Involvement of a Novel Mouse Hepatic Microsomal Esterase, ES46.5K, in the Hydrolysis of Phthalate Esters

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ES46.5K, a novel esterase from mouse hepatic microsomes (Watanabe K., *et al.*, *Biochem. Mol. Biol. Int.*, 31, 25—30 (1993)), catalyzed hydrolysis of phthalate esters. ES46.5K and mouse hepatic microsomes hydrolyzed diethyl-, dibutyl-, diisobutyl-, dioctyl- and diethylhexyl phthalates, whereas dicyclohexyl- and diphenyl phthalates having ring structure were not hydrolyzed by the enzymes. V_{max} (μmol/min/mg protein)/K_m (μM) ratios of ES46.5K for diethyl-, dibutyl-, diisobutyl-, dioctyl- and diethylhexyl phthalates were 291, 2786, 565, 51 and 57, respectively, while those of microsomes were 0.58, 0.83, 1.71, 0.05 and 1.10, respectively. The hydrolytic activity of ES46.5K was inhibited by diisopropylfluorophosphate and bis-*p*-nitrophenylphosphate. These results suggest that ES46.5K has high catalytic activity for phthalate esters and some role in the metabolism of phthalate esters in mice.

Key words hydrolysis; 46.5-kilodalton esterase (ES46.5K); microsome; esterase; phthalate ester

Carboxylesterase (EC 3.1.1.1) hydrolyzes xenobiotics containing an ester, thioester or amide.[1,2] It is well known that hydrolysis of xenobiotics is usually a detoxication process, because metabolites generally are more hydrophilic and more rapidly excreted. In the hydrolysis of phenacetin to phenetidine by carboxylesterase, however, the toxicity of the compound increases.[3] Multiple forms of carboxylesterase are present in numerous mammalian tissues and the highest levels of the enzymes are present in the hepatic microsomes.

The major metabolic pathway of phthalate esters, primary plasticizers used in polyvinyl chloride products, is the hydrolysis of diester to monoester which is suspected to be hepato-carcinogenic and teratogenic after chronic exposure or administration of high dosage.[4,5] Hydrolytic activity for phthalate esters has been detected in pancreas, liver, kidney, lung and mucosa of rat,[6] but the mammalian esterases or lipases involved in the metabolism of phthalate esters have not yet been identified.[7] We recently purified and characterized a novel 46.5-kilodalton esterase (called ES46.5K) from mouse hepatic microsomes. ES46.5K efficiently hydrolyzes lipophilic compounds such as 11-acetoxy-Δ^8-tetrahydrocannabinol (11-OAc-Δ^8-THC) and *p*-nitrophenyl acetate. N-Terminal amino acid sequence of ES46.5K has no homology to those of the known carboxylesterases (less than 20% up to 30 residues).[8,9] The only enzyme which has relatively high homology to ES46.5K is 2-acetylaminofluorene deacetylase (AAF-DAC) purified from human hepatic microsomes.[10] Inhibition by diisopropylfluorophosphate (DFP) suggests that serine may be located in the active site of the enzyme, although the physiological role of ES46.5K has not been elucidated. Further studies have been undertaken to characterize the function, structure and regulation of ES46.5K. The present study describes its comparative study with mouse hepatic microsomes on hydrolysis of phthalate esters.

MATERIALS AND METHODS

Animals and Enzymes Hepatic microsomes and various tissue homogenates (brain, lung, heart, pancreas, liver, spleen, adipose tissue, kidney and testis) were prepared from male ddN mice (25—30 g). ES46.5K was purified from hepatic microsomes of male ddN mice by the methods described previously.[11] Porcine liver carboxylesterase was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Chemicals Phthalate esters [diethyl (DEP), dibutyl (DBP), diisobutyl (DIBP) and diethylhexyl (DEHP)] and DFP were purchased from Wako Pure Chemical Ind. (Osaka, Japan). Other phthalate esters [dioctyl (DOP), dicyclohexyl, diphenyl and monobutyl (MBP)] were obtained from Tokyo Kasei Co. (Tokyo, Japan). Bis-*p*-nitrophenyl phosphate (BNPP) and phenylmethylsulfonyl fluoride (PMSF) were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Other chemicals used were of analytical reagent grade. Phthalate monoesters, major metabolite of phthlate diesters, were synthesized by reaction of phthalate anhydrous with the corresponding alcohols according to the method of Goggans and Copenhaver.[12]

