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Characteristics of Acetone Enhancement of Microsomal Cytochrome P450-dependent B(a)P Hydroxylation in 3-Methylcholanthrene-inducible Rat Liver Microsomes

Dong Wook Lee*, Ja Young Moon, Heung Bin Lim, Hyung Ok Sohn,
Young Gu Lee and Ki Hyun Park

Laboratory of Biochemistry, Korea Ginseng and Tobacco Research Institute

Yusung P. O. Box 7 Taeduk Science Town, Taejon 305-345, Korea

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ABSTRACT : Previously, we showed that acetone enhanced aryl hydrocarbon hydroxylase (AHH) activity in only 3-methylcholanthrene (MC)- or β-naphtoflavone (BNF)-inducible microsomes of rat liver. In the present study, the possible mechanism underlying acetone action on AHH was investigated in the liver microsomes from MC-pretreated rats. Other n-alkylketones except acetone did not increase AHH activity, which rather decreased significantly with the length of alkyl side chain. Acetone had no effect on the activity of NADPH-cytochrome P450 reductase or inhibited the formation of 3-OH benzo(a)pyrene (B(a)P) in nonenzymatic model ascorbic acid system. However, in cumene hydroperoxide (CuOOH)-supported B(a)P hydroxylation, acetone enhanced its velocity remarkably by 30% at the optimal concentration (30 μM CuOOH and 1.0% acetone). From these results, we conclude that acetone may facilitate the formation of an activated oxygen species or the insertion of oxygen into B(a)P molecule in CYP1A rich microsomes.

Key words : **Acetone, Benzo(a)pyrene hydroxylase, Cumene hydroperoxide, Cytochrome P450, 3-Methylcholanthrene, NADPH**

Microsomal cytochromes P450 (P450) catalyze a variety of mixed-function monooxygenase reactions that often result in detoxication of drugs and other xenobiotics including organic solvents (Casazza *et al.*, 1984; Pattern *et al.*, 1986; Toftgard *et al.*, 1986).

Acetone has been shown to enhance the P450-dependent monooxygenases in liver microsomes (Powis et al., 1977; Vore and Soliven, 1979; Moldeus and Gergely, 1980; Lee *et al.*, 1998). The enhancing effect is quite specific for the substrates as well as microsomes sources (Anders, 1971; Anders *et al.*, 1973; Powis *et al.*, 1977; Vore and Soliven, 1979; Moldeus and Gergely, 1980; Lee *et al.*, 1998). However, the mechanism underlying the action of acetone enhancement has not yet been elucidated although explanations such as alteration of the binding site of P450 (Backes and Canady, 1981), electron flow (Miwa *et al.*, 1978), oxygen consumption of the monooxygenase system (Anders and Gander, 1979), or change in microenvironment of the active site of P450 in the microsomal membrane (Lee *et al.*, 1998) have been put forth. In the present study, we investigated the possible mechanism of acetone enhancement on AHH activity in MC-pretreated rat liver microsomes.

* Corresponding author : *Korea Ginseng and Tobacco Research Institute, Shinsung-Dong, Yusung-Ku, Taejon 305-345, Korea*

MATERIALS AND METHODS

Chemicals. Benzo(a)pyrene (B(a)P), cumene hydroperoxide(CUOOH), sodium dithionite, and NADPH were purchased from Sigma Chemicals Co. (St. Louis, USA). 3-Methylcholanthrene (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 grade quality.

