Butylated Hydroxyanisole-stimulated NADPH Oxidase Activity in Rat Liver Microsomal Fractions*

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(Received for publication, April 4, 1983)

NADPH-dependent oxygen utilization by liver microsomal fractions was stimulated by the addition of increasing concentrations of butylated hydroxyanisole concomitant with the inhibition of benzphetamine N-demethylase activity. The apparent conversion of monooxygenase activity to an oxidase-like activity in the presence of the antioxidant was correlated with the partial recovery of the reducing equivalents from NADPH in the form of increased hydrogen peroxide production. The progress curve of liver microsomal NADPH oxidase activity in the presence of butylated hydroxyanisole was stimulated by the addition of the antioxidant was correlated with the apparent NADPH oxidase activity as measured by benzo[a]pyrene hydroxylase and benzphetamine demethylase activities (9). In the present study, addition of BHA to liver microsomes in the presence of NADPH and oxygen was shown to increase the utilization of molecular oxygen and NADPH but to decrease the apparent monooxygenase function of cytochrome P-450. These results were shown to be due to the formation of a BHA metabolite capable of interacting with NADPH-cytochrome c (P-450) reductase and directly linking the flavoprotein to oxygen reduction.

MATERIALS AND METHODS

Sprague-Dawley [Crl:CD(SD)BR] rats (200 g) purchased from the Charles River Breeding Laboratory (Wilmington, MA) were treated with phenobarbital in 0.9% saline (50 mg/kg) intraperitoneally on four consecutive days. The rats were fasted for 18 h prior to decapitation. Liver microsomal fractions were prepared as described by Remmer et al. (10). In all experiments, liver microsomal protein from phenobarbital-treated animals was used immediately after preparation. Rat liver microsomal NADPH-cytochrome c (P-450) reductase (EC 1.6.2.4) was prepared by the method of Yasukochi and Masters (11); the turnover number for cytochrome c reduction in 100 mM Tris-HCl, pH 7.7, at 25 °C was 1400 mol of cytochrome c reduced/min/mol of flavin. Analysis of the enzyme preparation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis demonstrated a single protein band upon staining with Coomassie blue (11). Butylated hydroxyanisole, menadione, and NADP(H) were obtained from Sigma. t-Butyldihydroquinone and metyrapone were purchased from Aldrich. t-Butyldihydroquinone was prepared by oxidizing t-butyldihydroquinone (1 mmol) with chromium trioxide (6 mmol) in pyridine (12). The melting point of this compound (58–59 °C) was identical with that reported by McKillop et al. (13). Upon mass spectral analysis, the t-butyldihydroquinone was noted to have a fragmentation pattern consisting of a parent peak (m/e 164), loss of CH₃ (m/e 149), and loss of CO (m/e 121). Identical increases in the microsomal NADPH oxidase activity were obtained with either commercially available BHA or BHA purified by hplc using a radial compression module (RCM-100) from Waters Associates Inc., (Milford, MA) with a Radial Pak A (C₁₈) column and a mobile phase of 50% methanol at a flow rate of 2 ml/min. The effluent was monitored at 270 nm and BHA was noted to elute at 22 min. BHA was dissolved in acetone and added in a volume of less than 0.1% of the volume of the reaction mixture; this volume of acetone did not alter the NADPH oxidase activity of microsomal cytochrome P-450 or NADPH-cytochrome c (P-450) reductase. The reaction mixtures consisted of 150 mM KCl, 10 mM MgCl₂, 3 mM sodium isocitrate, 0.8 IU of isocitrate dehydrogenase/ml, 150 µM NADP, microsomal protein or NADPH-cytochrome c (P-450) reductase.
peroxide formation is discussed in a later section. With 200 \mu M BHA, the stimulation of both NADPH oxidase activity (Fig. 1). At a concentration of 200 \mu M BHA, the stimulation of both NADPH oxidase activity (Fig. 1). In the presence of NADPH alone, an endogenous rate of 17 ± 1 nmol of O₂ consumed/min/mg of microsomal protein was obtained. This rate was increased to a maximal activity of 64 ± 6 nmol/min/mg in the presence of 200 \mu M BHA. The ability of BHA to alter monoxygenase function was also demonstrated by measuring the inhibition of benzphetamine N-demethylation activity in the presence of the antioxidant (Fig. 1). At a concentration of 200 \mu M BHA, greater than 80% of the N-demethylation activity of liver microsomes was inhibited. The fold stimulation of the NADPH oxidase activity was identical with the fold stimulation of oxygen utilization and hydrogen peroxide production seen with 200 \mu M BHA (Table I). The stimulation of both NADPH oxidation and oxygen utilization to yield at least one form of reduced oxygen supports the assumption that BHA can alter the monoxygenase activity of liver microsomes and enhance the NADPH oxidase activity. The stoichiometry of hydrogen peroxide formation is discussed in a later section.

