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Invited Review

Carboxylesterases: Structure, Function and Polymorphism

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Abstract − This review covers current developments in molecular-based studies of the structure and function of carboxylesterases. To allay the confusion of the classic classification of carboxylesterase isozymes, we have proposed a novel nomenclature and classification of mammalian carboxylesterases on the basis of molecular properties. In addition, mechanisms of regulation of gene expression of carboxylesterases by xenobiotics, and involvement of carboxylesterase in drug metabolism are also described.

Keywords: Carboxylesterase, Genetic polymorphism, Molecular structure, Classification

INTRODUCTION

The mammalian carboxylesterases (CarbEs, EC 3.1.1.1) comprise a multi-gene family whose gene products are localized in the endoplasmic reticulum (ER) of many tissues of human and animals. These enzymes efficiently catalyze the hydrolysis of a variety of ester- and amide-containing chemicals as well as drugs and chemicals to the respective free acids. They are involved in detoxification or metabolic activation of various drugs, pesticides, environmental toxicants and carcinogens. CarbEs also catalyze the hydrolysis of endogenous compounds such as short- and long-chain acyl-glycerols, long-chain acyl-carnitine, and long-chain acyl-CoA esters (Mentlein *et al.,* 1980; Mentlein and Heymann, 1984; Hosokawa, 1990; Maki *et al.,* 1991; Hosokawa and Satoh, 1996; Hosokawa *et al.,* 2001; Furihata *et al.,* 2004a; Furihata *et al.,* 2004b; Furihata *et al.,* 2005). We have reviewed the characteristics of CarbEs in relation to the metabolism of xenobiotics (Satoh and Hosokawa, 1995; Satoh and Hosokawa, 1998; Satoh and Hosokawa, 2006; Hosokawa *et al.,* 2007). Multiple isozymes of hepatic microsomal CarbE exist in various animal species (Hosokawa *et al.,* 1987; Hosokawa *et al.,* 1990; Hosokawa *et al.,* 1994), and some of these isozymes are involved in the metabolic activation of certain carcinogens, as well as being associated with hepatocarcinogenesis (Maki *et al.,* 1991).

Mammalian CarbEs are members of an α,β-hydrolase-fold family and are found in various mammalian species (Hosokawa, 1990; Kroetz *et al.,* 1993; Brzezinski *et al.,* 1994; Morgan *et al.,* 1994; Yan *et al.,* 1994; Yan *et al.,* 1995a; Yan *et al.,* 1995b; Yan *et al.,* 1995c; Kusano *et al.,* 1996; Brzezinski *et al.,* 1997; Langmann *et al.,* 1997a; Langmann *et al.,* 1997b; Ellinghaus *et al.,* 1998). It has been suggested that CarbEs can be classified into five major groups denominated from CES1 to CES5, according to the homology of the amino acid sequence (Satoh and Hosokawa, 1998; Satoh and Hosokawa, 2006; Hosokawa *et al.,* 2007), and the majority of CarbEs that have been identified to belong to the CES1 or CES2 family.

It has also been shown that striking species differences exist (Hosokawa *et al.,* 1990; Hosokawa *et al.,* 1994; Prueksaritanont *et al.,* 1996). For example, Inoue *et al.* (Inoue *et al.,* 1979a) showed that esterase activity in the dog intestine is very weak and produced no appreciable active band in a disk electrophoresis coupled with staining of esterase activity. On the other hand, esterase activities were observed in the intestines of other species (human, rat, mouse, guinea pig and rabbit) (Inoue *et al.,* 1979b; Prueksaritanont *et al.,* 1996; Mansbach and Nevin, 1998; Imai *et al.,* 2006; Satoh and Hosokawa, 2006) and found to produce a few active bands in an electrophoretic assay.

It is thought that CarbEs are one of the major determi-

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nants for pharmacokinetics and pharmacodynamics of ester-drugs or ester-prodrugs. Since the pharmacological data of ester prodrugs obtained from preclinical experiments are generally used as references for human studies, it is important to clarify the biochemical properties of each CarbE isozyme, including substrate specificity, tissue distribution, and transcriptional regulation.

This review addresses the significant differences of molecular structure and function of recently identified CarbEs., and proposes a novel nomenclature for mammalian CarbE isozymes that is based on the nucleotide sequences of the genes encoding the individual CarbE isozymes. In addition, the different structure-activity relationship of substrates with each CarbE family and genetic polymorphism of CarbE genes are also described.

