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## CONTRIBUTION OF HEPATIC CYTOCHROME P450 SYSTEMS TO THE GENERATION OF REACTIVE OXYGEN SPECIES

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**Abstract**—The rate of generation of reactive oxygen species (ROS) in hepatic microsomes was assayed using a fluorescent probe. This rate was stimulated in a manner proportional to the concentration of NADPH present. NADH could not be substituted for NADPH, and an inhibitor of mixed-function oxidases (SKF 525A) blocked stimulation by NADPH. This suggested the involvement of cytochrome P450 oxidase systems in ROS formation. Low molecular weight iron salts may not have been involved in the stimulated ROS formation since deferoxamine failed to eliminate the oxidative response to NADPH. Catalase only partially inhibited, and glutathione peroxidase did not significantly inhibit this response, implying that hydrogen peroxide does not play a key role. However, since NADPH-enhanced generation of reactive oxygen species was totally prevented by superoxide dismutase, superoxide was an obligatory intermediate. The presence of toluene, ethanol or phenobarbital did not enhance the production of NADPH-effected reactive oxygen species; free radical production was maximal in the absence of substrates subject to oxidation by cytochrome P450 enzymes. Hepatic cytochrome P450 oxidases are likely to contribute significantly to overall ROS formation, even under basal conditions where mixed-function oxidases are not induced.

**Key words:** cytochrome P450; reactive oxygen species; free radicals; superoxide; hydrogen peroxide; NADPH

The cytochrome P450 enzymes are important in the oxidative metabolism of xenobiotic agents. Their utilization of molecular oxygen for the combined oxidation of NADPH and target compounds makes these enzyme systems a potentially significant source of ROS† [1]. This group of enzymes may also play an important role in the physiological regulation of the oxidative degradation of several critical enzymes [2]. However, antioxidant activity has also been attributed to NADPH since it can inhibit microsomal lipid peroxidation under some circumstances [3]. This may be attributable to the role of NADPH in the regeneration of glutathione from glutathione disulfide by glutathione peroxidase [4]. Glutathione is a key source of intracellular reducing power [5]. Thus, NADPH is a cofactor in both oxidative and reductive reactions. The present study was intended to delineate the extent to which this enzyme series could contribute to the overall rate of generation of free radicals within the liver under basal conditions. The generation of reactive oxygen species was assayed by the use of a fluorescent probe, the utility of which has been documented previously [6]. The level of ROS production was determined in the presence and absence of cytochrome P450 substrates,

and the effect of an inhibitor of mixed-function oxidase was also studied. The study of various inhibitors of ROS generation allowed the identification of obligatory intermediates in this process. Such inhibitors included catalase, which degrades hydrogen peroxide; superoxide dismutase, which dismutates the superoxide radical; and an iron chelator, deferoxamine. In addition, the effects of the concurrent presence of several compounds that are potential substrates for mixed-function oxidases, namely toluene, ethanol and phenobarbital, were investigated.

### MATERIALS AND METHODS

**Materials.** DCFH-DA was purchased from Molecular Probes, Inc. (Eugene, OR). DCF, required for calibration, was obtained from Polysciences, Inc. (Warrington, PA). All other chemicals were obtained from the Sigma Chemical Co. (St. Louis, MO).

**Animals.** Male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) weighing 150–175 g were utilized. Rats were housed 4 per cage with wood chip bedding and maintained on a 12 hr light/dark cycle in a temperature controlled ( $20 \pm 1^\circ$ ) room. Food (Purina Laboratory Chow, St. Louis, MO) and water were provided *ad lib*.

