Hydroxyl radicals are not involved in NADPH dependent microsomal lipid peroxidation

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Summary. NADPH dependent H_2O_2 formation in microsomes in the presence of chelated iron leads to formation of hydroxyl radicals. Enhancement of hydroxyl radical generation (via ferric-EDTA or sodium azide) did not result in a concomitant increase in lipid peroxidation; rather, a decrease was observed. Moreover, the hydroxyl radical scavenger DMSO did not inhibit lipid peroxidation. This comparison of hydroxyl radical formation with lipid peroxidation suggests that hydroxyl radicals do not play a part in NADPH-dependent lipid peroxidation.

Key words. Lipid peroxidation; hydroxyl radicals; microsomes; cytochrome P-450.

Liver microsomes are frequently used to study NADPH-dependent lipid peroxidation. Xenobiotics can both inhibit and enhance lipid peroxidation. In order to apprehend the effects of xenobiotics on microsomal lipid peroxidation it is important to understand the mechanism of initiation and propagation of lipid peroxidation in the presence of NADPH but without xenobiotics. The respective roles of cytochrome P-450 and the flavoprotein NADPH dependent cytochrome P-450 reductase in microsomal lipid peroxidation are under debate[1-3]. Both the flavoprotein cytochrome P-450 reductase and the oxidase activity of cytochrome P-450 can give superoxide anion radicals which upon dismutation produce H_2O_2, ultimately leading to hydroxyl radicals. In microsomes cytochrome P-450 has been suggested to be the major source of hydroxyl radicals[2,4]. Also, the peroxidase activity of cytochrome P-450 may lead to formation of hydroxyl radicals[5]. Hydroxyl radicals are known to mediate oxidation of xenobiotics in microsomal incubates[6]. Because cytochrome P-450 is embedded in the microsomal membrane, the hydroxyl radicals formed in this environment may severely damage local unsaturated fatty acids, leading to lipid peroxidation. However, the prevalent theory that hydroxyl radicals play a role in lipid peroxidation is also challenged on the basis of experiments in different model systems[7,8]. The importance of hydroxyl radicals in the metabolism of xenobiotics as well as their presumed role in lipid peroxidation prompted us to this study. We found dissimilar optimal incubation conditions for NADPH-dependent microsomal lipid peroxidation and hydroxyl radical formation. This strongly indicates that hydroxyl radicals are not involved in NADPH-dependent microsomal lipid peroxidation.

Materials and methods. Pretreatment of rats and preparation of hepatic microsomes. Male Wistar rats (T.N.O., Zeist, The Netherlands) 200–250 g received i.p. injections of phenobarbital dissolved in saline. The phenobarbital treatment consisted of 3 daily injections of 80 mg/kg. On day 4 the rats were killed by decapitation. Livers were removed and homogenized (1:2 w/v) in ice-cold phosphate buffer (50 mM, pH 7.4) containing 0.1 mM EDTA. Microsomes were prepared as described[9]. Washing and final resuspension was done in the phosphate buffer without EDTA.

Assay methods. Microsomes (0.7–1.0 mg protein/ml) were incubated (37 °C) in 50 mM phosphate buffer (pH 7.4) containing 0.5 mM $NADP^+$, 4.2 mM glucose-6-phosphate, 0.3 IU/ml incubate of glucose-6-phosphate dehydrogenase (all from Sigma Chem Co.) and 4.2 mM $MgCl_2$. Production of formaldehyde from 33 mM dimethylsulphoxide (DMSO) was used to detect hydroxyl radicals in the microsomal incubates[10]. An aliquot of 1 ml of the reaction mixture was stopped with 1 ml ice-cold 15% trichloroacetic acid. After mixing and centrifugation the supernatant was assayed for formaldehyde[11]. Lipid peroxidation was determined by measuring thiobarbituric acid reactive material (ΔA 535–600 nm) in an aliquot (1 ml) of the incubation mixture[9]. Microsomal protein was assayed[12], using crystalline bovine serum albumin as standard.

