

Restoration of hydroperoxide-dependent lipid peroxidation by 3-methylcholanthrene induction of cytochrome P-448 in hepatoma microsomes

Silvia Borrello, Tommaso Galeotti*, Guglielmo Palombini and Giorgio Minotti

Institute of General Pathology, Catholic University, Largo F. Vito 1, 00168 Roma, Italy

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Microsomal membranes from the slow-growing Morris hepatoma 9618A catalyze, in the presence of *t*-butyl hydroperoxide, lower rates of lipid peroxidation than rat liver microsomes. The cytochrome P-450 content of hepatoma microsomes is about 40% that of the liver. SKF 525-A, an inhibitor of mixed-function oxidase, produces in hepatoma microsomes a P-450 type I binding spectrum similar to that of hepatic microsomes. The concentration of the inhibitor required for half-maximal spectral change is about 2 μ M in both microsome types. SKF 525-A or ethylmorphine inhibit lipid peroxidation of normal and tumor microsomes to the same extent (about 60%). Treatment of the tumor-bearing rats with 3-methylcholanthrene increases the hepatoma cytochrome P-450 to values comparable to those of control membranes, although the hemoprotein has a peak in the CO-reduced difference absorption spectrum at 448 nm. The cytochrome P-448 induction is accompanied by an almost complete restoration of the hydroperoxide-dependent lipid peroxidation.

(Morris hepatoma 9618A)	Lipid peroxidation	Cytochrome P-448	3-Methylcholanthrene	Microsome
		<i>t</i> -Butyl hydroperoxide		

1. INTRODUCTION

Neoplastic transformation of the liver is characterized by a decrease in the vulnerability of the intracellular and plasma membranes to peroxidative agents [1]. The most remarkable changes are observable in membranes isolated from fast-growing hepatomas, where the major limiting factor appears to be the lipid substrate availability (low phospholipid content and degree of fatty acid unsaturation). Evidence has been provided, however, that microsomes from the slow-growing hepatoma 9618A, although they have a normal lipid composition, may fail to propagate lipid peroxidation initiated by O_2^- radicals [2] or even to be resistant to the entire process (initiation and propagation reactions) when it is promoted by

organic hydroperoxides (ROOH) [3]. In both circumstances cytochrome P-450 deficiency, rather than an increase in the protective effect of membrane antioxidants, seems to be the rate-limiting step. This is in accordance with the proposed implication of the hemoprotein in the oxidation of lipid hydroperoxides (LOOH), formed during the initiation step, to propagating radicals [4] as well as in the oxidation of ROOH to initiating radicals [5].

In the present study we have reexamined the lipid peroxidation of hepatoma 9618A microsomes by using a ROOH-dependent system, with the aim of verifying the previous conclusions on the involvement of cytochrome P-450 as ROOH and LOOH oxidase. For this purpose, the effect on *t*-butyl hydroperoxide (*t*-BuOOH)-induced peroxidation of mixed-function oxidase inhibitors or substrates has been tested, comparatively, in rat

* To whom correspondence should be addressed

liver and tumor microsomes. Furthermore, experiments have been performed in order to reconstitute the normal level of cytochrome P-450 in the hepatoma membranes. Such a condition was afforded by the use of 3-methylcholanthrene (MC), whose administration to the tumor-bearing rats induces an elevation of the hemoprotein in the form of cytochrome P-448. Membranes from MC-induced hepatomas should thus be able to catalyze rates of hydroperoxide-dependent lipid peroxidation similar to those of rat liver membranes.