**Tissue preparation.** Rats were decapitated, and the liver was removed and frozen at  $-70^\circ$ . Tissue was placed into screw-capped microcentrifuge tubes, stored at  $-20^\circ$  for 24 hr, and subsequently stored at  $-70^\circ$  until preparation. Tissue was then weighed

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† Abbreviations: ROS, reactive oxygen species; DCFH-DA, 2',7'-dichlorofluorescein diacetate; DCF, 2',7'-dichlorofluorescein; and DCFH, 2',7'-dichlorofluorescein.

and homogenized in 10 vol. of 0.32 M sucrose and centrifuged at 1800 *g* for 10 min. The resulting supernatant fraction was then centrifuged at 31,500 *g* for 10 min to pellet the crude mitochondrial fraction. A further centrifugation at 105,000 *g* for 30 min yielded the microsomal fraction (P3). The P3 pellet was resuspended in HEPES buffer to a concentration of 0.2 g-equiv/mL containing about 0.33 mg protein/mL. The composition of the HEPES buffer was (mM): NaCl, 120; KCl, 2.5; NaH<sub>2</sub>PO<sub>4</sub>, 1.2; MgCl<sub>2</sub>, 0.1; NaHCO<sub>3</sub>, 5.0; glucose, 6.0; CaCl<sub>2</sub>, 1.0; and HEPES, 10; pH 7.4.

**Assay for reactive oxygen species formation.** Reactive oxygen species were assayed using DCFH-DA, which is de-esterified to the ionized free acid, DCFH [7]. DCFH is capable of being oxidized to the fluorescent DCF by reactive oxygen. P3 suspensions were diluted in 9 vol. of HEPES buffer. This was then incubated with 5  $\mu$ M DCFH-DA (added from a stock solution of 1.25 mM in ethanol) at 37° for 15 min. After this loading with DCFH-DA, the aliquots were incubated for a further 15 min in the presence of various enzymes or pharmacological inhibitors. This relatively short incubation period allowed the reaction rate to be determined under linear conditions. At the beginning and at the end of incubation, fluorescence was monitored on a Farrand Spectrofluorometer, with the excitation wavelength at 488 nm (bandwidth 5 nm), and the emission wavelength at 525 nm (bandwidth 20 nm). The rate of generation was linear over the incubation period [8].

Autofluorescence of fractions was corrected for by the inclusion in each experiment of parallel blanks with no DCFH-DA. The correction for autofluorescence was always less than 11% of the total. Oxygen reactive species formation was quantitated from a DCF standard curve (0.05 to 1.0 mM), and results were expressed as nanomoles of DCF formed per hour per milligram of protein.

**Protein determination.** Protein concentration was assayed using the method of Bradford [9].

**Statistical analyses.** Differences between groups were assessed by one-way analysis of variance followed by Fisher's Least Significant Difference Test. The acceptance level of significance was  $P < 0.05$ , using a two-tailed distribution. Each value presented was obtained from 5–6 individual rats.

## RESULTS

ROS production by the liver microsomes was elevated several-fold in the presence of NADPH, and this stimulation was concentration dependent (Fig. 1). A concentration of 0.2 mM NADPH was used in subsequent experiments. NADPH could not be substituted for NADPH in effecting hepatic ROS generation, indicating the P450 source of this stimulated pro-oxidant activity. This was substantiated by the finding that an inhibitor of several mixed-function oxidases, SKF 525A, largely blocked NADPH-stimulated ROS production (Fig. 2).

We have looked for obligatory intermediates in the formation of the short-lived strongly pro-oxidant species that acts on DCFH [10]. The NADPH-stimulated production of hepatic microsomal ROS

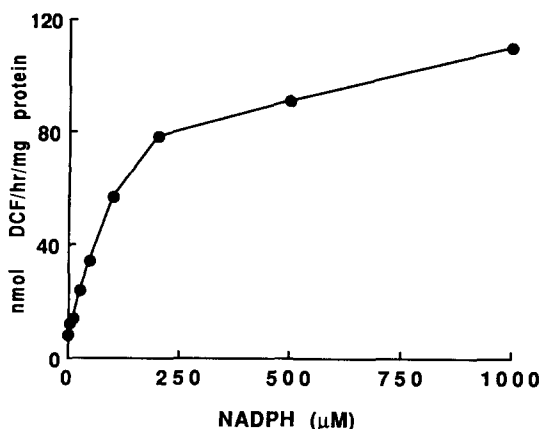


Fig. 1. Effect of various concentrations of NADPH upon ROS formation in liver microsomes. Each value is the mean of three separate determinations within 14% of each other.