Enzyme Assay A typical incubation mixture consisted of phthalate esters (dissolved in 2 to 10 μl of acetone) and the enzyme source (2 mg organ equivalent, 0.3 μg protein of ES46.5K or 80 μg protein of hepatic microsomes) mixed in 100 mM Tris–HCl buffer (pH 8.0) and incubated at 37 °C for 5 or 10 min. Phthalate monoesters formed were extracted with 5 ml of diethyl ether. After evaporation of the solvent, phthalate monoesters were methylated with diazomethane in diethyl ether, and then the organic solvent was evaporated to dryness and the residue was dissolved in 50 μl of ethyl acetate. Methylated phthalate esters were determined by gas chromatography (GC). The conditions used were as follows: apparatus, a Shimadzu GC-16A equipped with a flame ionization detector; column, 5% SE-30 on Chromosorb W (60—80 mesh, 3 mm × 2 m); column temperature 190 °C; under these conditions, the

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Table 1. Tissue Distribution of Hydrolytic Activities for Phthalate Esters in Mice

Organ	Phthalate ester hydrolytic activities (nmol/min/mg protein)				
	DEP	DBP	DIBP	DOP	DEHP
Liver	99.6±13.9	73.5±11.2	26.9±3.7	6.3± 0.7	3.7±0.3
Intestine	57.4± 8.7	54.3± 1.2	23.2±2.3	20.8± 6.3	18.7±1.9
Kidney	19.3± 3.1	22.9± 3.2	9.0±1.3	11.1± 5.2	4.5±0.4
Lung	16.9± 3.0	0.5± 0.5	2.0±0.1	2.2± 0.9	4.3±0.4
Pancreas	6.3± 0.7	85.5±13.6	16.0±1.9	9.5± 0.8	18.5±1.3
Adipose t.	5.9± 2.5	1.6± 1.6	1.7±0.1	27.3±10.1	5.6±0.6
Spleen	0.5± 0.2	3.3± 0.7	1.6±0.4	5.7± 2.2	2.7±0.4
Testis	2.7± 0.4	0.1± 0.1	1.5±0.4	2.0± 1.7	3.3±0.3
Brain	N.D.	N.D.	0.2±0.2	0.6± 0.1	1.5±1.5
Heart	1.1± 0.4	N.D.	0.8±0.2	2.0± 1.6	2.8±0.3

N.D. = <0.1. Adipose t. = adipose tissue. $N=3$.

retention times of methyl ethyl phthalate, methyl butyl phthalate, methyl isobutyl phthalate, methyl octyl phthalate and methyl ethylhexyl phthalate were 1.8, 3.6, 3.4, 5.1 and 4.8 min, respectively. Kinetic parameters were determined from double reciprocal plots of enzyme activity and substrate concentration (5 to 1000 μM). Under these conditions, the regression lines for the plots were linear in all cases. Inhibitory effects of several esterase inhibitors were determined to know the contribution of ES46.5K to the hydrolysis of phthalate esters in mice. Protein was determined by the method of Lowry *et al.*[13] with bovine serum albumin as the standard.

RESULTS AND DISCUSSION

Distribution of Phthalate Ester Hydrolytic Activities A few reports have been published on the metabolism of DEHP and DBP, but no published data are available on the hydrolysis of phthalate esters by purified enzyme from mice. Okada and Tamemasa reported that ^{14}C-labeled DBP administered orally to mice was rapidly metabolized and excreted, and that MBP was a major metabolite in liver and kidney.[14] Albro and Lavenhar described that pancreas, intestine, liver and lung were active in hydrolyzing DEHP in rats.[15]

The present study demonstrated that kidney together with those organs was also active in hydrolyzing the phthalate esters in mice. Hydrolytic activities in mouse organ homogenates for 5 kinds of phthalate esters are shown in Table 1. Hydrolytic activities toward the phthalate esters on brain, heart, spleen and testis were less than those of other organs. DBP hydrolytic activity was comparatively higher in liver, pancreas, small intestine and kidney. Phthalate ester hydrolytic activity for liver homogenates was seemingly dominanted among all mouse homogenates tested. However, the activities of DBP and DOP were highest in pancreas and adipose tissue, respectively. The relatively high hydrolyzing activity of liver in the present study suggests that the enzymes ES46.5K and carboxylesterase may have a major role in the hydrolysis of phthalate esters, although the role of intestinal enzymes is considered to be also important for the metabolism of the phthalate esters.