2. MATERIALS AND METHODS

Morris hepatoma 9618A was propagated by subcutaneous transplantation into both hind legs of inbred rats of the Buffalo strain. Details concerning the growth and isolation of the tumor are reported elsewhere [6,7]. Tumor-bearing rats were treated intraperitoneally with 3-methylcholanthrene (30 mg/kg body wt) in corn oil for 3 days [8] or orally with sodium phenobarbital (1 mg/ml drinking water) for 5 days [9]. The animals were killed 24 h after treatment. Normal male rats (150–200 g) of the same strain were used as controls. Rat liver and hepatoma microsomes were isolated in 0.25 M sucrose, 0.5 mM EGTA, 5 mM Hepes (pH 7.4) as in [10]. The fractions were washed once by centrifugation in 0.15 M KCl, 50 mM Tris-HCl (pH 7.5) and suspended in the same medium. Proteins were estimated by the biuret method [11].

Lipid peroxidation was performed at 37°C in capped flasks containing 0.2–0.5 mg protein/ml, 0.11 mM EDTA, 0.15 M KCl, 50 mM Tris-HCl (pH 7.5). The reaction was started by the addition of 0.25 or 2 mM *t*-BuOOH and was allowed to run for the time indicated in a Dubnoff metabolic shaker. Lipid peroxidation was measured as malondialdehyde (MDA) formation, by the thiobarbituric acid assay at 535 nm [12]. The content of cytochrome P-450 or P-448 was calculated from CO-reduced difference absorption spectra, performed in a dual-wavelength/split-beam Aminco-Chance spectrophotometer, using an extinction coefficient of $91 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ [13]. Changes in absorbance induced by the addition of increasing concentrations of SKF 525-A to aerobic microsomes (2 mg protein/ml) were recorded at

room temperature at two fixed wavelengths by means of the same spectrophotometer.

Ethylmorphine was a generous gift from Dr M.A. Iorio of the Istituto Superiore di Sanità, Laboratory of Pharmaceutical Chemistry (Roma, Italy). HEPES and thiobarbituric acid were obtained from Sigma (St. Louis, MO). SKF 525-A was a product of Smith, Kline and French Laboratories (Philadelphia, PA). 3-Methylcholanthrene was purchased from Eastman Kodak Company (Rochester, NY). Sodium phenobarbital was obtained from BDH (Poole, England). All other chemicals were products of E. Merck (Darmstadt) of reagent grade quality.

3. RESULTS

Fig.1 shows the carbon monoxide difference absorption spectra of anaerobic rat liver (A) and Morris hepatoma 9618A (a) microsomes, together with the spectral changes induced by addition of an excess of SKF 525-A to aerobic suspensions of the corresponding membranes (B and b). Both the CO spectra have a peak at 450 nm, indicative of cytochrome P-450. The interaction of SKF 525-A

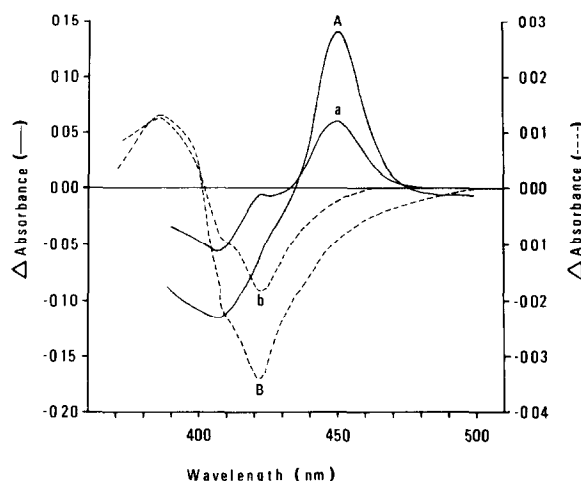


Fig.1. Room temperature difference absorption spectra of cytochrome P-450 recorded in the presence of CO (—) and SKF 525-A (---) in rat liver (A, B) and hepatoma 9618A (a, b) microsomal suspensions. The proteins (2 mg/ml) were suspended in 0.15 M KCl, 50 mM Tris-HCl (pH 7.5). CO was added to $\text{Na}_2\text{S}_2\text{O}_4$ -reduced microsomes and SKF 525-A ($100 \mu\text{M}$) to aerobic microsomes.