Characterization of a BHA Metabolite—The progress curve of liver microsomal NADPH oxidation in the presence of BHA had a small lag in pyridine nucleotide oxidation, suggestive of the formation of a metabolite which might enhance the NADPH oxidase activity (Fig. 2A). A similar small lag was noted when one measured oxygen utilization (data not shown). After 20 min, the reaction mixture was extracted with ethyl acetate, the organic phase was evaporated to dryness, and the residue was dissolved in a minimal volume of acetone. Addition of an amount of the extract (equivalent to 3 ml of the original reaction mixture) to a 3-ml cuvette containing liver microsomes in the presence of NADPH and oxygen led to an enhanced NADPH oxidase activity (3.2-fold, Fig. 2A) without a lag phase similar to the result obtained with the quinone, menadione (17).

Addition of the microsomal extract as described above to solutions containing NADPH and purified NADPH-cytochrome c (P-450) reductase greatly stimulated (50-fold) the NADPH oxidase activity of the flavoprotein (Fig. 2B). The concentration of pure flavoprotein used (51 nm) was similar to the concentration of NADPH-cytochrome c (P-450) reductase in a 1 mg/ml solution of liver microsomes from pheno-
solution of the hydroquinone was allowed to stand at room temperature for at least 15 min prior to its addition to the cuvette. The metabolite(s) of BHA may be the t-butylhydroquinone or its oxidation product, t-butylhydroquinone. Quinones such as menadione have been shown to interact directly with the flavoprotein reductase and subsequently reduce molecular oxygen to superoxide anion radical or hydrogen peroxide (17).

In order to demonstrate that t-butylhydroquinone and t-butylquinone are formed during the metabolism of BHA, we used hplc techniques to separate the hydroquinone and other metabolites from BHA as described under "Materials and Methods.” The reverse phase hplc elution profiles for the ethyl acetate extracts of reaction mixtures containing NADPH, BHA, and liver microsomes incubated for 20 min are shown in Fig. 3. A significant amount of material which stimulated the NADPH oxidation activity of the flavoprotein was found to elute from normal and reverse phase hplc columns with retention times identical with t-butylhydroquinone. The mass spectra of this active metabolite was identical with that of the authentic t-butylhydroquinone (Fig. 4). The fragmentation pattern consisted of a parent peak (m/e 168), loss of CH₃ (m/e 151), and loss of CO (m/e 123).

A second metabolite was observed (Fig. 3) which also stimulated the NADPH oxidase activity of the purified flavoprotein.

![Fig. 2. Stimulation of the NADPH oxidase activity of rat liver microsomal fractions or purified NADPH-cytochrome c (P-450) reductase.](image-url)

**Fig. 2.** Stimulation of the NADPH oxidase activity of rat liver microsomal fractions or purified NADPH-cytochrome c (P-450) reductase. The rate of NADPH oxidation with liver microsomes (0.2 mg/ml) from phenobarbital-treated rats in the presence of 140 μM NADPH and 2 mM 5'-AMP was measured by the loss of absorbance at 340 nm (A). The effect of various compounds was observed: BHA (200 μM), menadione (Kᵢ, 100 μM), or a volume of the ethyl acetate extract of a 20-min reaction mixture containing NADPH, oxygen, BHA, and liver microsomes equivalent to 3 ml of reaction mixture (MET). The NADPH oxidase activity of purified rat liver microsomal NADPH-cytochrome c (P-450) reductase (51 nM) was measured at 340 nm in the presence of 140 μM NADPH (B). The effect of various compounds were observed: BHA (200 μM), HQ (50 μM), Kᵢ (100 μM), or a volume of the ethyl acetate extract of a reaction mixture containing NADPH, BHA, oxygen, and liver microsomes equivalent to 3 ml of reaction mixture (MET). A volume of the ethyl acetate extract of a 20-min reaction mixture containing only BHA, oxygen, and liver microsomes equivalent to 3 ml of reaction mixture (minus NADPH) gave the same results with the pure flavoprotein as did BHA alone. The arrows at 0 and 5 min represent the addition of NADPH and effector, respectively.

