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Acetone Enhancement of Cumene Hydroperoxide-supported Microsomal Cytochrome P450-dependent Benzo(a)pyrene Hydroxylation

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***In vitro* effects of acetone on cytochrome P450 (P450)-dependent benzo(a)pyrene (B(a)P) hydroxylation supported by cumene hydroperoxide (CuOOH) or NADPH/O_2 systems were studied using 3-methylcholanthrene-pretreated rat liver microsomes. The maximal rate of B(a)P hydroxylation at constant concentration (80 μM) of the substrate was observed in the presence of 30 μM CuOOH. However, at concentrations higher than 30 μM CuOOH the hydroxylation rates were rapidly decreased. In contrast to CuOOH, at a concentration of 200 μM NADPH, B(a)P hydroxylation rate reached a plateau. At concentrations higher than 200 μM NADPH, the rates of substrate hydroxylation were maintained at the maximal rate with no inhibition. Acetone at 1% (v/v) enhanced both CuOOH- and NADPH/O_2-supported B(a)P hydroxylation at the optimal concentrations of the cofactors. At concentrations higher than 1% (v/v) acetone, substrate hydroxylation was sterero specific under the support of these two cofactors; it was strongly enhanced with 30 μM CuOOH, but rather inhibited in the 200 μM NADPH/O_2 system. The lipid peroxidation rate induced during CuOOH-supported P450-dependent B(a)P hydroxylation was increased as CuOOH concentrations were increased. Acetone in the concentration range of 2.5~7.5%(v/v) inhibited lipid peroxidation during CuOOH supported B(a)P hydroxylation. The finding that CuOOH-supported B(a)P hydroxylation is greatly enhanced by acetone suggests that acetone may contribute more to the activation of oxygen (for the insertion of oxygen into the substrate) in the presence of CuOOH than with NADPH/O_2. Acetone may also contribute to the partial inhibition of destruction of microsomal membranes by lipid peroxidation.**

Keywords: Acetone, Benzo(a)pyrene hydroxylase, Cumene hydroperoxide, Cytochrome P450, NADPH.

Introduction

Cytochromes P450 (P450) comprise a family of hemoproteins which catalyze the oxidation of exogenous chemicals such as water-insoluble drugs, organic solvents (Casazza *et al.*, 1984; Pattern *et al.*, 1986; Toftgard *et al.*, 1986), and carcinogens, as well as endogenous compounds such as steroids, fatty acids, and prostaglandins (Guengerich, 1991; Porter and Coon, 1991). During the oxidation reaction by NADPH/O_2, two electrons are transferred from NADPH to the heme iron of P450 via NADPH-P450 reductase (Ballou *et al.*, 1974; Guengerich *et al.*, 1975). The heme iron forms an oxyferro complex with molecular oxygen and electrons received from NADPH P450 reductase are transferred to the oxygen (Estabrook *et al.*, 1971; Blake and Coon, 1981a). Electron transfer to the oxygen results in the production of an activated oxygen which then inserts into the substrate. Alternatively, organic hydroperoxides are also able to support the substrate oxidation in the absence of NADPH and P450 reductase (Nordblom *et al.*, 1976; Blake and Coon, 1981a; 1981b; Vaz *et al.*, 1990). Although sharing a common ternary peroxide complex (Nordblom *et al.*, 1976; White *et al.*, 1980; Larroque and van Liehr, 1986), the mechanisms of the NADPH/O_2- and hydroperoxide-supported reactions may differ in certain aspects (van Lier and Rousseau, 1976). Since the hydroperoxide-supported P450 substrate reaction only involves a single protein and two substrate molecules, it provides a valuable and simple

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model system for investigating the nature of the activated oxygen intermediate involved in P450 catalyzed oxidation (White *et al.*, 1980; Blake and Coon, 1980; 1981a; 1981b).

Many investigators have studied the effect of acetone on the P450-dependent monooxygenases in microsomes (Powis *et al.*, 1977; Vore and Soliven, 1979; Moldeus and Gergely, 1980; Lee *et al.*, 1998a; 1998b). Although their studies were focused on the basis of alteration of the binding site of P450 (Backes and Canady, 1981), electron flow (Miwa *et al.*, 1978), or oxygen consumption of the monooxygenase system (Anders and Gander, 1979), the mechanism underlying the action of acetone is not yet clearly understood.

