Stimulation of microsomal lipid peroxidation by iron and cysteine

Characterization and the role of free radicals

Andrew J. F. SEARLE and Robin L. WILLSON

Department of Biochemistry, Brunel University, Uxbridge, Middlesex UB8 3PH, U.K.

(Received 24 November 1982/Accepted 1 March 1983)

The stimulation of microsomal lipid peroxidation by FeSO_4 and cysteine has been investigated. Although both FeSO_4 and cysteine alone promoted an increase in malonaldehyde production, when these agents were added together to microsomes the resultant level of malonaldehyde was greater than the sum of the amounts formed by these pro-oxidants when acting individually. A further indication of an interaction between FeSO_4 and cysteine was shown by the inhibitory action of chelating agents. Stimulation of peroxidation was shown to be independent of microsomal protein, including cytochrome *P*-450. The system has been characterized for the effects of cysteine, Fe^{2+} and O_2 concentrations, pH, temperature and antioxidants. The results indicate that the high level of peroxidation attained with this system, its non-enzymic character and the involvement of hydroxyl radicals make it particularly useful for the investigation of the action of antioxidants. Furthermore it may also be a model of the way in which decompartmentalized, delocalized or 'free' iron initiates peroxidation *in vivo*.

Peroxidative decomposition of cellular lipids has been associated with functional disturbances in various subcellular structures (Slater, 1972; Tappel, 1973). In the case of the liver, the microsomal fraction has been shown to be particularly sensitive to peroxidation and as such has been used extensively to investigate the antioxidant activity of many naturally occurring and synthetic compounds (Wills, 1969; Slater, 1978, 1979).

It has long been known that iron, incorporated in enzymes or acting non-enzymatically, can catalyse peroxidation and that this can be further stimulated by the additional presence of ascorbic acid (Wills, 1969; Willson, 1977, 1979). We have briefly reported that the addition of cysteine rather than ascorbate can also stimulate microsomal peroxidation and this has since been confirmed (Malvy *et al.*, 1980; Tien *et al.*, 1982). In the present paper a more detailed characterization of the peroxidizing system and its potential usefulness in assessing antioxidant activity is described.

Materials and methods

Liver microsomes prepared from male Wistar rats (Slater & Sawyer, 1971) were stored as pellets at -20°C until required. Storage of the pellets had no discernible effect on levels of peroxidation. For each

experiment they were suspended in 0.033 M-Tris buffer (pH 7.4), containing 0.1 M-KCl, to give a stock suspension at a final concentration of 15 mg of protein/ml. Protein content was determined by the method of Lowry *et al.* (1951).

Peroxidation of 1.5 ml of a 1 mg/ml microsomal suspension in Tris/KCl was stimulated by the addition of portions of freshly prepared stock solutions of cysteine (5 mM) and FeSO_4 (50 μM) in double-distilled water. These gave final concentrations of 500 μM and 5 μM respectively. When inhibitors were investigated, they were added to the microsomal suspension before the addition of cysteine and FeSO_4 . Samples were incubated at 37°C and the peroxidation was stopped after 30 min by the addition of 3 ml of ice-cold 10% (w/v) trichloroacetic acid. After centrifugation, triplicate 2 ml samples of the supernatant fluid were removed and added to 2 ml of 0.67% (w/v) thiobarbituric acid in distilled water. The samples were then incubated for 15 min in a boiling-water bath, cooled and the absorbance measured at 535 nm in a 1 cm cuvette. Where different experimental procedures were necessary, for example, when the effects of scavengers were studied, these are described in the appropriate Figure or Table legend. Each point in the Figure represents the mean of three measurements and the error bars give their range.

Desferrioxamine mesylate (Desferal) was kindly donated by CIBA-Geigy. 6-Hydroxy-1,4-dimethyl-carbazole and 9-hydroxyellipticine were gifts from Professor C. Paoletti and colleagues of the Institut Gustav Roussy, Villejuif, France.