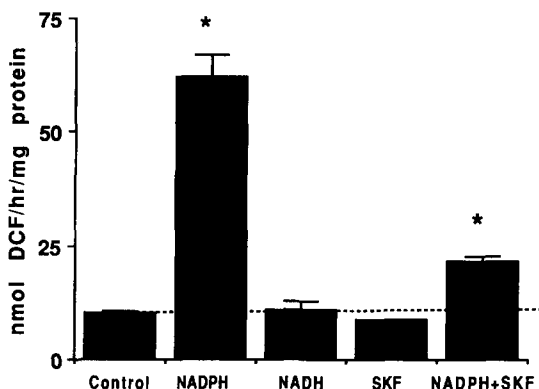


Fig. 2. Hepatic microsomal ROS generation in the presence of NADH (0.2 mM), NADPH (0.2 mM), and SKF 525A (50  $\mu$ M). Values are the means  $\pm$  SEM of 5–6 individual determinations. Key: (\*) significantly different from control ( $P < 0.05$ ).

was partially inhibited in the presence of catalase (3500 U/mL) (Fig. 3). This inhibition was prevented completely by the concurrent presence of an inhibitor of a catalase, 100  $\mu$ M sodium azide (data not shown), implying that hydrogen peroxide was an intermediate in the oxidation of a significant fraction of DCFH. While we previously found sodium azide (100  $\mu$ M) to stimulate basal ROS generation in hepatic microsomes [11], this agent did not enhance NADPH-effected ROS significantly. A 1 mM concentration of sodium azide is known to scavenge hydroxyl radicals [12], and this property may account for the failure of azide to consistently enhance the rates of ROS generation. Since glutathione peroxidase has a higher affinity for hydrogen peroxide than catalase, the effect of this enzyme upon NADPH enhancement of ROS generation was also examined.

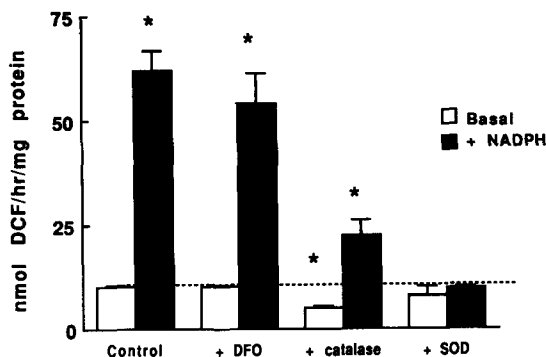


Fig. 3. NADPH-stimulated microsomal ROS production in the presence of superoxide dismutase (200 U/mL), catalase (3500 U/mL) and deferoxamine (100  $\mu$ M). Values are the means  $\pm$  SEM of 6 individual determinations. Key: (\*) significantly different from basal level in the absence of NADPH ( $P < 0.05$ ).

However, in this case the NADPH-dependent excess ROS generation was not depressed significantly (data not shown). Superoxide dismutase (200 U/mL) completely blocked the stimulatory effect of NADPH, revealing a key requirement for the superoxide anion in the promotion of oxidation of the DCFH probe. However, enhancement of ROS by NADPH was not inhibited significantly by 100  $\mu$ M deferoxamine, a potent inhibitor of iron-promoted ROS generation (Fig. 3). A parallel result was obtained when another iron chelator, diethylenetriaminepentaacetic acid (DETAPAC), was substituted for deferoxamine (data not shown). Thus NADPH-stimulated ROS production did not appear to require the presence of low molecular weight iron.