In the previous study, we reported that acetone, in a limited range of concentration, specifically enhanced B(a)P hydroxylation in 3-methylcholanthrene (MC)- or β-naphtoflavone-inducible rat liver microsomes, but not in uninduced- or phenobarbital-induced ones (Lee *et al.*, 1998a). In order to elucidate the enhancing mechanism of B(a)P hydroxylation by acetone in the present study, we investigated the effect of acetone on the hydroxylation rate of B(a)P supported by NADPH/O_2 or by cumene hydroperoxide (CuOOH) using liver microsomes from Sprague-Dawley rats induced with MC.

Materials and Methods

Chemicals B(a)P, cumene hydroperoxide, sodium dithionite, and NADPH were purchased from Sigma Chemicals Co. (St. Louis, USA). MC was purchased from Eastman Kodak Co. (Rochester, USA). Acetone (purity 99.7%) was purchased from Merck Co. (Darmstadt, Germany). Other chemicals used were of the highest quality grade commercially available.

CYP1A1/1A2 Induction For the induction of CYP1A1/1A2, male Sprague-Dawley rats (150–160 g) were administered with MC (20 mg/kg in corn oil) by intraperitoneal injections for three consecutive days as described elsewhere (Kim *et al.*, 1990; Moon *et al.*, 1998). Control rats were maintained *ad libitum* without receiving any chemicals and the inducer-treated rats were fasted overnight before sacrificing. Microsomes were prepared by a differential centrifugation as described previously (Moon *et al.*, 1986; Lee and Park, 1989). The content of microsomal P450 was measured as described by Omura and Sato (1964).

Enzyme assays The NADPH/O_2-supported B(a)P hydroxylation was assayed according to the procedure of Nebert and Gelboin (1968) with slight modification as previously described (Lee *et al.*, 1998a). The reaction mixture contained 100 μmol potassium phosphate (pH 7.4), 5 μmol $MgCl_2$, 0.1 μmol EDTA, 0.4 μmol NADPH, and microsomes in a final volume of 1.0 ml. After a preincubation of 5 min at 37°C, the reaction was initiated by the addition of 80 nmoles B(a)P in 20 μl methanol, and the mixture was incubated for 10 min at 37°C. The activity was expressed in turnover numbers, i.e., nmol of phenolic products formed/min/nmol of cytochrome P450 (Nebert and Gelboin, 1968). Similar conditions were used for assaying the CuOOH-supported B(a)P hydroxylation. The reaction mixture contained 100 μmol potassium phosphate, pH 8.0, 5 μmol $MgCl_2$, 0.1 μmol EDTA, 80 nmol B(a)P, and microsomes in a final volume of 1 ml. After preincubating at 37°C for 5 min, the reaction was initiated by the addition of varying concentrations of CuOOH in 10 μl distilled water and allowed to proceed for 10 min.

Assay for lipid peroxidation Lipid peroxidation of the microsomal membrane during the reaction of B(a)P hydroxylation supported by CuOOH or NADPH/O_2 was measured using the method of Andersen *et al.* (1993). After incubating for an appropriate time at 37°C for the substrate hydroxylation, aliquots of the reaction mixtures (0.2 ml) were directly mixed with 1.5 ml of 20% (v/v) acetic acid, 0.225 ml of 8.1% (w/v) SDS, and 1 ml of 1.2% (w/v) thiobarbituric acid in a total volume of 3 ml and boiled for 30 min. After cooling to room temperature, absorbance of the samples was read at 532 nm for measuring the amount of thiobarbituric acid reactive substances (TBARS) formed. Lipid peroxidation was expressed as the level of malondialdehyde (MDA) calculated from the calibration curve using 1,1,3,3-tetramethoxypropane.

Statistics Values are presented as mean ± SD. The student *t*-test was performed on the individual sets of data. Differences were considered to be significant when $p<0.05$.