Cysteine, adrenaline, dihydroxyphenylalanine, mannitol, thiourea, superoxide dismutase, catalase, β -carotene, diphenylfuran, propyl gallate, ADP, NADPH, diethylenetriaminepenta-acetic acid (DTPA) and sodium maleate were obtained from Sigma (London) Chemical Co. Diazabicyclo-octane was purchased from Aldrich Chemical Co. Ethylenediamine-*NN'*-bis-(*o*-hydroxyphenylacetic acid) (EHPG) was supplied by Fluorochem. *t*-Butyl alcohol was obtained from Koch-Light. All other chemicals were supplied by BDH and were of either AnalaR, or the best available, grade. All solutions were made up in either double-glass-distilled or Millipore-filtered water.

Results

Effect of incubation time, pH, Fe²⁺ and cysteine concentration

Portions of stock cysteine and FeSO₄ solutions were added to microsomal suspensions and the reaction was stopped at various times. The yield of malonaldehyde was found to increase steadily up to 30 min but then became constant (Fig. 1).

The yields measured after 30 min incubation in the presence and absence of iron and cysteine are given in the legend to Fig. 1. In the absence of iron or cysteine very little malonaldehyde was produced. The presence of either iron or cysteine increased the yield but the most marked effect was seen when the incubations took place with both present. Indeed the amount of malonaldehyde produced clearly exceeded that which would be expected if the peroxidation brought about by Fe²⁺ and cysteine were simply additive.

A concentration of at least 10 μ M-Fe²⁺ was needed to give the highest level of peroxidation, but at about 30 μ M there was a sharp decrease in malonaldehyde production. However, when incubation mixtures containing different iron concentrations were saturated with O₂, higher malonaldehyde yields were found at all Fe²⁺ concentrations used (Fig. 2).

The level of peroxidation increased steadily with increasing cysteine concentration up to a concentration of 0.8 mM but then remained constant (results not shown). Although optimum results were obtained with 10 μ M-FeSO₄ and 0.8 mM-cysteine, subsequent studies were undertaken using 5 μ M-FeSO₄ and 0.5 mM-cysteine in order to provide data consistent with that published previously (Malvy *et al.*, 1980).

Altering the pH of the incubating medium had a pronounced effect on malonaldehyde production; the

Fig. 1. *Effect of incubation time on malonaldehyde production*

Microsomes (1 mg of protein/ml) were incubated at 37°C in 0.033 M-Tris buffer, pH 7.4 (containing 0.1 M-KCl) containing final concentrations of 500 μ M-cysteine and 5 μ M-FeSO₄. In the absence of iron or cysteine only 2 nmol of malonaldehyde was produced per mg of protein. With iron alone and with cysteine alone about 7 and 5 nmol of malonaldehyde/mg of protein respectively were produced. However, with both iron and cysteine present about 33 nmol of malonaldehyde/mg of protein was measured.

yield-pH curve showed two maxima at pH 5 and 7 (Fig. 3). When Tris/maleate buffers were used a slightly reduced level of malonaldehyde production was observed.

Effect of temperature

An approximately linear increase in malonaldehyde concentration with increasing temperature was observed up to 50°C, above which the amount of malonaldehyde declined (Fig. 4). Above 50°C, the microsome suspensions were seen to coagulate and settle out. However, when microsomes were boiled for 15 min, cooled, homogenized and resuspended to the same volume, and then incubated with Fe²⁺-cysteine, they continued to produce amounts of malonaldehyde corresponding to approx. 75% of normal values.

Effect of cytochrome P-450 inhibitors and surfactants

Two cytochrome P-450 inhibitors, CO and metyrapone, at concentrations known to inactivate the enzyme, had little or no effect on peroxidation,

Fig. 2. Effect of FeSO_4 concentration on malonaldehyde production

Microsomes (1 mg of protein/ml) were incubated for 30 min at 37°C in Tris/KCl buffer (see Fig. 1) containing final concentrations of $500\text{ }\mu\text{M}$ -cysteine and FeSO_4 as indicated. ●, Air-saturated and stopped; O, gassed with O_2 throughout the incubation.

whereas SKF 525A ($500\text{ }\mu\text{M}$), which also possesses surfactant activity, almost completely abolished any malonaldehyde formation. The surfactant sodium dodecyl sulphate at a concentration of 10 mM also markedly inhibited peroxidation, although at lower concentrations ($100\text{ }\mu\text{M}$ and 1 mM) an increase occurred.