Comparison of Hydrolytic Activity for Phthalate Esters between ES46.5K and Mouse Hepatic Microsomes We recently purified and characterized a novel 46.5-kilodalton esterase from mouse hepatic microsomes.[11] This novel esterase (ES46.5K) efficiently hydrolyzes 11-OAc-Δ^8-THC, acetate of an active metabolite of Δ^8-THC. Mentlein and Butte[7] reported the hydrolysis of phthalate esters by carboxylesterases purified from rat and human hepatic microsomes. The dibutyl-, diallyl- and dibutyl phthalates were hydrolyzed by the esterases, whereas strong hydrophobic diesters like DEHP were not significantly hydrolyzed. The present study demonstrated that ES46.5K and mouse hepatic microsomes hydrolyzed DEP, DBP, DIBP, DOP and DEHP.

Table 2. Kinetic Constants on Phthalate Esters Hydrolysis of ES46.5K and Mouse Hepatic Microsomes

Phthalate ester	Hepatic microsomes			ES46.5K		
	K_m (μM)	V_{max} (μmol/min/mg)	V_{max}/K_m ×1000	K_m (μM)	V_{max} (μmol/min/mg)	V_{max}/K_m ×1000
DEP	703	0.41	0.58	68	19.8	291
DBP	408	0.34	0.83	7	19.5	2786
DIBP	105	0.18	1.71	17	9.6	565
DOP	418	0.02	0.05	382	19.3	51
DEHP	9.1	0.01	1.10	10	0.57	57

$N=3$.

Table 2 shows kinetic constants for phthalate esters hydrolysis of ES46.5K and mouse hepatic microsomes. Kinetic analyses indicated that K_m (μM) for DEP, DBP, DIBP, DOP and DEHP were 703, 408, 105, 418 and 9.1, respectively, and V_{max} (μmol/min/mg protein) for these phthalate esters were 0.41, 0.34, 0.18, 0.02 and 0.01, in the hepatic microsomes, whereas the values in ES46.5K were 68, 7, 17, 382 and 10 (μM), and 19.8, 19.5, 9.6, 19.3 and 0.57 (μmol/min/mg protein), respectively. V_{max}/K_m ratios for ES46.5K were 50—3300 times higher than those for the hepatic microsomes indicating that the catalytic efficiency of ES46.5K was markedly higher than that of the microsomes. ES46.5K did not hydrolyze phthalates having ring structure such as dicyclohexyl phthalate or diphenyl phthalate (data is not shown). Monophthalate esters were also not hydrolyzed even after prolonged incubation periods (30 min assays). The results suggest that the structure of two aliphatic esters is essential for the inter-

Table 3. Effects of Various Inhibitors on Hydrolytic Activities of DBP

Inhibitor	mM	Mouse hepatic microsomes	ES46.5K	Porcine hepatic carboxylesterase
BNPP	0.1	72	62	1[a]
	1.0	39	47	1
DFP	0.01	0	0	0
	0.1	0	0	0
PMSF	0.1	69	74	52
	1.0	58	15	10

a) % of control. $N=2$.

action of the substrates with an active site of ES46.5K.

It is therefore concluded that ES46.5K and mouse hepatic microsomes hydrolyzed only one ester bound from phthalate diesters. This result was the same as that of Mentlein and Butte using rat carboxylesterases[7]; namely the activities of ES46.5K in hydrolyzing the phthalate esters were higher than those of the rat and human carboxylesterase. Moreover, it was interesting that K_m value of ES46.5K in hydrolyzing the DBP was lower than those of the rat and human. However, DEHP hydrolyzing activity of ES46.5K was lower than those of pancreatic lipase reported by Albro and Thomas.[6] Difference in K_m values of the microsomes and ES46.5K for DEP, DBP and DIBP suggests that other esterases are present in the mouse liver microsomes.

Effects of Various Inhibitors on Hydrolysis Activities of DBP As shown in Table 3, effects of various inhibitors on hydrolytic activities of DBP. DFP (0.01 mM), which is known to be a typical inhibitor of serine hydrolase, completely inhibited the activities of all tested enzymes. BNPP (1.0 mM), which is known to be a typical inhibitor of carboxylesterase, completely inhibited the activity of the carboxylesterase from porcine hepatic microsomes, whereas it exhibited a somewhat lower inhibitory effect on ES46.5K and hepatic microsomes of mice. Thus, the inhibition of liver microsomal hydrolysis of DBP by BNPP was parallel to that of ES46.5K but not to that of carboxylesterase from porcine liver. PMSF (0.1 mM) inhibited 30—50% of the hydrolytic activity of all enzyme sources. These results suggest that hydrolysis of DBP by mouse liver microsomes is catalyzed by an enzyme that is not significantly inhibited by BNPP. Since ES46.5K has high catalytic activity to hydrolyze phthalate esters, the esterase is most likely participating in metabolism of such environmental pollutants in mice.

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