Results

Effect of CuOOH or NADPH on P450-dependent B(a)P hydroxylation activity in MC-pretreated rat liver microsomes Our preliminary studies served to characterize CuOOH-dependent B(a)P hydroxylation (Lee *et al.*, 1998b). Figure 1 demonstrates the effect of CuOOH or NADPH on P450-dependent B(a)P hydroxylase activity in MC-pretreated rat liver microsomes. The hydroxylation rate of the substrate was almost linearly increased at low concentrations of CuOOH (<30 μM), indicating that P450 inactivation was not significant (Fig. 1A). The maximal rate of B(a)P hydroxylation at 80 μM of the substrate was observed at 30 μM CuOOH and for a 10 min reaction time. At high concentrations of CuOOH (>30 μM), the hydroxylation rate of B(a)P dropped drastically, suggesting that the inactivation of P450 had become an important consideration (Fig. 1A). In contrast to CuOOH, NADPH drastically increased the substrate hydroxylation rate at 0.24 mM NADPH and reached to a plauteau at 1.2 mM NADPH. The hydroxylation rate was maintained at concentrations higher than 1.2 mM NADPH with no decrease (Fig. 1B).

Comparison of acetone effect on CuOOH- and NADPH/O_2-supported B(a)P hydroxylation The effect of acetone on the rate of CuOOH- and NADPH/O_2-supported B(a)P hydroxylation in liver microsomes from MC-pretreated rats was studied (Fig. 2). Characteristically, acetone in a limited concentration range increased the rate of B(a)P hydroxylation supported by the NADPH/O_2

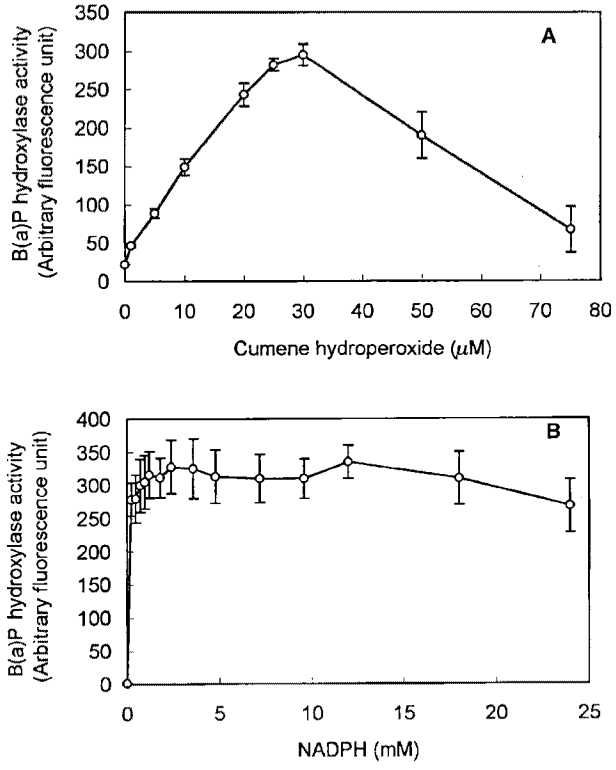

Fig. 1. Sensitivity of CuOOH or NADPH for B(a)P hydroxylase activity in MC-pretreated rat liver microsomal system. A; CuOOH-sensitivity, B; NADPH-sensitivity.

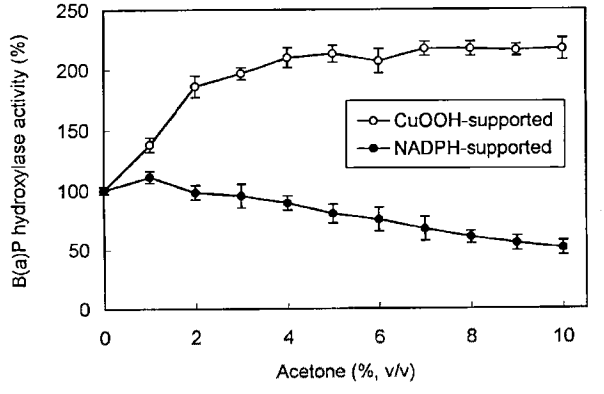

Fig. 2. Acetone effects on CuOOH- or NADPH-supported B(a)P hydroxylation in MC-pretreated rat liver microsomes.

system; at only 1% (v/v), it was enhanced maximally (11%), but substrate hydroxylation was inhibited with increasing concentrations of acetone. The addition of 1% (v/v) acetone to the CuOOH-supported system resulted in a great enhancement (38%) of B(a)P hydroxylation and the enhancing effect of acetone was dependent upon the concentration of acetone up to 2% (v/v). At concentrations higher than 3%, hydroxylation rates were not affected.

