of metabolism.

There is also another line of evidence which indicates that inhibition of LP by drug substrates is independent of drug metabolism. If substrate inhibition of LP involved competition for reducing equivalents from NADPH, there should be a direct correlation between the rate at which a substrate is metabolized and its ability to inhibit lipid peroxidation. However, no such correlation exists. Ethylmorphine is metabolized much more rapidly by rat liver microsomes than is benzo[*a*]pyrene [33]. Yet, benzo[*a*]pyrene is a more potent inhibitor of lipid peroxidation than is ethylmorphine. Thus, consumption of NADPH via drug metabolism does not appear to be the mechanism by which inhibition of lipid peroxidation occurs.

In addition to demonstrating that substrate inhibition of microsomal lipid peroxidation does not require drug metabolism, our results indicate that the inhibition is due to the antioxidant properties of the substrates. These antioxidant effects are probably not due to contamination of the drug preparations. We have established the purity of benzo[*a*]pyrene by using high-pressure liquid chromatography, and the compound eluted as a single peak with no apparent impurities. In addition, the aniline and aminopyrine we used were 99 and 97 per cent pure, respectively. Thus, the antioxidant effects are probably caused by the drug molecules themselves. Other investigators have reached similar conclusions. For example, Matsushita and Ibuki [29] have reported that aminopyrine is a powerful antioxidant. Also Jansson [34] has proposed that inhibition of lipid peroxidation by drug substrates may be due to their antioxidant effects, but antioxidant properties were not directly measured in that study. Our data, however, clearly demonstrate that the drug substrates used in the present study are antioxidants, and it is this property of the substrates which is probably responsible for the inhibition of hepatic microsomal lipid peroxidation.

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