NOVEL CLASSIFICATION AND NOMENCLATURE OF MAMMALIAN CarbEs

According to the classification of esterase by Aldridge (Aldridge, 1993), the serine superfamily of esterase, i.e., acetylcholinesterase (AChE), butyrylcholinesterase (BuChE) and CarbE, falls into the B-esterase group. CarbE isozymes were initially classified by their substrate specificities and pI. However, the classification is ambiguous in overlapping substrate specificities. A single esterolytic reaction is frequently mediated by several kinds of enzyme. Recent studies on esterases as with other enzymes concerned with xenobiotic metabolism, have afforded evidence of multiple forms.

It seems almost impossible to classify these CarbE isozymes based on their substrate specificity along with the lines of the International Union of Biochemistry (I.U.B.) classification, because the individual hydrolases exhibit properties of CarbE, lipase or both. Mentlein *et al.* (Mentlein *et al.,* 1984) proposed to classify these hydrolases as "unidentified CarbEs" (EC 3.1.99.1 to 3.1.99.x). Based on high homology and similarity of amino acid sequence alignment of the encoding genes, we tried to classify CarbE isozymes into five families, CES 1, CES 2, CES 3, CES4 and CES 5 (Satoh and Hosokawa, 1998; Satoh and Hosokawa, 2006) (Fig. 1).

The CES 1 family includes the major form of CarbE isozymes (more than 60% homology of human CES). Thus, they could be divided into eight subfamily: CES1A, CES1B, CES1C, CES1D, CES1E, CES1F, CES1G, CES1H. Most of all CES1 family, except CES1G, are mainly expressed in liver. The CES 1A subfamily includes the major forms of human CarbEs, and the major isoforms of rat, dog rabbit, and mouse CarbE. The CES1B subfamily includes the major isoforms of rat, mouse and hamster CarbE, and CES1C includes the major isoforms of dog, cat and pig CarbE (Robbi and Beaufay, 1987; Ovnic *et al.,* 1991b; Zschunke *et al.,* 1991; Yan *et al.,* 1995c; Hosokawa and Satoh, 1996; Potter *et al.,* 1998; Mori *et al.,* 1999; Hosokawa *et al.,* 2001; Furihata *et al.,* 2004a). The CES 1H subfamily includes RL1 (CES1H4), mouse ES 4 (CES1H1) and hydrolase B (CES1H3) and C (CES1H2), which catalyze long-chain acyl-CoA hydrolysis (Hosokawa *et al.,* 1987; Yan *et al.,* 1994; Yan *et al.,* 1995c; Hosokawa and Satoh, 1996; Robbi *et al.,* 1996). Members of the CES 1G family are not retained in ER, which are secreted to blood from liver (Ovnic *et al.,* 1991b; Yan *et al.,* 1995b). These families are all secretary type CarbEs. It is interested that CES1G family are found in only rats and mice, but not in humans, and they are all a secretary type of CESs. Although high level of CES1 activity is detected in the blood of rats and mice, but no activity is detected in human blood.

In contrast, the CES2 family is mainly expressed in small intestine. It includes human intestinal CarbE (CES2A1) (Schwer *et al.,* 1997; Humerickhouse *et al.,* 2000; Imai *et al.,* 2006; Taketani *et al.,* 2007; Yang *et al.,* 2007; Shi *et al.,* 2008), rCES2 (CES2A10) (Furihata *et al.,* 2005), rat intestinal CarbE RL4 (rCES2) (CES2A6) (Furihata *et al.,* 2003), rabbit form 2 (Ozols, 1989) and hamster AT51 (CES2A11) (Sone *et al.,* 1994). CES3 includes ES-male (CES3A2) and human CES3 (CES3A1) (Aida *et al.,* 1993; Sanghani *et al.,* 2004). Human CES3 (CES3A1) has about 40% amino acid sequence identity with both CEA1A1 and CES2A1, and is expressed in the liver and gastrointestinal tract at an extremely low level in comparison with CES1A1 and CES2A1 (Sanghani *et al.,* 2004).