These results that acetone enhances B(a)P hydroxylation supported by both NADPH/O_2 and CuOOH suggest that acetone may facilitate the formation of an activated oxygen species or the insertion of oxygen into the substrate. That CuOOH-supported B(a)P hydroxylation is enhanced more strongly by acetone than NADPH/O_2-supported B(a)P hydroxylation suggests that the solvent may contribute more to the production of activated oxygen to be inserted to the B(a)P molecule in the presence of CuOOH than with NADPH/O_2. The action of CuOOH as an oxygen donor for P450-dependent B(a)P hydroxylation may also contribute to our results.

Effect of acetone on microsomal lipid peroxidation during B(a)P hydroxylation We investigated whether the acetone-enhancing effect on B(a)P hydroxylation in MC-pretreated rat liver microsomes was due to the inhibition of lipid peroxidation of the microsomal membrane by acetone. As shown in Fig. 3, during the substrate hydroxylation by CuOOH, TBARS levels were increased with increasing concentrations of CuOOH, indicating the occurrence of lipid peroxidation in the microsomes. Under the condition of 2% acetone concentration, TBARS formation was inhibited at concentrations of 25, 50, and 75 μM CuOOH by 10, 7, and 9%, respectively. However, at 150 μM CuOOH, acetone had no effect on the inhibition of TBARS formation or rather slightly enhanced the formation. TBARS production in NADPH/O_2-supported B(a)P hydroxylation was not detected at concentrations even higher than 20 mM NADPH (data not shown). These results indicate that CuOOH easily induces lipid peroxidation because of a potent trigger whereas NADPH does not form enough

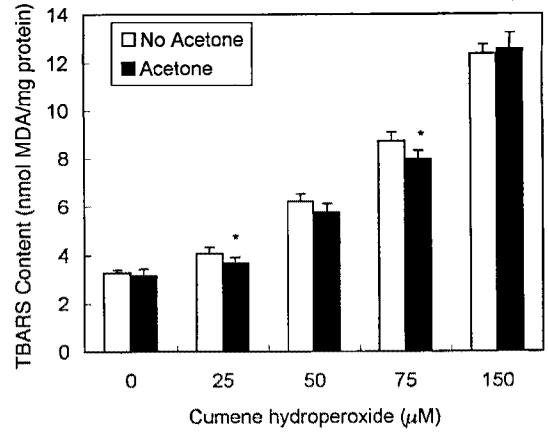

Fig. 3. Comparison of the inhibitory effect of acetone on lipid peroxidation induced during CuOOH-supported B(a)P hydroxylation in MC-pretreated rat liver microsomes. B(a)P hydroxylation supported by CuOOH in the presence of 2% (v/v) acetone was applied as described in Materials and Methods. The results shown are means ± SD of three experiments performed in duplicate. *$p<0.05$ for no acetone vs acetone.

reactive oxygen species (ROS) for lipid peroxidation since there are no available metals in the reaction system. However, acetone in limited concentrations protected from lipid peroxidation partially, as shown in Fig. 3. Incubation of acetone, up to 10% (v/v), with various concentrations of MDA in the absence of P450 produced no detectable TBARS formation (data not shown).

Discussion

In the present work, the *in vitro* effect of acetone enhancement on the P450-dependent B(a)P hydroxylation in MC-pretreated rat liver microsomes was investigated using NADPH/O_2 or CuOOH as a cofactor. Our finding that the differential effect of acetone was observed in the NADPH/O_2- and CuOOH-supported B(a)P hydroxylation may be potentially helpful, although partially, in elucidating the enhancing mechanism of the hydroxylation of B(a)P by acetone.