The effects of chemicals known to serve as substrates for different cytochrome oxidases, upon NADPH-stimulated ROS, were tested. These

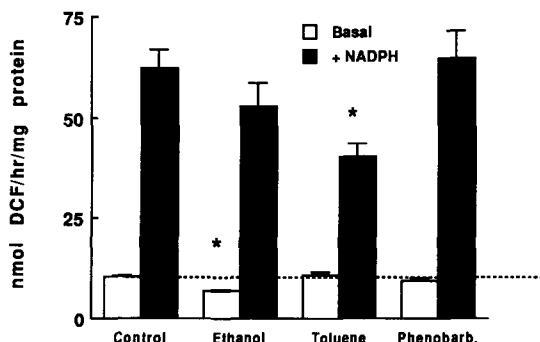


Fig. 4. NADPH-stimulated ROS synthesis in the presence of substrates for cytochrome P450 enzymes: 100 mM ethanol, 22 mM toluene and 100 mM phenobarbital. Values are the means  $\pm$  SEM of 5–6 individual determinations. Key: (\*) significantly different from the corresponding control value ( $P < 0.05$ ).

included phenobarbital (100 mM), ethanol (100 mM), and toluene (22 mM) [13–16]. While ethanol quenched basal microsomal ROS generation at the concentration used, the other substrates did not alter this function significantly. NADPH-effected oxidative activity was not enhanced by any of these substrates, but toluene significantly depressed the response to NADPH (Fig. 4).

## DISCUSSION

Many reactive oxygen species are implicated as intermediaries, enabling the oxidation of DCFH. However, it is likely that the more stable oxidants, such as nitric oxide, superoxide and hydrogen peroxide, act indirectly by conversion to a short-lived, reactive species that is the direct oxidant. Thus, hydrogen peroxide and superoxide, in the presence of iron, can give rise to the hydroxyl radical by way of the Fenton and Haber–Weiss reactions. The precursor status of hydrogen peroxide, superoxide and nitric oxide is suggested by ability of iron chelators to inhibit the oxidation of DCFH [8, 10, 17]. There has been considerable difficulty and controversy concerning unequivocal identification of these potent terminal agents.

The cytochrome P450 enzyme source of NADPH-stimulated ROS generation is supported by the finding that, when brain microsomes were tested in an analogous manner, NADPH had no stimulatory capacity [12]. Cerebral tissues are known to possess very low levels of mixed-function oxidases [18].

It is possible, however, that NADPH–cytochrome P450 reductase rather than cytochrome P450 was the source of peroxide. Such peroxide could then form ROS by interaction with cytochrome P450-associated heme [19]. This interaction could also be sensitive to SKF 525A, perhaps by exclusion of DCFH from the P450 binding site. The failure of an iron chelator, deferoxamine, to inhibit NADPH-stimulated DCFH oxidation would be in accord with this concept. Since both cytochrome P450 and cytochrome P450 reductase are low in brain [20], the failure of brain ROS production to be enhanced by NADPH does not allow distinction between these two possible sites of response to NADPH. While we found ROS generation to be enhanced markedly by NADPH, another group using liver microsomes found NADPH stimulation of hydroxyl radical generation to occur only in the presence of azide [12]. This implies that catalase may play a key role in the regulation of levels of induced ROS. However, in the present study, exogenous catalase, even at very high levels, was not able to inhibit NADPH-catalyzed ROS formation completely. Glutathione peroxidase had no significant effect upon this stimulated ROS formation, further suggesting that  $H_2O_2$  is not a major intermediary in this process.

Some of these apparent inconsistencies may be related to the different means by which ROS are assayed. Measurement of secondary changes involving oxidative damage to water-soluble proteins may yield different data than the use of a lipophilic probe, which is directly oxidized to a fluorescent derivative by ROS [21]. In addition, the ultimate oxidizing species are very short-lived and are probably unable

to leave the compartment within which they are generated.

