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Human Cytochrome P450's are Pro-oxidants in Iron/Ascorbate-initiated Microsomal Lipid Peroxidation

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We have examined the effect of human cytochrome P_{450}'s (1A1, 1A2, 3A4, 2A6, 2B6, 2D6, 2E1) on ascorbate/iron-induced lipid peroxidation. Using microsomes prepared from human lymphoblastic cells enriched in recombinant cytochrome P_{450} isoenzymes, we have shown that the degree of peroxidation is a function of the amount of P_{450} present rather than the presence of any specific isoenzyme. Incorporated P_{450} increased the amount of peroxidation products by up to 2.1-fold compared to the control microsomes with no P_{450}. It is therefore concluded that cytochrome P_{450}'s play a significant role in ascorbate/iron peroxidation.

Key words: Lipid peroxidation, microsomes, cytochrome P_{450}, cultured cells

INTRODUCTION

Many factors such as cigarette smoke, pollutants, drugs and polycyclic hydrocarbons are known to induce cytochrome P_{450}, and this may result in an increased rate of activation of pre-carcinogens to carcinogenic intermediates.[1] Furthermore, induction of P_{450} may lead to an increase in the potential for cellular free radical-mediated damage. For example, rat livers containing elevated levels of P_{450} after feeding of phenobarbital showed far greater susceptibility to microsomal lipid peroxidation when compared to control microsomes from untreated rats.[2]

An understanding of the full role of cytochrome P_{450} in microsomal lipid peroxidation is complicated by the fact that cytochrome P_{450}'s may function in several ways: (i) Generation of metabolic intermediates of xenobiotics (eg CCl_4) capable of initiating peroxidation; (ii) Reduction of redox active compounds with generation of oxygen radicals capable of initiating peroxidation; (iii) Peroxidase activity using lipid hydroperoxides as substrates, which is NADPH-independent and results in the formation of peroxyl, alkoxyl and hydroxyl radicals; (iv) A source of oxygen radicals, mainly $O_2^{\bullet -}$, which in the presence of non-haem iron can initiate peroxidation; (v) As an antioxidant by facilitating the reduction of lipid peroxides (all reviewed in [3]).

Microsomal lipid peroxidation can be induced by iron/ascorbate,[4] NADPH,[4] hydroperoxides[5] or nitrofurantoin,[5] and cytochrome P_{450} may have

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different roles in peroxidation depending on the method of initiation. However, the role of P_{450} is most frequently studied in relation to NADPH-induced microsomal lipid peroxidation. This form of peroxidation is also dependent upon the presence of ADP-iron and is believed to function directly via the cytochrome P_{450} reductase-cytochrome P_{450} electron transport chain,[6,7] and hence is frequently referred to as 'enzymic' microsomal lipid peroxidation. Studies where different purified isozymes have been incorporated into liposomes containing reductase indicate that different isozymes of P_{450} have specific influences on microsomal lipid peroxidation.[8,9]

To investigate further the role of P_{450} in microsomal lipid peroxidation, we have used microsomes from a panel of human B lymphoblastoid cells, engineered to express individual cDNAs for specific human cytochrome P_{450}'s (reviewed in[10]). These cells and extracts derived from them were originally produced for toxicological testing purposes, and have been used extensively to this end.[11] The microsomes contain levels of P_{450} reductase and cytochrome b5 which compare favourably to those found in human liver cells and are generally higher than those used for peroxidation studies involving reconstitution of liposomes. The microsomes are commercially available and in this paper we describe in detail the properties of the microsomes, and carry out extensive characterisation of their peroxidisability. One advantage for this study is that the content of P_{450} varies for each isoenzyme, from between 10 pmol/mg protein to 160 pmol/mg protein. Furthermore, these levels are similar to the amount of *individual* P_{450} isozymes present in human liver, which is approx 10–50 pmol/mg protein, although the total amount of cytochrome P_{450} in human liver is 750 pmol/mg protein (see Table 2). The cell microsomes support iron/ascorbate-induced lipid peroxidation and we have used them to demonstrate the role of cytochrome P_{450} in this form of peroxidation. Specifically we show that (a) the degree of peroxidation is increased by the presence of P_{450}, and (b) that this increase is proportional to the total amount of P_{450} and is not dependent on the presence of individual or specific isoenzymes.