Preparation of MC-inducible rat liver microsomes. To prepare the CYP1A-rich liver microsomes, male Sprague-Dawley rats (150-160g) were administered with MC (20 mg/kg in corn oil) by intraperitoneal injection for three consecutive days as described elsewhere (Moon *et al.*, 1998). Microsomes were prepared by a differential centrifugation as described previously (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.*, 1998). The reaction mixture contained 100 μ moles potassium phosphate (pH 7.4), 5 μ moles $MgCl_2$, 0.1 μ mole EDTA, 0.4 μ mole NADPH, and microsomes in a final volume of 1.0 ml. After a preincubation for 5 min at 37 °C, the reaction was initiated by the addition of 80 nmoles B(a)P in 20 μ l methanol, and the mixture was incubated for 10 min at 37 °C. The activity was expressed as nmoles of phenolic products per mg protein per min (Nebert and Gelboin, 1968). Similar conditions were used for assaying the CuOOH-supported B(a)P hydroxylation. The reaction mixture contained 100 μ moles potassium phosphate, pH 8.0, 5 μ moles $MgCl_2$, 0.1 μ mole EDTA, 80 nmoles 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 30 nmols CuOOH dissolved in 10 μ l distilled water and allowed to proceed for 10 min. NADPH-P450 reductase activity was deter- mined by monitoring the reduction rate of P450 at 37 °C for 3-4 min (Gigon *et al.*, 1969).

Formation of 3-OH B(a)P by model ascorbic acid system. The model ascorbic acid system was applied for the test of B(a)P hydroxylation as a nonenzymatic system as described previously (Udenfriend *et al.*, 1954). Reaction mixture consisted of 142 μ M of ascorbic acid, 15 μ M of ferrous sulfate, and 80 μ M of EDTA and 80 μ M of B(a)P as a substrate in 3 ml of 0.1 M phosphate buffer, pH 5.5. The reaction was started by adding B(a)P at 37 °C for 10 min. This model system was also applied for the investigation of acetone effect on B(a)P hydroxylation.

RESULTS

Effect of ketones on NADPH/O_2-supported B(a)P hydroxylation In order to test whether the ketone group in acetone is an essential factor for the activation of P450-dependent B(a)P hydroxylation, a series of n-alkylketones was applied to the assay system. Table 1 shows the effect of methyl *n*-alkyl ketones on P450-dependent B(a)P hydroxylation supported by NADPH and molecular oxygen in the liver microsomes from MC-pretreated rats. Acetone at 1% (v/v) enhanced B(a)P hydroxylation in the MC-inducible microsomes, which is in agreement with the data already shown previously (Lee *et al.*, 1998). However, other ketones at the same concentration

Table 1. Effect of various methyl n-alkyl ketones on the P450-dependent B(a)P hydroxylation in MC-pretreated rat liver microsomes

Methyl n-alkyl ketones	Relative Activity (%)
Control	100
Acetone	111
Methyl ethyl ketone	48
Methyl propyl ketone	39
Methyl butyl ketone	5

The activities were measured at 1% (v/v) of organic solvents. The values are mean of three repeated experiments.

(1%, v/v) inhibited B(a)P hydroxylation and the inhibition was increased by the carbonyl compounds depending on the length of the side chain. In other words, the inhibitory potency of P450-dependent B(a)P hydroxylation by methyl *n*-alkyl ketones was dependent on their hydrophobicity. These results indicate that carbonyl group of the n-alkyl ketones is not an essential factor for the acetone enhancement in B(a)P hydroxylation.

Effect of acetone on NADPH-P450 reductase activity. Effect of acetone on NADPH-P450 reductase activity was investigated whether the enhancement of B(a)P hydroxylation by acetone resulted from the activation of the reductase which transfers electrons from NADPH to P450. As seen in Table 2, acetone at up to 2% (v/v) did not give any effect on the activity of NADPH-P450 reductase in the liver microsomes of rats pretreated with MC. This result indicates that the enhancement of B(a)P hydroxylation by acetone in MC-inducible microsomes was not due to the stimulation of electron flow.

Table 2. Effect of acetone on the activity of NADPH-P450 reductase in MC-pretreated rat liver microsomes

Acetone (%, v/v)	Activity (μmole/min/mg protein)	Relative Activity (%)
0	0.081	100
0.5	0.080	99
1.0	0.081	100
2.0	0.080	99

The values are mean of three repeated experiments.