Inhibition of NADPH-effected ROS formation by superoxide dismutase suggested that superoxide is an obligatory intermediate. We have found previously that the oxidation of DCFH, the dye precursor employed in this study, cannot proceed by a direct interaction with superoxide anion or with hydrogen peroxide, but that a highly reactive oxidant intermediate is required [10]. There is some evidence that oxygenated intermediates of cytochrome P450 can decompose in a minor side reaction and release superoxide [4]. Metal ion-catalyzed reactions of the Haber–Weiss and Fenton type can lead to the formation of very active oxidant species such as the hydroxyl radical or the ferryl ion. However, the lack of effect of deferoxamine upon NADPH-stimulated microsomal ROS activity reported here suggests that such ROS formation may not be mediated by the presence of low molecular weight iron. This is in contrast to other reports on liver microsomes where deferoxamine was found to be a potent inhibitor of NADPH-dependent oxidation of ethylene glycol or ketothiomethylbutyrate [22, 23]. It is possible that the covalently sulfur-bound iron within some cytochrome P450 enzymes is unavailable for chelation and may yet be capable of catalyzing redox cycling. Results obtained with chelators are not easily interpreted. While ferric iron chelation can depress ROS formation, chelation of the ferrous form can enhance ROS [24, 25]. Stimulation of oxidative events in the intact animal or isolated preparation by deferoxamine has also been reported [26, 27]. Thus, the possibility of redox cycling of deferoxamine-bound iron, or of protein-bound iron, cannot be ruled out. However, using specifically-prepared iron-free media, hepatic microsomal suspensions are still capable of the formation of hydroxyl radicals [12]. Superoxide is in equilibrium with the very reactive perhydroxyl radical,  $\text{HO}_2^{\cdot}$ . In aqueous media at physiological pH, very little perhydroxyl is present, but its proportion is increased within a lipid matrix [28]. This represents another mechanism by which the need for iron in the microsomal generation of active oxygen species by oxidases could be circumvented. The possibility of the subsequent formation of a reactive  $\text{NADP}^{\cdot}$  radical [29] may be yet another means by which classical Fenton chemistry is not essential in the formation of ROS.

Iron dependence of NADPH-dependent ROS generation is pronounced after ethanol induction of the specific cytochrome P450 IIEI enzyme [30, 31]. It may be that NADPH-dependent oxidation of a chemical differs qualitatively from NADPH oxidation in the absence of a substrate. Under the latter circumstances, Fenton chemistry may not be essential for the formation of reactive oxidant species by cytochrome P450. This may account for the failure of substrates for mixed-function oxidases to enhance ROS formation, either in the presence or absence of NADPH. Other reports have also indicated that these enzymes can be more potent in ROS production in the absence of exogenous substrates [32, 33]. Ethanol-inducible cytochrome P450 IIEI oxidase is especially capable of effecting oxidative events since it has a high rate of oxidase activity in the absence

of substrate [34, 35]. This enzyme has been proposed to play a significant role in oxygen-mediated tissue toxicity [36]. This enzyme subclass is not inhibited by SKF 525A [37]. Since we found SKF 525A to block completely NADPH-dependent ROS generation, it follows that cytochrome P450 IIEI contributed little to the NADPH-stimulated ROS production observed here.

Cytosolic levels of NADPH in the liver are in the range of 100–200  $\mu\text{M}$  [38, 39]. Thus, a significant amount of free-radical generation may occur by this means in the hepatic cell under basal metabolic conditions. Although SKF 525A alone significantly reduced ROS production by isolated microsomes in the absence of exogenous NADPH, this result may not completely reflect the magnitude of the cytochrome P450 contribution in the intact cell where a significant concentration of NADPH is present. Other sources of reactive oxygen, including arachidonic acid catabolism and xanthine oxidase, may not contribute as much pro-oxidant activity as the NADPH-stimulated microsomal oxidases. Even in the uninduced state, hepatic mixed-function oxidases are likely to constitute a major source of total intracellular production of free radicals.

The data suggest that the mixed-function oxidase systems make a significant contribution to overall hepatic ROS generation. This contribution does not depend on the presence of xenobiotic substrates and may occur in the absence of free low molecular weight iron compounds.

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