MATERIALS AND METHODS

Materials

B-lymphoblastoid cell microsomes containing defined amounts of individual P_{450}'s were obtained from Gentest Inc., 6, Henshaw St., Woburn, MA 01801, USA. Microsomes were obtained in 1 ml aliquots, suspended in 100 mM potassium phosphate buffer pH 7.0 at a protein concentration of 10 mg/ml. P_{450} activities of the specific microsomes were provided by data from Gentest and some were checked by ourselves (see below). Microsomes arrived in dry-ice and were stored subsequently at –70°C. Aliquots of non-diseased human liver were kindly supplied by the Transplant Unit of the Addenbrookes Hospital, Cambridge U.K. The liver was stored at –40°C.

ADP, NADPH and all biochemicals required for the various enzyme assays were obtained from Sigma Chemical Company, Poole, U.K. All other chemicals were of Analar grade and were obtained from BDH, Poole, U.K.

Methods

Preparation of Human Liver Microsomes

Liver microsomes were isolated according to the method of Lambert.[12] The buffer used throughout was 20 mM Bis Tris Propane pH 7.0, containing 250 mM sucrose and 150 mM NaCl. Microsomes were freed of contaminating cytosolic proteins by washing with 150 mM Tris pH 8.0,[12] before being aliquoted and stored at –70°C in 250 mM sucrose at a protein concentration of 10–20 mg/ml. Microsomes were used within two weeks of preparation.

Washing of Cell Microsomes

The cell microsomes, as obtained, were washed to remove phosphate ions and cytosolic components

which would interfere with subsequent assays. Aliquots of cell microsomes (0.5 ml) were suspended in 20 mM Bis Tris Propane buffer (10 ml) pH 7.0 containing 150 mM NaCl. By trial and error, we found that the best washing procedure involved centrifugation at 50,000 g for 10 min. The resulting pellet was suspended in a total volume of 0.5 ml of the same buffer and used within 8 hours of preparation.

Protein and Enzyme Assays

Cytochrome P_{450} 1A1 was assayed fluorimetrically as 7-ethoxyresorufin-O-deethylase activity.[13] P_{450} 3A4 activity was monitored by hplc using the testosterone B-hydroxylase assay.[14] Lactate dehydrogenase (LDH), glutamate dehydrogenase (GDH), NADPH cytochrome P_{450} reductase, acid phosphatase and nucleoside diphosphatase (NDPase) were assayed as described by Lambert and Freedman.[15] For the NDPase assays, the washed cell microsomes were dialysed against assay buffer to remove all traces of phosphate from the membranes which would have interfered with the assay. Protein concentrations were determined by the bicinchoninic acid method,[16] using bovine serum albumin as a standard protein.

Lipid composition

Estimations of saturated and unsaturated lipid were kindly performed by Prof. R. J. Hamilton and colleagues of the John Moores University, Liverpool. Microsome samples were derivatized and analyzed by gas-liquid chromatography on a BD1 capillary column from J and W Inc., Folsom, CA, USA.

Lipid Peroxidation of Microsomes

Prior to analysis, liver microsomes were desalted on a PD-10 column (Pharmacia, U.K.) equilibrated in 20 mM Bis Tris Propane buffer pH 7.0 containing 150 mM NaCl, to remove sucrose which interferes with the TBARS assay.[17] The cell microsomes were washed as described above.

Of several assay buffers tested, Bis Tris Propane proved the most satisfactory. We found that phosphate significantly inhibited both peroxidising systems with liver microsomes thus supporting the need for its removal from the cell microsomes. At a concentration of 50 mM, phosphate buffer completely inhibited lipid peroxidation, although such concentrations and higher have been used by other authors.[18-20]

For a typical peroxidation assay, microsomes (0.4 mg protein) were suspended in 20 mM Bis Tris Propane buffer pH 7.0 containing 150 mM NaCl, 0.8 mM ADP and 0.5 mM $FeCl_3$ (freshly prepared in water). Peroxidation was initiated with either ascorbate (final concentration 0.05 mM) or NADPH (final concentration 0.4 mM). The total volume was 0.4 ml. Variations in reactant concentrations and further additions are as detailed in the figures and tables. Samples were incubated at 37°C for 40 min, and the reaction terminated by the addition of 0.8 ml of 20% (w/v) trichloroacetic acid/0.4% thiobarbituric acid/0.25 M HCl and 0.01 ml of butylated hydroxytoluene (5% w/v) in ethanol. The production of malondialdehyde was measured by the reaction with thiobarbituric acid as described by Buege and Aust.[17]

Treatment of 2D6 microsomes with carbon monoxide

Control and 2D6 cell microsomes (0.5 ml) were washed as described above before being diluted to 1 mg protein/ml 150 mM NaCl. Carbon monoxide was gently bubbled through aliquots (0.5 ml) of both control and 2D6 microsomes for 15 min at room temperature. Treated and untreated microsomes were then assayed for susceptibility towards lipid peroxidation as detailed above.