Results and discussion. Ferric-EDTA results in a concentration-dependent increase in microsomal NADPH dependent hydroxyl radical generation from H_2O_2 indicated by an increase in DMSO oxidation. A convenient concentration of ferric-EDTA was used (final concentration of 0.025 mM, 1:2 molar ratio) in order to be able to investigate the effect of both inhibition and enhancement

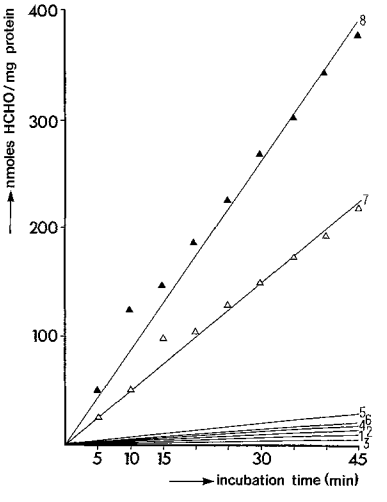

Figure 1. Time course of the oxidation of DMSO (expressed as nmol formaldehyde formed/mg protein) in liver microsomes obtained from phenobarbital pretreated rats supplemented with a NADPH regenerating system. Further additions (1) control; (2) sodium azide (1 mM); (3) DMSO (33 mM); (4) DMSO and sodium azide; (5) ferric-EDTA (1:2 molar ratio, final concentration of 0.025 mM); (6) ferric-EDTA and sodium azide; (7) ferric-EDTA and DMSO; (8) ferric-EDTA and DMSO and sodium azide. Data show one representative experiment performed in duplicate.

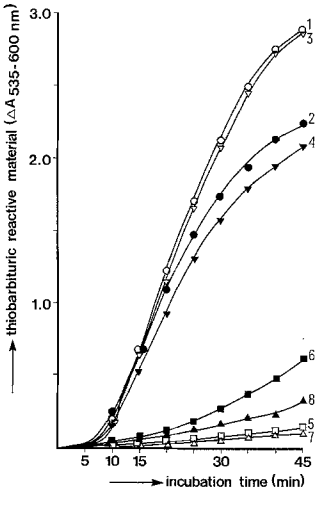

Figure 2. Time course of production of thiobarbituric acid reactive material, measured as $\Delta A_{535-600\ nm}$ in 1 ml of the incubation mixture. Further as in figure 1.

of hydroxyl radical production. Ferric-EDTA stimulates DMSO oxidation (lines 3 and 7, fig. 1). The addition of sodium azide resulted in a further increase (line 7 and 8, fig. 1). Both without ferric EDTA (line 3, fig. 1) and in the presence of sodium azide alone (line 4, fig. 1) the oxidation of DMSO is minimal.
Microsomal NADPH dependent lipid peroxidation is most prominent without any addition at all (line 1, fig. 2). The hydroxyl scavenger, DMSO, did not inhibit lipid peroxidation. Ferric-EDTA resulted in an inhibition of lipid peroxidation. Remarkably, sodium azide decreased lipid peroxidation both in the absence and in the presence of ferric-EDTA.
Comparison of the optimal conditions for microsomal hydroxyl radical formation and lipid peroxidation in this parallel experimental set-up shows: 1) Higher production of hydroxyl radicals, via ferric-EDTA, does not result in an increase in lipid peroxidation. Rather, a decrease in lipid peroxidation is observed. 2) DMSO in a concentration that is able to scavenge hydroxyl radicals (fig. 1) did not inhibit lipid peroxidation (fig. 2). 3) Addition of sodium azide resulted in an increase in hydroxyl radical formation and a concomitant decrease in lipid peroxidation.
Azide is used in order to inhibit endogenous microsomal catalase, thereby leading to a rise in microsomal H_2O_2 concentration. This does not give stimulation of lipid peroxidation, thus confirming the notion that H_2O_2 is not involved in a rate-limiting step in NADPH-dependent lipid peroxidation[2,13]. The observed inhibition by azide may be the result of its scavenging effect of singlet oxygen. Our observations strongly indicate that hydroxyl radicals play no part in NADPH-dependent lipid peroxidation.