Effect of acetone on CuOOH-supported B(a)P hydroxylation. The effect of acetone on the rate of CuOOH- supported B(a)P hydroxylation in liver microsomes from MC-pretreated rats was studied (Table 3). The rate of the CuOOH-supported reaction was lowered by 71% compared with that of the NADPH/O_2-supported one. Characteristically, acetone at 1% (v/v) increased the rate of B(a)P hydroxylation supported by NADPH/O_2 system by 11%. Whereas, addition of acetone (1%, v/v) resulted in a great enhancement of B(a)P hydroxylation by 38%.

The results that acetone at 1% (v/v) 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 in enzymatic system. Our another result that acetone enhanced more strongly CuOOH-supported B(a)P hydroxylation than NADPH/O_2-supported B(a)P hydroxylation suggests that the solvent may more contribute to the activation of oxygen to be inserted to B(a)P molecule in the presence of CuOOH than in that of NADPH/O_2. Action of CuOOH as an oxygen donor for P450-dependent B(a)P hydroxylation may be also another contribution to our results.

Effect of acetone on the formation of 3-OH B(a)P in model ascorbic acid system. In order to test whether acetone enhances the formation of 3-OH B(a)P in nonenzymatic system, it was applied in the model ascorbic acid system as described in Materials and Methods. Table 3 shows the results of acetone effect on the formation of 3-OH B(a)P by the model ascorbic acid system. Even though statistically insignificant, acetone at 1% (v/v) rather decreased the formation rate of 3-OH B(a)P by 10%.

Table 3. Effects of acetone on B(a)P hydroxylation in nonenzymatic system or MC- pretreated rat liver microsomal P450- dependent systems

System	Acetone (-)	Acetone (+)	Enhancement (%)
NADPH/O_2-supported[1)]	1.73	1.92	11
CuOOH-supported[1)]	0.50	0.69	38
Model Ascorbic Acid System[2)]	186±44	170±41	-9

Acetone was added to become 1% (v/v) of final concentration. [1)]Activities are expressed as nmol of 3-OH B(a)P/ min/mg protein. [2)]Activity is expressed as pmol of 3-OH B(a)P produced/min.

DISCUSSION

In the present study, we investigated the *in vitro* effect of acetone on P450-dependent B(a)P

hydroxylation and its related system using MC-pretreated rat liver microsomes and obtained some clues on the mechanism underlying acetone action. In the first, we identified that acetone had no effect on the electron transport enzyme, NADPH-P450 reductase, and had the enhancing effect on the B(a)P hydroxylation in only limited concentration of the solvent. Our interest was toward on the ketone group of acetone as an essential factor for the enhancement of AHH. However, all carbonyl compounds tested except acetone did not increase B(a)P hydroxylation, rather strongly inhibited AHH activity with the length of side chain as well as their hydrophobicity. Therefore, we reached a conclusion that the ketone group in acetone is not an important factor for the enhancement of AHH activity in MC-microsomes.

The finding that differences in the effect of acetone observed in the NADPH- and CuOOH-supported B(a)P hydroxylation as well as in model ascorbic acid system may be potentially useful in elucidating the enhancing mechanism of the hydroxylation of B(a)P by acetone. The data obtained from the model ascorbic acid system indicated that acetone enhances B(a)P hydroxylation in only enzymatic systems.

The potential site for acetone action is thought to be oxygen activation or the oxygen insertion step into substrate in CYP 1A-induced microsomes. Akhrem *et al.*, (1977) reported 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 another possibility, CuOOH itself may also contribute to the enhancing effect of acetone on the P450-dependent B(a)P hydroxylation in MC-pretreated rat liver microsomes by acting as an oxygen donor.

More detailed experiments are in progress, quantitating each of the effect of CuOOH and NADPH on liver microsomal P450-dependent B(a)P hydroxylation with the concentrations of acetone. These experiments may help to clarify the exact mechanism of the differential enhancement of acetone observed in this study.

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