RESULTS

Characterisation of the cell microsomes

Isolation of the microsome fraction from the engineered human lymphoblastoid cells has been well

TABLE 1 Characterisation of the various microsomal preparations

Characteristic	Human liver microsomes[a]	Cell microsomes (washed)[b]	Recovery (%)[c]
LDH[d]	90–101	49–75	3–9
Reductase[e]	78–96	33–44	74–83
P_{450} 1A1[f]	n/d	105	76
P_{450} 3A4[f]	n/d	510	75
Acid Phosphatase[g]	25–32	20–33	n/d
GDH[h]	0–1	0–1	n/d
NDPase (+)[i]	992–1340	640–1350	n/d
NDPase (–)	174–249	320–710	n/d
Protein (mg/ml)	n/d	6.4–7.1	64–71

Assays were performed as described in the Methods section.
a) values represent the range from three separate experiments.
b) values represent the range obtained across all microsome preparations, (total = 8) except for the P_{450} data which are from individual experiments.
c) Percentage recovered relative to untreated cell microsome preparations. The values for the cell microsomes are shown after washing, and the recovery refers to the yield after washing (the data for unwashed microsomes are not shown).
d) μmoles pyruvate reduced/min/mg protein.
e) μmoles cytochrome c reduced/min/mg protein.
f) pmoles substrate/min/mg protein.
g) μmoles p-nitrophenol phosphate hydrolysed per min per mg protein.
h) μmoles of a-ketoglutarate reduced per min per mg protein.
i) μmoles of inorganic phosphate liberated per min per mg protein in the presence (+) or absence (–) of Triton X-100.
n/d = not determined

documented.[21] Similarly the microsomes have been thoroughly characterised regarding activities of enzymes associated with xenobiotic metabolism.[10] Although peroxidation of the cell microsomes has been reported before,[22] no detailed characterisation of this novel system was undertaken. To redress this lack of information, a basic biochemical characterisation of the cell microsomes was performed.

Washing the microsomes removed over 90% of the cytosolic contamination from the cell microsomes as evidenced by LDH content (Table 1). Both the washed cell microsomes and the liver microsomes were essentially free from mitochondrial contamination, as determined by GDH content. Lysosomal contamination of the cell microsomes, as judged by acid phosphatase activity, was similar to that found in liver microsomes.

Microsomal integrity was assessed by determining the levels of NDPase[23] in the presence and absence of detergent (Triton X-100). This enzyme is located in the microsomal lumen and in fully intact vesicles no activity can be measured due to substrate impermeability. Total activity can only be measured after membrane disruption (e.g. by detergent treatment). NDPase activity indicated that the microsomes from human liver were about 80% intact, as expected.[23] The cell microsomes were 50% intact, which probably reflects the harsher methods needed to prepare microsomes from cells compared to liver.[12] The amount of cytochrome P_{450} reductase in the cell microsomes was about half that found in human liver microsomes. Data in Table 2 shows that the content of **specific** isozymes in the cell microsomes was similar to the levels of each specific isoenzymes found in human liver. However, since the cells only contain one form of P_{450}, and human liver contains many, the total P_{450} levels in the cell microsomes were substantially lower than those found in liver microsomes.

Lipid Composition

To confirm that any difference in lipid peroxidisability between microsomes from various

TABLE 2 Lipid peroxidation of the various microsomal preparations

Microsomes	P_{450} content (μmoles/mg)	Iron/ascorbate peroxidation (nmoles MDA/mg protein)	NADPH/iron peroxidation (nmoles MDA/mg protein)
Human liver	750 ± 100	32.5	29.0
Control cells	1	17.8	0.7
1A1 cells	25	23.0	0.8
1A2 cells	40	27.1	1.1
3A4 cells	30	24.0	0.9
2A6 cells	60	28.6	1.1
2B6 cells	55	30.3	1.0
2D6 cells	160	37.6	1.1
2E1 cells	10	21.3	1.5

P_{450} contents of cell microsomes were provided by Gentest Corp. Data for human liver microsomes represents typical levels of P_{450}[30].