![Fig. 3. The reverse phase hplc profile of BHA metabolites.](image-url)

**Fig. 3.** The reverse phase hplc profile of BHA metabolites. The conditions of the hplc analysis are as described under “Materials and Methods.” The elute was extracted with ethyl acetate, dried, and redisolved in the same volume used for addition of the sample into the hplc system. A constant volume of the original material or each fraction was added to 3 ml of the purified flavoprotein in the presence of 140 μM NADPH to measure the stimulated NADPH oxidation activity. A, standards. B, ethyl acetate extract of a 20-min incubation mixture of 2 mg/ml of liver microsomes from phenobarbital-treated rats, NADPH, oxygen, and 200 μM BHA. C, relative stimulation of the NADPH oxidase activity with purified NADPH cytochrome c (P-450) reductase (51 nM).
Stoichiometry of Hydrogen Peroxide Formation - It has been established that in the absence of any exogenously added substrates, liver microsomes catalyze the oxidation of NADPH to yield hydrogen peroxide (18). With rat liver microsomes, a 1:1 stoichiometry of pyridine nucleotide oxidation to oxygen utilization was noted and approximately 50% of both NADPH and oxygen utilized could be accounted for as hydrogen peroxide (19). These results were further substantiated by our data demonstrating that the oxidation of 96 μM NADPH utilized 92 μM molecular oxygen and formed approximately 48 μM hydrogen peroxide (Table II). The presence of 200 μM BHA, which stimulated maximally the consumption of oxygen, altered slightly the ratio of reducing equivalents utilized per mol of hydrogen peroxide formed (95 μM NADPH and 91 μM oxygen are utilized to form 54 μM hydrogen peroxide, Table II). The increased stoichiometry (from 0.50 to 0.57 in the presence of BHA) demonstrated that not only was the rate of hydrogen peroxide production stimulated (Table I), but the recovery of reducing equivalents from NADPH was apparently increased (p = 0.06).

Addition of t-butylhydroquinone (50 μM) and menadione (5 μM) which stimulated microsomal oxygen utilization and hydrogen peroxide production by 3- to 4-fold increased the stoichiometry from 0.50 in the absence of effectors to 0.64 and 0.61 in the presence of HQ and K3, respectively. Since quinones are known to interact directly with NADPH-cytochrome P-450) reductase, t-butylhydroquinone (or its oxidation products) and menadione directly linked NADPH oxidation to hydrogen peroxide production to give a stoichiometry of hydrogen peroxide produced to NADPH oxidized of 1.01 and 0.98, respectively (Table II). The lack of complete recovery of reducing equivalents as hydrogen peroxide in the presence of liver microsomes cannot be explained to date. Based on the work of others (19, 20), it is clear that either reducing equivalents most likely are utilized for metabolism of endogenous substrates, for formation of some reduced oxygen species other than superoxide anion radical or hydrogen peroxide or for some unknown chemical reaction with a component of liver microsomes. The quantities of hydrogen peroxide produced in the presence of liver microsomes and NADPH by BHA, t-butylhydroquinone, or menadione demonstrate the partial uncoupling of the flavoprotein reductase and the subsequent linking of NADPH oxidation to oxygen reduction without involvement of the heme protein, cytochrome P-450.

Effect of Monoxygenase Inhibitors on the NADPH Oxidase Activity of Liver Microsomes - Werringloer has demonstrated that the endogenous production of hydrogen peroxide by liver microsomes is effectively inhibited by carbon monoxide and suggested that cytochrome P-450 may serve as the terminal oxidase for this process (19). In the presence of 1 mM metyrapone, a known inhibitor of cytochrome P-450, the BHA-
that the interaction of BHA with liver microsomal cytochrome consumption, and hydrogen peroxide formation. BHA does by identical fold increases in NADPH oxidation, oxygen

Fig. 2. The values represent the average ± S.D. or 3 preparations of rat liver microsomes.

The incubation mixture containing 140 μM NADPH, 2 mM $5'$-AMP, and 0.2 mg/ml of liver microsomal protein was as described in Fig. 2. The values represent the average ± S.D. or 3 preparations of rat liver microsomes.

Metyrapone was added simultaneously with BHA or t-butylhydroquinone, the amount of inhibition was less than 5 and 15%, respectively. Addition of 15 μM dicoumarol had no effect on either the rates or extents of NADPH oxidation or H$_2$O$_2$ formation ruling out a role of microsomal detoxification pathways, i.e. increased conjugation and excretion of proximal carcinogens (8) may also contribute to the anticarcinogenic activity of this antioxidant.

### References

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