Table 1

Cytochrome P-450 absorbance changes induced by SKF 525-A and carbon monoxide in rat liver and hepatoma 9618A microsomes

Microsomes	ΔA (420–500 nm) SKF-complex	ΔA (450–490 nm) CO-complex	$\frac{\Delta A (420-500 \text{ nm})}{\Delta A (450-490 \text{ nm})}$
Rat liver	0.013 ± 0.001 (5)	0.063 ± 0.003 (5)	0.204 ± 0.013 (5)
Hepatoma	0.008 ± 0.0006 (5)	0.025 ± 0.001 (5)	0.305 ± 0.015 (5)

ΔA values were determined from difference absorption spectra of SKF-treated aerobic microsomes and CO-treated anaerobic microsomes, similar to those shown in fig.1, and are expressed for a microsomal protein content of 1 mg/ml. Values are means $\pm$ SE (number of observations)

with the oxidized hemoprotein causes the appearance of characteristic spectra, termed type I [14], similar in control and tumor microsomes, with an absorption maximum at 385 nm and a trough at 422 nm. It can be seen that the amount of cytochrome P-450 is considerably lower (about 2.5-fold) in hepatoma microsomes, whereas the difference in the magnitude of the SKF 525-A-induced spectral change between the two types of membrane appears to be less pronounced. This appears more clearly from the mean values of the absorbance changes of the type I compound and of the CO-complex of the reduced pigment, reported in table 1, the ratio between such values being higher in the hepatoma.

Fig.2 illustrates the double reciprocal plots of changes in absorbance at 422 relative to 450 nm caused by consecutive additions of SKF 525-A to aerobic suspensions of rat liver and hepatoma microsomes. Extrapolation of the plots to the abscissa allows one to calculate the concentration of the inhibitor needed to obtain the half-maximal spectral change, a constant which has been termed the 'spectral dissociation constant' [14]. Such a concentration does not differ significantly in the two microsome types (rat liver, 2.2 μ M; hepatoma, 2.4 μ M).

The above data indicate that the tumor P-450 hemoprotein, although being diminished, may be interacting with SKF 525-A in a manner similar to that of liver. Information about the extent to which cytochrome P-450 is implicated in the hepatoma microsomal lipid peroxidation should thus be given by the use of inhibitors, such as SKF 525-A, and/or substrates of mixed-function ox-

idase. Fig.3 presents the inhibitory effect obtained by such drugs on the *t*-BuOOH-induced lipid peroxidation of control and hepatoma membranes. The range of concentration employed as well as the degree of inhibition (about 60%) were comparable in control and tumor membranes for SKF 525-A and ethylmorphine. Conversely, progesterone was less effective in hepatoma microsomes.

Microsomal monooxygenase activities are induced in several lines of Morris hepatomas by treatment of the tumor-bearing rats with phenobarbital (PB) or MC [15–17]. Induction of such activities is parallel to the increase in cyto-

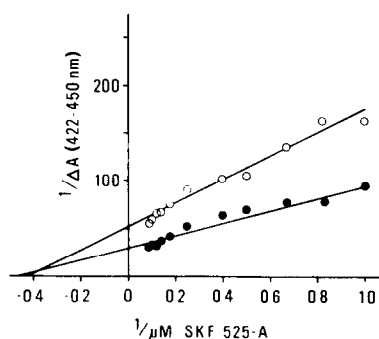


Fig.2. Double reciprocal plots of changes in absorbance following the addition of increasing concentrations of SKF 525-A to aerobic rat liver (●) and hepatoma 9618A (○) microsomes. The measurements were performed in a dual-wavelength spectrophotometer at 422–450 nm, using 2 mg microsomal proteins per ml of the KCl/Tris-HCl medium. Each point is the mean of 5–10 determinations.