Microsomes (0.4 mg protein) were subjected to enzymic and non-enzymic lipid peroxidation as described in the methods section. Cell microsomes were 'washed' prior to analysis. Lipid peroxidation values represent the mean of at least five separate experiments. The precision of the assay was calculated to have a S.E.M. of 8%.

sources was not due to large variations in lipid composition, analysis was performed on three samples. The results showed that control microsomes, microsomes from cells containing 2D6 and human liver microsomes had very similar proportions of unsaturated fatty acids (Table 3).

Effect of iron concentration on lipid peroxidation

Figure 1 shows the influence of Fe(III) concentration upon ascorbate-induced lipid peroxidation. Maximal peroxidation of liver microsomes could be obtained with as little as 0.05 mM iron; this was also the case for NADPH-induced peroxidation. With the cell microsomes, however, a much larger amount (10-fold) was needed for maximal iron/ascorbate-dependent peroxidation. It may be, therefore, that the cell microsomes contain some component(s) capable of sequestering iron. As the microsomes were centrifuged and resuspended prior to analysis, any iron-binding component must be membrane associated.

Effect of ascorbate on lipid peroxidation

Ascorbate is known to function as both a pro- and antioxidant.[24,25] Our data (Figure 2) with both liver and cell microsomes showed that maximal peroxidation was achieved with about 0.1 mM ascorbate and that higher concentrations diminished the degree of peroxidation. The range of ascorbate concentrations used by other workers to stimulate lipid peroxidation is variable (0.5–1.0 mM).[26–28]

TABLE 3 Lipid analysis of cell microsomes and human liver microsomes

Microsomes	Saturated (X: 0)	Monounsaturated (X:1)	Polyunsaturated (X:n+1)
Control cells	40	14	27
2D6 cells	45	8	30
Human liver	40	11	30

Samples were analysed by gas chromatography as detailed in the Methods section. The values are expressed as percentages of the total area of chromatograms.

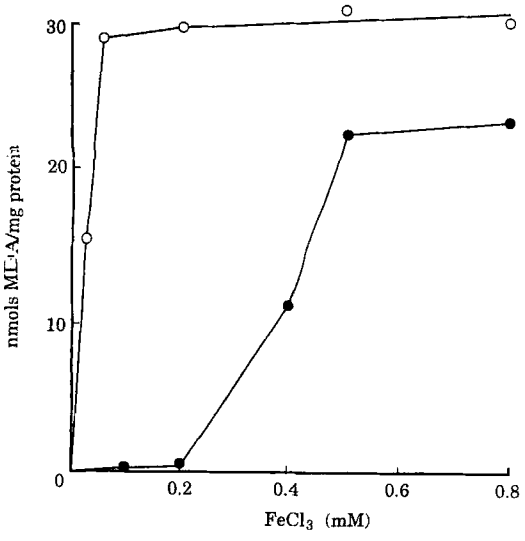

FIGURE 1 Influence of iron concentration upon the degree of iron ascorbate-induced peroxidation. Lipid peroxidation assays were performed in 150 mM KCl in the presence of 0.05 mM ascorbate as described in the Methods section. (○), human liver microsomes; (●), washed cell microsomes (data is from 3A4 microsomes and is representative of values obtained with the other cell microsomes). Assays were performed in triplicate and the data varied by no more than 10%. Production of TBARS is expressed relative to a malondialdehyde standard.