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0014-4754/86/050555-02$1.50 + 0.20/0

A comparison of cycloartenol and lanosterol biosynthesis and metabolism by the Oomycetes

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Summary. Representative members in each of the four orders of Oomycetes (*Phytophthora cactorum,* Peronosporales; *Lagenidium callinectes, L. giganteum,* Lagenidiales; *Saprolegnia ferax,* Saprolegniales; *Apodachylella completa,* Leptomitales) have been examined for their ability to synthesize and polycyclize squalene-oxide (SO) to a tetracyclic product and to differentiate between cycloartenol and lanosterol metabolism to sterols. *P. cactorum* and *L. giganteum* failed to synthesize or metabolize SO, cycloartenol or lanosterol. While the other three fungi synthesized sterols via SO and lanosterol, a minor metabolism of added cycloartenol to the 4,4-desmethyl-14α-methylcyclosteroid dehydropollinastanol was observed.
Key words. Oomycetes; squalene-oxide; cycloartenol; lanosterol; sterol metabolism.

Fungal evolution has classically been assessed through comparisons of specific phenotypic characters and life cycle events coupled with a few biochemical parameters, such as, in the sequencing of intermediates in the sterol pathway[1,2]. Dissociation between a cycloartenol- and a lanosterol-based steroid pathway is known to phylogenetically link organisms having an evolutionary history of photosynthesis, e.g. cycloartenol biosynthesis and metabolism, with those which lacked such an ancestry. Since the fungal progenitors are generally believed to have been, at one time, photosynthetic organisms[3,4] which subsequently lost the chloroplastic system, it seems logical that the Oomycetes, a primitive group believed to be closely allied with the Phaeophyte[5] or Chrysophyte algae[3], may have retained the genes for the cycloartenol route. In fact, there are reports which seemingly support this dogma[6,7]. However, as we now unequivocally show, the Oomycetes either utilize a lanosterol-based pathway or fail to synthesize sterols de novo: no vestigial SO-cycloartenol cyclase or cyclosteroid isomerase was operational in the various mycelia. When the five Oomycetes were examined by GLC and GC-MS, only *L. callinectes, S. ferax,* and *A. completa* produced detectable levels of sterols and lanosterol. While the synthesis of SO and its cyclization are implied in the three latter fungi, detailed studies with *P. cactorum* and *L. giganteum* failed to show mycelial conversion of [2-^{14}C]acetate or [^{3}H]squalene to SO or cyclize [^{14}C]SO to a tetracyclic product. Thus, the ability to synthesize sterols is variable in Oomycetes, as it apparently is in the slime molds[8,9]. In oomycetous fungi which demonstrably produce lanosterol[10,11], the possibility of it having been formed subsequent to cycloartenol synthesis could not be ruled out. Therefore, with *S. ferax, P. cactorum,* and *L. giganteum* we performed on each of the mycelia two sets of additional experiments with ^{14}C and ^{3}H substrates to trap the putative cycloartenol intermediate and to test whether cycloartenol could be directly isomerized to lanosterol.
When 0.056 μCi/flask of [2-^{14}C]acetate and nonradioactive cycloartenol (10 ppm) were fed to *S. ferax,* lanosterol was isolated in radiochemically pure form. While significant mycelial absorption of cycloartenol was evident by GLC of the mycelial extacts, no significant radioactivity eluted with cycloartenol in HPLC. However, as shown in figure 1, an HPLC peak which may have been cycloartenol was evident in the *S. ferax* incubation with [^{14}C]SO (sp. act. 3.6×10^6 dpm/mg; synthesized by incubating the GL-7 yeast sterol auxotroph with [^{14}C]-acetate and purified by HPLC as described in Nes and Heupel[12] and 10 ppm tridemorph (a specific inhibitor of the cyclosteroid isomerase[13]). The HPLC fractions from the latter feed corresponding to lanosterol and cycloartenol, were collected and diluted with nonradioactive carrier to produce a starting sp. act. for lanosterol, 1.42×10^4