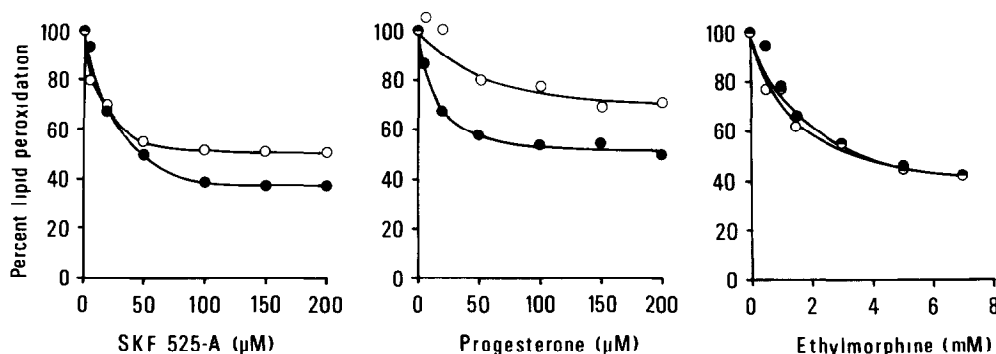


Fig.3. The effect of various drugs on *t*-BuOOH-dependent lipid peroxidation of rat liver (●) and hepatoma 9618A (○) microsomes. The experimental conditions were: 0.2 mg protein/ml and 0.25 mM *t*-BuOOH in the presence of SKF 525-A; 0.5 mg protein/ml and 2 mM *t*-BuOOH in the presence of progesterone or ethylmorphine. Incubation time: 20 min. The 100% values of lipid peroxidation, expressed as nmol MDA/20 min per mg protein, correspond to 7.44 ± 0.54 (9) and 2.42 ± 0.26 (13) (SKF 525-A experiment); 12.1 ± 0.71 (4) and 3.90 ± 0.26 (14) (progesterone experiment); 10.20 ± 0.29 (8) and 4.70 ± 0.05 (4) (ethylmorphine experiment), for rat liver and hepatoma microsomes, respectively.

chrome P-450 content. Phenobarbital administration to Buffalo rats bearing the hepatoma 9618A did not increase, however, the level of the tumor microsomal pigment (not shown). Instead, as shown in fig.4, treatment of the rats with MC caused the appearance of a cytochrome, with an ab-

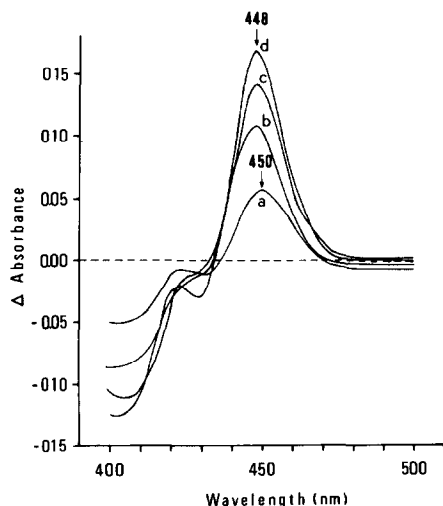


Fig.4. Difference absorption spectra of hepatoma 9618A cytochrome P-450 and P-448. Microsomal membranes were isolated from hepatomas of untreated (a) or MC-treated (b,c,d) rats. The cytochrome contents were 0.33 (a), 0.63 (b), 0.78 (c) and 0.93 (d) nmol/mg protein. Experimental conditions are described in section 2.

sorption peak at 448 nm in the CO-reduced form, similar to the hepatic species induced by various polycyclic hydrocarbons, known as cytochrome P₁-450 [18] or cytochrome P-448 [19]. The induced levels of the hemoprotein in the hepatoma microsomes were not always the same, ranging from 0.2 to 0.93 nmol/mg protein (see figs 4 and 5). This allowed us to establish a relationship between the cytochrome content and the extent of