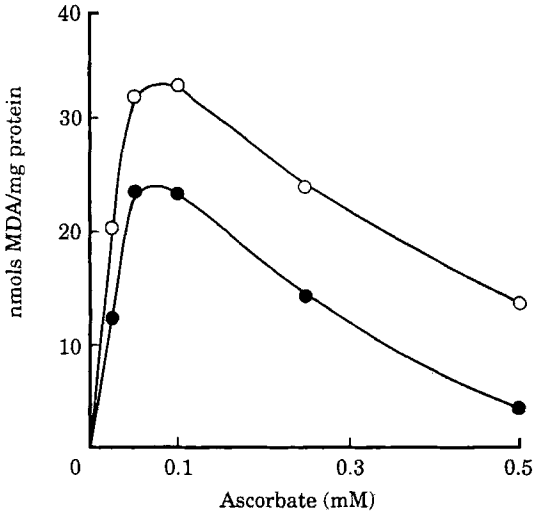

FIGURE 2 Influence of ascorbate concentration upon the degree of iron ascorbate-induced peroxidation. Lipid peroxidation assays were performed in 150 mM KCl in the presence of 0.5 mM Fe(III) as described in the Methods section. (○), human liver microsomes; (●), washed cell microsomes (data is for 3A4 microsomes and is representative of the data obtained with the other cell microsomes). Assays were performed in triplicate and varied by no more than 10%. Production of TBARS is expressed relative to a malondialdehyde standard.

Effect of P_{450} content on iron/ascorbate-induced lipid peroxidation

The levels of iron/ascorbate-induced peroxidation in cell microsomes containing various amounts and isoenzymes of cytochrome P_{450} are shown in Table 2. When the amount of the individual P_{450}'s was plotted against the extent of peroxidative damage (Figure 3), a striking correlation was found. This demonstrates that it is the total level of P_{450}, and not the nature of any specific isozyme, that determines the contribution of the cytochrome P_{450}'s to the rate of peroxidation.

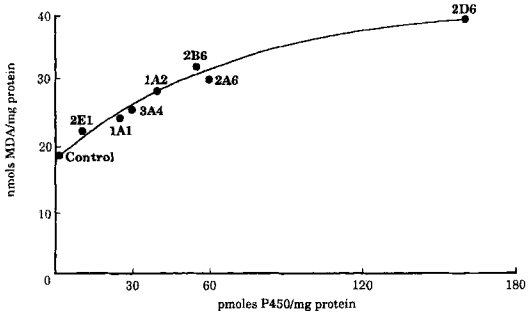

FIGURE 3 Relationship between iron ascorbate-induced lipid peroxidation of cell microsomes and the amount of P_{450}, based on data from Table II.

Effect of carbon monoxide treatment on lipid peroxidation

To confirm that the increased levels of ascorbate/iron-induced peroxidation shown in Figure 3 were due to P_{450} content, control and 2D6 cell microsomes were subjected to carbon monoxide, an inhibitor of P_{450}, prior to peroxidation. The results (Table 4) showed that CO treatment did not affect peroxidation levels in control microsomes, but did reduce peroxidative damage in 2D6 microsomes to levels similar to those found in control microsomes. This is strong evidence

TABLE 4 Influence of carbon monoxide treatment on lipid peroxidation

Source of Microsomes	Iron/ascorbate-induced peroxidation (nmoles MDA/mg protein)
Control cells	18.5
Control cells + CO	19.2
2D6 cells	35.8
2D6 cells + CO	22.2

Carbon monoxide treatment and lipid peroxidation assays were performed as described in the Methods section. Data represent the means of three assays which varied by less than 10%.

that it is the P_{450}'s themselves which are responsible for the increase in peroxidation.

Effect of lipid peroxidation on P_{450} activity

During peroxidation of liver microsomes, P_{450} activity is known to be destroyed.[30] To determine if this occurred with the cell microsomes, P_{450} 1A1 was assayed after iron/ascorbate-induced peroxidation. By direct assay, 1A1 activity was decreased by 65% after peroxidation for 40 min compared to microsomes containing P_{450} 1A1 incubated with either iron or ascorbate alone.

NADPH-induced lipid peroxidation

Under conditions that readily facilitated NADPH-induced lipid peroxidation in liver microsomes, no significant peroxidation was found with any of the cell microsomes (Table 2). Increasing the amount of microsomes assayed up to 5-fold did not stimulate peroxidation. It is known that NADPH and ADP-Fe(III) can induce lipid peroxidation in liposomes containing reductase in the presence of specific amounts of EDTA-Fe(III).[29] However addition of such amounts of EDTA-iron chelate to control cell microsomes did not facilitate peroxidation.