Effect of oxygen radical scavengers

When propan-2-ol, *t*-butyl alcohol, sodium formate, thiourea, mannitol and benzoate were included (1 mM) in peroxidizing suspensions very little reduction in the yield of malonaldehyde was observed (Table 1). At a concentration of 50 mM , thiourea and sodium benzoate reduced the yield by approximately one-half and an even greater reduction was observed when lower amounts of iron ($1\text{ }\mu\text{M}$) and cysteine ($250\text{ }\mu\text{M}$) were used to stimulate peroxidation. This increase in effectiveness at lower iron and

Fig. 3. Effect of buffer pH on malonaldehyde production. Microsomes (1 mg of protein/ml) were incubated for 30 min at 37°C in 0.1 M -Tris buffer (■) or 0.1 M -Tris/maleate buffer (○) containing final concentrations of $500\text{ }\mu\text{M}$ -cysteine and $5\text{ }\mu\text{M}$ - FeSO_4 .

Fig. 4. Effect of incubation temperature on malonaldehyde production

Microsomes (1 mg of protein/ml) in Tris/KCl buffer (see Fig. 1) containing final concentrations of $500\text{ }\mu\text{M}$ -cysteine and $5\text{ }\mu\text{M}$ - FeSO_4 were incubated for 30 min at the temperatures indicated.

cysteine concentrations was not observed with mannitol. Diazabicyclo-octane, diphenylfuran and β -carotene (all at 10 mM) were more effective at inhibiting malonaldehyde production. Indeed the true activity of diphenylfuran and β -carotene may not have been realised owing to their incomplete dissolution.

Table 1. *Effect of singlet oxygen and hydroxyl radical scavengers on yields of malonaldehyde produced from peroxidizing microsomes*

Microsomes (1 mg of protein/ml) were incubated for 30 min at 37°C in Tris/KCl buffer (see Fig. 1). The 100% value corresponds to 33 ± 1 nmol of malonaldehyde/mg of protein.

Additive present during incubation	Additive concn. (mM) ...	Malonaldehyde yield (% of control)			
		1	10	50	50*
Diazabicyclo-octane		93	69±		
Diphenylfuran†		93	84±		
β-Carotene†		93	87±		
Mannitol		100	97	99	99
Propan-2-ol		94	92	94	
t-Butyl alcohol		98	98	92	
Sodium formate		96	95	91	
Thiourea		93	76	51±	39±
Sodium benzoate				50±	20±

* 1 μM-FeSO₄/250 μM-cysteine rather than the normal 5 μM-FeSO₄/500 μM-cysteine respectively.

† Added as a suspension.

‡ Significantly different from control at $P < 0.01$.

Table 2. *The effect of antioxidants and chelating agents on malonaldehyde production*

Microsomes (1 mg of protein/ml) were incubated for 30 min at 37°C in Tris/KCl (see Fig. 1) containing final concentrations of 500 μM-cysteine and 5 μM-FeSO₄. *n* is the number of estimates of the IC₅₀. Each value was based on the triplicate assay of at least seven concentrations of a compound. Where more than one estimate of the IC₅₀ was obtained the range is given.

Compound	IC ₅₀ (μM)	<i>n</i>
EDTA	26 ± 2	2
DTPA	8	1
EHPG	3	1
Desferal	8	1
Propyl gallate	3 ± 1	3
Butylated hydroxytoluene	7	1
Butylated hydroxyanisole	5	1
Dihydroxyphenylalanine	82	1
Adrenaline	50	1
Promethazine	11 ± 2	3
Chlorpromazine	91	1
Metiazinic acid	160	1
9-Hydroxyellipticine	9	1
6-Hydroxy-1,4-dimethyl-carbazole	0.4 ± 0.1	5

Superoxide dismutase and catalase added to the microsomes before stimulation showed no significant inhibition; and when NaN₃ was added at a concentration sufficient to inhibit any contaminating catalase no effect was observed.