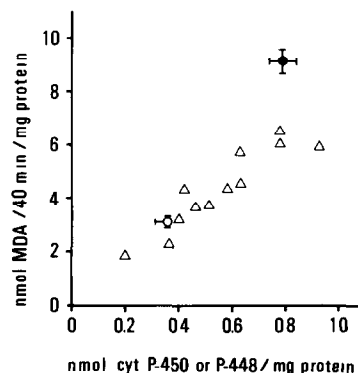


Fig.5. Relationship between cytochrome P-450 or P-448 content and *t*-BuOOH-induced lipid peroxidation in microsomes isolated from rat liver (●) and hepatomas 9618A of untreated (○) or MC-treated (Δ) rats. The peroxidation reaction mixture contained 0.2 mg protein/ml and 0.25 mM *t*-BuOOH. P-450 and MDA values are means $\pm$ SE of 7 experiments for rat liver and 4 experiments for hepatoma 9618A of untreated rats.

malondialdehyde production in a *t*-BuOOH-induced peroxidative system (fig.5). It appears clearly that hepatoma microsomes with increasing cytochrome levels catalyze proportionally higher rates of lipid peroxidation.

4. DISCUSSION

Microsomes isolated from slow-growing hepatomas, although they are able to catalyze reasonable rates of lipid peroxidation, appear to be less susceptible to this process, when compared to normal liver microsomes [1]. Such resistance, which is more evident in the ROOH- or in the NADPH-dependent systems, has been ascribed to deficiency of cytochrome P-450 [3,7]. Indeed, microsomes from Morris hepatoma 9618A, which is a slow-growing, highly differentiated carcinoma, lack about 60% of the liver cytochrome P-450. That the amount of the hemoprotein still present participates to a great extent in the *t*-BuOOH-dependent peroxidative activity of these microsomes, by functioning as a ROOH and LOOH oxidase, is suggested by the marked inhibitory effect elicited by aminopyrine, a drug hydroxylation substrate [3]. In the present work confirmatory evidence for the involvement of cytochrome P-450 in the hepatoma 9618A microsomal lipid peroxidation was obtained by the experiments performed with the inhibitor SKF 525-A and two mixed-function oxidase substrates, progesterone and ethylmorphine. SKF 525-A has a binding affinity for cytochrome P-450 similar in hepatoma and liver microsomes, as indicated by spectral titration experiments. However, comparison of the magnitude of the spectral changes observed in the presence of CO and SKF 525-A in the two microsomal systems reveals that in hepatoma membranes the maximal binding capacity of the P-450 hemoprotein is proportionally lower for CO than for SKF 525-A. *t*-BuOOH-dependent lipid peroxidation of control and tumor membranes is inhibited to the same extent (about 60%) in the presence of either SKF 525-A or ethylmorphine; conversely, progesterone is less efficient in the hepatoma (30% vs 50% inhibition). As already suggested for the effect observed in the presence of aminopyrine [3], progesterone and ethylmorphine would inhibit lipid peroxidation by competing for *t*-BuOOH. Indeed, these drugs are capable of

reducing hydroperoxides to the corresponding alcohols, in a cytochrome P-450-catalyzed reaction, via a peroxidase-type mechanism [4,20-22]. Among them only progesterone seems to interact differently with the hemoprotein in the two microsome types, a result which, combined with that of the decreased CO binding capacity of the hepatoma pigment, might suggest the existence in the tumor membranes of cytochrome P-450 isoenzymes with somewhat altered functional properties.

If cytochrome P-450 is implicated in the ROOH-dependent hepatoma microsomal lipid peroxidation, by reacting with ROOH and LOOH to yield initiating and propagating radicals, respectively, but its low availability is the rate-limiting step in the process, restoration of normal hemoprotein levels should remove the resistance to ROOH. This was obtained by treatment of the tumor-bearing rats with 3-methylcholanthrene which induced the appearance of amounts of cytochrome P-448 comparable to those of liver cytochrome P-450. These results strongly support our hypothesis that cytochrome P-450 deficiency limits the lipid peroxidation of slow-growing hepatoma microsomes and also indicate that other forms of the hemoprotein, such as cytochrome P-448, may participate in microsomal peroxidation.

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