Many investigators have proposed the kinetic models of substrate hydroxylation of P450 supported by CuOOH (Rahimtula *et al.*, 1974, Nordblom *et al.*, 1976; Koop and Hollenberg, 1980; Yu *et al.*, 1996). The models describe that P450 binds to the substrate and hydroperoxide and forms a ternary hydroperoxide complex. Protonation and subsequent loss of a molecular water or organic alcohol from this ternary hydroperoxide complex leaves an activated oxygen species which rapidly attacks the substrate. The complex is also believed to exist in the reaction of P450 supported by NADPH/O_2.

The reason that CuOOH is effective in the inactivation of P450 at concentrations higher than 30 μM is probably that CuOOH is a hydroperoxide, which easily induces lipid peroxidation. Thus, the inactivation of P450-dependent B(a)P hydroxylation supported by CuOOH at higher concentrations was accompanied by the lipid peroxidation of microsomal membranes (Fig. 3).

There is a possibility that the differential effect of acetone enhancement in the NADPH/O_2- and CuOOH-supported B(a)P hydroxylation may result from the use of multiple P450 isoforms of microsomes and differential metabolic reactions of the cofactors to the P450 isoforms. In fact, metabolism of B(a)P supported by NADPH/O_2 and by CuOOH shows a marked difference in the profiles of their products (Capdevila *et al.*, 1980; Wong *et al.*, 1986), which supports our discussion in this study. According to Wong *et al.* (1986), NADPH/O_2-supported B(a)P metabolism in MC-induced rat liver microsomes yields a major increase in 3-hydroxy B(a)P, dihydrodiols, and quinones. On the other hand, the major metabolites of B(a)P are dependent on CuOOH concentration.

Differences in degree of lipid peroxidation in NADPH/O_2- and CuOOH-induced systems in MC-pretreated rat liver microsomes might be due to the differential induction of CYP 1A1 and 1A2 by MC. Guengerich (1987) reported that MC induces much higher levels of CYP1A1 than CYP1A2 in hepatic microsomes. Ohmori *et al.* (1993) reported that CYP1A2 catalyzed NADPH/O_2-induced lipid peroxidation more effectively than CYP1A1 did. In contrast to NADPH/O_2-induced lipid peroxidation, that of CuOOH was much higher by CYP1A1 than CYP1A2. They also reported that CYP1A1 is more active in catalyzing CuOOH-induced lipid peroxidation than CYP1A2 and catalyzes the formation of TBARS to a greater extent than CYP1A2.

It is known that hydroxyl radical scavengers do not inhibit NADPH-induced lipid peroxidation catalyzed by CYP1A1 and CYP1A2 (Ohmori *et al.*, 1993). Previous investigation (Ohmori *et al.*, 1993) showed that CYP1A2 produces more of the superoxide anion radical than CYP1A1, and the reduction rate of CYP1A2 is 24-fold faster than that of CYP1A1. Weiss and Estabrook (1986) have demonstrated that the ferrous form of P450 is not involved in peroxide-dependent lipid peroxidation, suggesting that CYP1A2 acts mainly as an initiating factor in the organic peroxide-independent lipid peroxidation step.

Acetone has a stereospecifically accessible oxygen atom for binding to the ferric ion of P450, forming the 6-coordinated species, which may facilitate the oxygen activation or the oxygen insertion into the substrate in the CuOOH-supported B(a)P hydroxylation (Anders and Gander, 1979). Akhrem *et al.* (1977) concluded that oxygen insertion into the substrate is the rate-limiting step in hydroperoxide-supported substrate hydroxylation. Although no clear evidence could be made, it would appear that the probable mechanism of acetone enhancement, in the case of CuOOH-supported B(a)P hydroxylation, is to facilitate either the oxygen insertion or the transfer of the oxygen to the substrate as well as its scavenging activity for ROS at a limited concentration.

Further studies may be required to elucidate the enhancement of CuOOH-supported B(a)P hydroxylation by acetone in terms of the kinetic mechanism of the reaction in the liver microsomal P450 system.

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