Effect of chelating agents

The chelating agents EDTA, DTPA, EHPG and Desferal all inhibited peroxidation to some extent, as

reflected in the corresponding values for IC₅₀ values (i.e. the concentration of compound needed to be added to the incubation mixture to inhibit malonaldehyde production by 50%) obtained from concentration-inhibition plots (Table 2).

Effect of antioxidants

The lipid-soluble phenolic compounds propyl gallate, butylated hydroxytoluene, butylated hydroxyanisole, 9-hydroxyellipticine and 6-hydroxy-1,4-dimethylcarbazole inhibited peroxidation strongly (Table 2). The water-soluble catecholamines adrenaline and dihydroxyphenylalanine (Dopa) were an order of magnitude less effective. The inhibitory activity of the phenothiazines promethazine, chlorpromazine and metiazinic acid varied considerably.

Discussion

The present results clearly show that the level of peroxidation that occurs when liver microsomes are incubated with iron and cysteine depends on (a) having both agents together and (b) their concentration. The inhibition of peroxidation by high concentrations of iron has been previously observed in systems stimulated by NADPH and iron (Kornbrust & Mavis, 1980). However, other studies with this system failed to show such an effect (Wills, 1969). The fact that present results indicate that O₂ limitation was at least partly responsible for the diminished peroxidation at high levels of iron may be relevant.

Clearly, the interaction of iron and cysteine is critical for effective peroxidation. DTPA, EHPG and Desferal have a high affinity for iron in

agreement with their good inhibitory action. The relatively poor inhibitory activity of EDTA may be because it does not prevent the redox cycling of iron and the concomitant production of superoxide and hydroxyl radicals still occurs (Halliwell, 1978; May *et al.*, 1978; Gutteridge *et al.*, 1979; Cohen & Sinet, 1982).

The finding that heat-denatured microsomes are still capable of being peroxidized by Fe^{2+} –cysteine and that at high temperatures the microsomes coagulate supports the idea that the lowering of peroxidation at incubation temperatures $>50^\circ\text{C}$ is more likely to be due to a physical change of the vesicles rather than a loss of enzyme activity, although the possibility that an increased rate of O_2 consumption by the Fe^{2+} –cysteine system resulting in insufficient O_2 being available cannot be ruled out. The possibility that cytochrome *P*-450 is involved is ruled out by the finding that peroxidation is not inhibited by pretreatment with CO or by metyrapone. Although SKF 525A is an effective inhibitor of cytochrome *P*-450 and this compound strongly inhibited peroxidation, this may be due to its reported antioxidant ability (Miles *et al.*, 1980) or surfactant action (Lee *et al.*, 1968). In the latter instance the phospholipid arrangement of the microsomal vesicles may be disrupted in such a way as to prevent access to either the Fe^{2+} or cysteine components. The experiments with sodium dodecyl sulphate suggest that the disorganization caused by high concentrations of the surfactant is sufficient to prevent oxidative attack. The stimulatory effects of the lower concentrations of the detergent could be the result of the microsomes being solubilized or dispersed into smaller vesicles, which present an increased surface area for peroxidative attack (Wills, 1969).

Regarding the mechanism of stimulation, *esr.* studies have shown unequivocally that solutions containing FeSO_4 , cysteine and O_2 can produce hydroxyl radicals and that these can react with other compounds present (Searle & Tomasi, 1982). Furthermore hydroxyl radicals can be trapped even in the presence of a high concentration of Tris buffer (25 mM), which is a good scavenger of hydroxyl radicals. Thus the involvement of hydroxyl radicals in the stimulatory action cannot be ruled out. Microsomal suspensions contain many components, including Tris buffer, which can react rapidly with $\text{OH}^\bullet$ and only a small proportion of the hydroxyl radicals generated are likely to react directly with peroxidizable lipid. The fact that protection by hydroxyl-radical scavengers does not occur to any great extent, except with benzoate or thiourea at high concentrations and when low amounts of iron and cysteine are present, is therefore not surprising. A mechanism for the production of superoxide and hydroxyl radicals from Fe^{2+} –