The CES4 family includes carboxylesterase-like urinary excreted protein (CAUXIN) (CES4A2), which is excreted as a major urinary protein in cat urine (Miyazaki *et al.,* 2006a; Miyazaki *et al.,* 2006b). The CES 5 family includes 46.5-kDa CarbE isozymes (Probst *et al.,* 1991), which have a different structure from the structures of isozyme in other CarbE families. Esterase (ES) 46.5-kDa from mouse liver (Watanabe *et al.,* 1993) and amide hydrolase of monkey liver (Kusano *et al.,* 1996) probably belong to this family. These groupings are similar to the results of phylogenic analysis (Fig. 1).

STRUCTURE AND CATALYTIC MECHANISM OF CarbE ISOZYMES

It has been shown that several proteins of the endoplasmic reticulum (ER) lumen have a common carboxy-termi-

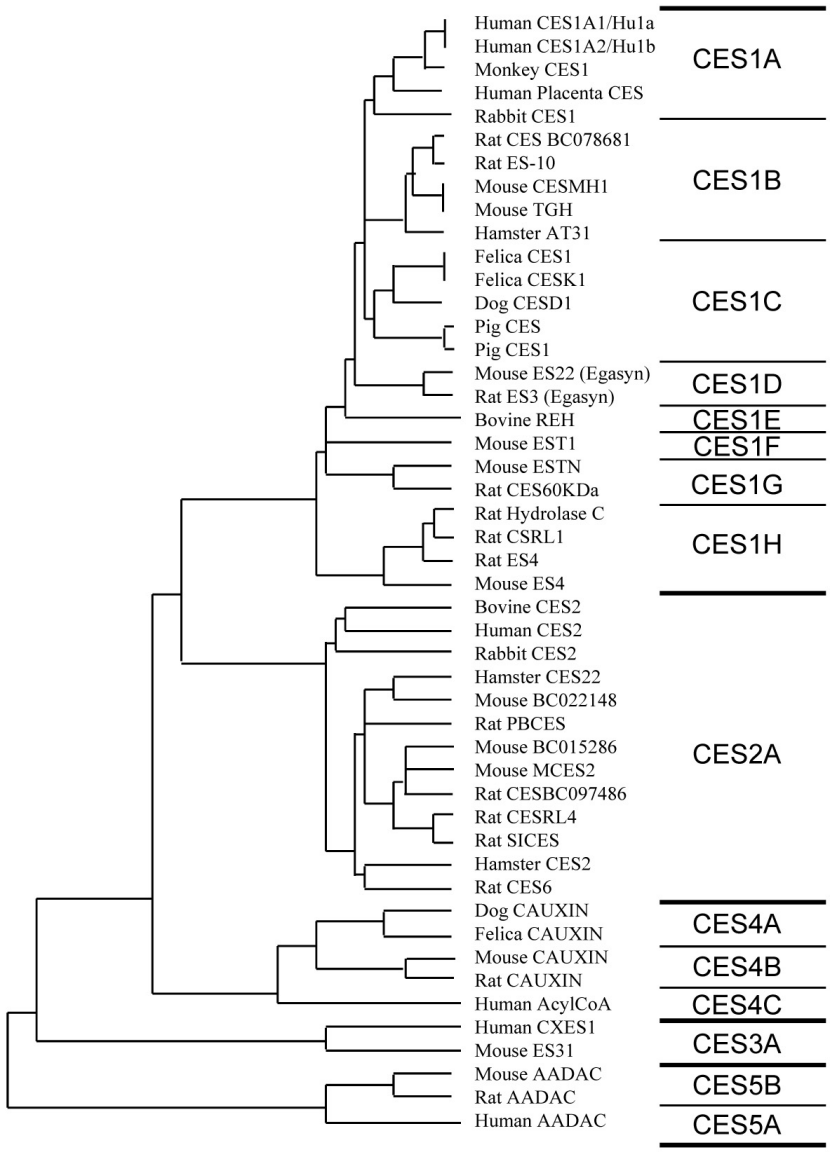


Fig. 1. Phylogenic tree and nomenclature of CarbE families. CarbE isozymes are classified into five families, CES1, CES2, CES3, CES4 and CES5. Each family is also divided into subfamilies.

nal sequence, KDEL-COOH, and that the structural motif is essential for retention of the protein in the luminal side of the ER through the KDEL receptor bound to the ER membrane (Pelham, 1990; Robbi and Beaufay, 1991; Tang and Kalow, 1995).