DISCUSSION

The recent commercial availability of human lymphoblastoid cell microsomes expressing specific P_{450}s offered an opportunity for addressing the role of P_{450}'s in lipid peroxidation in a biological membrane. The cell microsomes supported lipid peroxidation as stimulated by iron-ascorbate, indicating that the lipid itself was readily peroxidisable. As boiled microsomes and lipid vesicles alone can support iron-ascorbate induced peroxidation, this process has been viewed as non-enzymic, and consequently the possible role of P_{450} has not been well studied.

Cytochrome P_{450}, through its peroxidase activity, can readily catalyse the breakdown of LOOH,[3] and thereby catalyse LOOH-dependent lipid peroxidation. It is important to note that this reaction does not require NADPH, molecular oxygen or reductase, and culminates in the destruction of the P_{450}. Again whether all P_{450} isozymes catalyse LOOH breakdown equally is not known. With cell microsomes freed of possible contaminants, the content of P_{450} increased the amount of ascorbate/iron-induced peroxidation. The maximum amount of this increase, compared to control microsomes with no P_{450}, was two-fold. The relationship between peroxidation and the content of P_{450} was confirmed by inhibition using carbon monoxide, which abolished most of the increase in peroxidation. The correlation is independent of the individual P_{450}, and so our results suggest that it is the *total* amount of P_{450} rather than the amount of any *specific* P_{450} that is significant in exacerbating iron ascorbate-induced peroxidation. The liver microsomes had 5-fold the total levels of P_{450} compared to the microsomes containing 2D6 and yet displayed similar peroxidisability. Although it may not be possible to directly compare cell microsomes with liver microsomes, the role of P_{450} in peroxidation is illustrated by experiments on rat liver microsomes from animals fed phenobarbital. This led to elevated levels of P_{450} (via phenobarbital induction), which showed far greater levels of LOOH-dependent lipid peroxidation than control liver microsomes.[2]

Although iron/ascorbate can readily induce peroxidation in liposomes alone, we would predict that the degree of peroxidation would be

greater if P_{450} was added, but this experiment has never been performed to our knowledge. Iron/ascorbate-peroxidation is often termed as 'nonenzymic', based on the ability to obtain peroxidation even with boiled liver microsomes.[4] However comparison between boiled and unboiled microsomes is limited since the lipid may be in a different physical state after boiling. Therefore, the term 'nonenzymic' for iron ascorbate-induced microsomal lipid peroxidation is not strictly correct, since P_{450} clearly plays a role. It is also obvious and well-known that peroxidation occurs in the absence of any cytochrome P_{450} but that the presence of P_{450} aggravates it. The results presented here show that, in the presence of biochemically similar microsomes, P_{450} exacerbates the rate of peroxidation, and this effect is dependent on the total amount of cytochrome P_{450}.

None of the cell microsomes supported NADPH-induced lipid peroxidation under conditions that readily facilitated peroxidation of liver microsomes. As the lipid was readily peroxidised with iron/ascorbate (see above) it is unlikely to be the lipid per se that was protective. Furthermore the ratio of polyunsaturated to saturated lipids of the cell microsomes was not dissimilar to that of the liver microsomes. The low level of total P_{450} in the cell microsomes relative to liver microsomes is also unlikely to be the reason for this protection for reasons described as follows: In reconstitution experiments, lipid peroxidation is readily generated with about 100 pmol P_{450}[9,29] but using cell microsomes with equivalent amounts of specific P_{450}'s, no NADPH-induced peroxidation was detected. With P_{450} 2D6, no peroxidation was detected even with 320 pmol P_{450}. Furthermore, the addition of EDTA-Fe(III) chelate, which can substitute for P_{450} in linking NADPH oxidation to ADP-Fe(III) reduction in reconstitution experiments,[29] failed to stimulate peroxidation. This again implies that the low rate of peroxidation was not because of insufficient P_{450}. Nor was the protection of the cell microsomes against NADPH-induced lipid peroxidation due to a lack of reductase. In reconstitution studies, peroxidation is observed with less than 1 unit of reductase/sample,[8,9,29] yet in this study over 20 units of reductase were typically present. The cells and microsomes derived from them have been used for toxicology testing. This has been shown to be mediated via the recombinant cytochrome P_{450} and the constitutive NADPH cytochrome P_{450} reductase, which shows that these components are correctly positioned in the membrane. Therefore it is likely that the low rate of peroxidation using NADPH/iron is due to an agent that interferes at a different stage of peroxidation, as described above. The nature of the protection is not known and merits further investigation.

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