cysteine– O_2 complexes through the intermediate formation of Fe^{2+} –cysteine–superoxide and Fe^{2+} –cysteine– H_2O_2 complexes has been outlined previously (Willson & Searle, 1975; Willson, 1977, 1979, 1982) and the presence of superoxide radicals confirmed by using Nitroblue Tetrazolium and superoxide dismutase (Cheeseman *et al.*, 1981). The rate of formation of hydroxyl radical production by such a mechanism will vary immensely depending not only on the concentrations of Fe^{2+} , cysteine and O_2 but also on pH. The acid dissociation constants of the reactions:

and

(where RSH is cysteine) are 4.8 and 8.35 respectively (Jocelyn, 1972; Bielski & Allen, 1977). At pH 4.8 the dismutation of superoxide to H_2O_2 is particularly rapid ($k = 8.9 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$; Bielski & Allen, 1977) and this could explain why one peak of peroxidation occurs at a pH value close to 5.0. At high pH the reaction of OH^- with Fe^{3+} becomes increasingly important and it is perhaps not surprising that the pH profile for malonaldehyde production is complex.

The inhibition observed with propyl gallate, butylated hydroxyanisole and butylated hydroxytoluene is consistent with their high absolute rates of reaction with peroxy radicals. The particularly high inhibitory activity of 6-hydroxy-1,4-dimethylcarbazole compared with promethazine, whose rate of reaction with the organic trichloromethylperoxy radical ($\text{CCl}_3\text{O}_2^\bullet$) is similar ($k_{\text{CCl}_3\text{O}_2^\bullet + \text{HDC}} = 8.3 \times 10^8 \text{ M}^{-1} \cdot \text{s}^{-1}$ where HDC represents 6-hydroxy-1,4-dimethylcarbazole, and $k_{\text{CCl}_3\text{O}_2^\bullet + \text{PMZ}} = 4.5 \times 10^8 \text{ M}^{-1} \cdot \text{s}^{-1}$, where PMZ represents promethazine, Packer *et al.*, 1980), may be due to a more favourable access to sites vulnerable to oxidation. Problems of accessibility may also be responsible for the lack of any protective effect by superoxide dismutase or catalase, although the absence of any inhibition by the latter enzyme may be due to the effect of cysteine on its activity (Seabra & Deutsch, 1955).

It has been suggested that the superoxide anion is involved in maintaining iron in the reduced state (Van Hemmen & Meuling, 1977; Halliwell, 1978). In the present system cysteine itself can serve this purpose. Therefore any changes in the rate of dismutation of superoxide to form H_2O_2 do not necessarily lead to lowering of the rate of hydroxyl radical production, indeed they might even lead to an increase.

The fact that the compounds diphenylfuran, diazabicyclo-octane and β -carotene show some inhibition of malonaldehyde production and that these are known singlet-oxygen scavengers does not necessarily imply that this species is involved.

Pulse-radiolysis experiments have shown that these compounds too can react with organic peroxy radicals (Packer *et al.*, 1981). Thus the nature of the actual initiating species has not been established. Evidence from these and related studies (Searle & Tomasi, 1982; Rowley & Halliwell, 1982) does not rule out the possibility that hydroxyl radicals are involved in increasing the extent of initiation.

Fe^{2+} , however, can also stimulate peroxidation by catalysing the decomposition of hydroperoxides to reactivate alkoxy radicals, which can also act as propagating radicals. Thus the Fe^{2+} -cysteine complex may stimulate peroxidation in a similar manner, the resulting ferric complex being reduced by cysteine. It has been suggested that the ferryl or perferryl species may be involved in iron-stimulated lipid peroxidation (Tien *et al.*, 1982). The present work throws no further light on this proposal.

Whatever the mechanism, this study has clearly demonstrated the ease with which the Fe^{2+} -cysteine system can induce a high level of lipid peroxidation in rat liver microsomes. The system not only provides a useful non-enzymic means of studying the effectiveness of inhibitors and antioxidants against radical-mediated damage but may also serve as a model for the study of the manner by which decompartmentalized, delocalized or 'free' iron may initiate peroxidation *in vivo*.

We are grateful to the Cancer Research Campaign for financial support.

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