Korza and Ozols (Korza and Ozols, 1988) and Ozols (Ozols, 1989) established the primary structures of two microsomal esterases purified from rabbit liver and designated them 60-kDa esterase forms 1 and form 2, respectively. These two forms of CarbE have the consensus sequence for the ER retention tetra-peptide (HTEL or HIEL in the one-letter code) that is recognized with the luminal side of the KDEL receptor. The HXEL-COOH motif is also essential for retention of the protein in the luminal side of the ER through the KDEL receptor bound to ER membrane (Pelham, 1990; Robbi and Beaufay, 1991; Tang and Kalow, 1995). Robbi *et al.* (Robbi *et al.,* 1990) reported cDNA cloning of rat liver CES1B4 (ES-10). That was the first report to show that cDNA of liver CarbE has the consensus sequence of the ER retention tetrapeptide (HVEL-COOH).

Later, Robbi and Beaufay (Robbi and Beaufay, 1994) isolated a cDNA clone of another rat liver CES1D2 (ES-3, egasyn) which encoded the consensus sequence of the ER retention tetrapeptide (HTEL-COOH). The other clone encoded egasyn, an accessory protein of β-glucuronidase in the liver microsomes (Medda *et al.,* 1987). Egasyn is identical to CarbE, and it binds β-glucuronidase *via* its CarbE active site. In the case of rat and mouse, the carboxyl terminal amino acid sequence of clone rat CES-

60KDa (CES1G1) and mouse Es-N (CES1G2) is HTEHK-COOH, which can not bind to KDEL-receptor, and these isozymes are secreted to blood (Ovnic *et al.,* 1991b).

CarbEs have a signal peptide of 17 to 22 amino acid residues of N-terminal amino acid, including hydrophobic amino acid. In CES1 family, exon1 encodes a signal peptide (Ovnic *et al.,* 1991a; Shibata *et al.,* 1993). In a case of CES1 family, a bulky aromatic residue (Trp) followed by a small neutral residue (Gly) directly precedes the cleavage site (von Heijne, 1983). CarbEs have four Cys residues that may be involved in specific disulfide bonds. Among them, Cys98 is the most highly conserved residue in many CarbE isozymes. Cygler *et al.* (Cygler *et al.,* 1993) reported an important alignment of a collection of related amino acid sequences of esterase, lipase and related proteins based on X-ray structures of *Torpedo californica*AChE and *Geotrichum candidum* lipase.

According to literature, Ser_{203}, Glu_{336} and His_{450} form a catalytic triad, and Gly_{124}-Gly_{125} may be part of an oxyanion hole (Fig. 2). These residues are also highly conserved among CarbE isozymes. Site-specific mutation of Ser_{203} to Thr_{203}, Glu_{336} to Ala_{336}, or His_{450} to Ala_{450} greatly reduced the CarbE activity towards substrates. Therefore, the mutagenesis confirmed a role of Glu_{336} and His_{450} in forming a putative charge relay system with active site Ser203 (Satoh and Hosokawa, 1998).

Frey *et al.* (1994) reported that the formation of low barrier hydrogen bonds between His and Asp (Glu for CarbE) facilitates nucleophilic attack by the β-OH group of Ser on the acyl carbonyl group of peptide in chymotrypsin. The catalytic triad in the tetrahedral addition intermediate is stabilized by the low barrier hydrogen bonds. According to their theory, we speculated that the low barrier hydrogen bond between Glu_{336} and His_{450} facilitates nucleophilic attack by the β-OH group of Ser_{203} on the carbonyl group of the substrate in CarbE (Fig. 2).

The mechanism of CarbE could thus be divided into next steps. 1) The enzyme substrate complex form, positioning the substrate in the correct orientation for reaction. 2) Hydrolysis of the ester bond starts with an attack by the oxygen atom of the hydroxy group of Ser_{203} on the carbonyl carbon atom of the ester bond. 3) The hydrogen bonds between the negatively charged oxygen of the tetrahedral intermediate and the N-H group of Gly_{123} and Gly_{124} stabi-

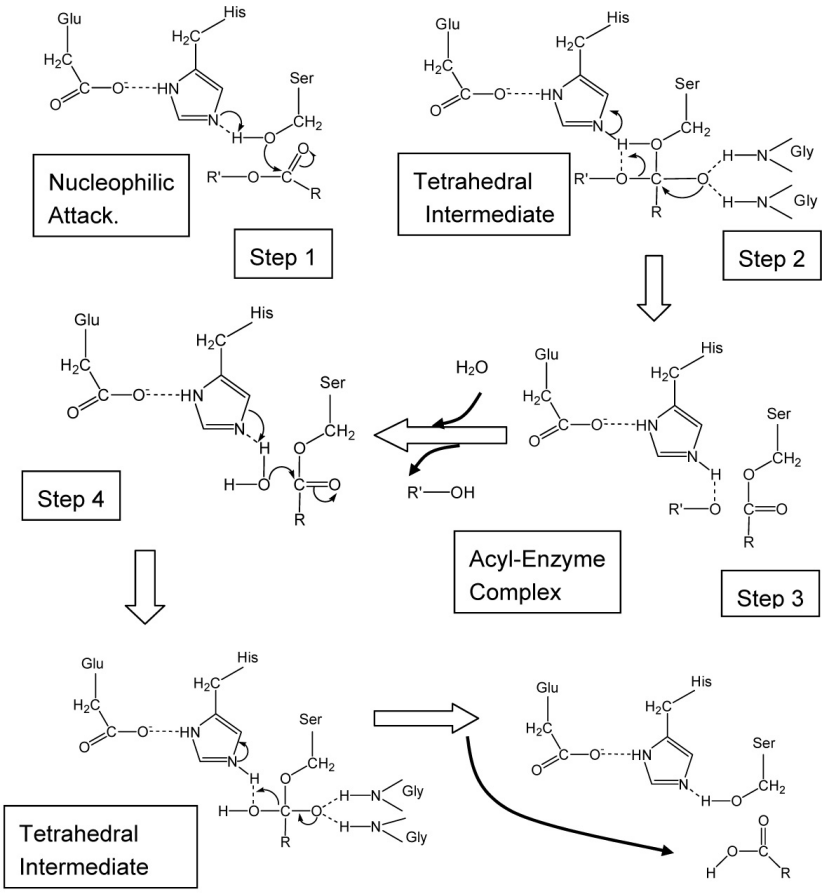


Fig. 2. Proposed mechanism for the action of CarbE. Conformation of the Ser-His-Glu catalytic triad in CarbE.

lize the negatively charged oxygen (O^-). This configuration, in which negatively charged carboxyl oxygen is hydrogen bonded to two N-H group, is called oxyanion hole.

In the general acid-catalyzed step, the ester bond breaks, and the leaving group picks up a proton from the imidazolium ion of His_{450}. The acyl portion of the original ester bond remains bound to the enzyme as an acyl-enzyme intermediate. The alcohol component (R'-OH) diffuses away, completing the acylation stage of the hydrolytic reaction. 4) A water molecule attacks the acyl-enzyme intermediate to give a second tetrahedral intermediate. 5) His_{450} then donates the proton to the oxygen atom of Ser_{203}, which then releases the acid component of the substrate. The acid component diffuses away and the enzyme is ready for catalysis.

The tetrahedral transition state is stabilized by the formation of low barrier hydrogen bonds between His_{450} and Glu_{336}. The low barrier hydrogen bonds facilitated mechanism includes weak hydrogen bonds between the oxyanion (O^-) and peptide N-H bonds contributed by Gly_{123} and Gly_{124}, which stabilize the tetrahedral adduct on the substrate side of the transition state (Fig. 2). Formation of the acyl-enzyme complex in the next step requires removal of a proton from His_{450}, so that the tetrahedral intermediate is disrupted in the acyl-enzyme intermediate. When the unbound portion of the alcohol group of the first product of the substrate has diffused away, a second step which the deacylation step is essentially the reverse of the acylation step occurs, with a water molecule substituting for the alcohol group of the original substrate.

It is of interest that the sequences required for the hydrolytic capability at the catalytic triad (Glu, His, Ser) of CarbE, AChE, BuChE, and cholesterol esterase are highly conserved. This is a common structure of α,β-hydrolase-fold families, which are responsible for the hydrolysis of endogenous and exogenous compounds. Furthermore, these elements are strongly conserved among orthologous CarbEs of the mouse, rat, rabbit, monkey and human.

A three-dimensional model for human CarbE has been proposed on the basis of crystal structure coordinates of AChE and overlapping active sites with pancreatic lipase and CarbE (Alam *et al.,* 2002). The modeled structure shares the overall folding and topology of the proteins identified in the recently published crystal structures of the rabbit (Bencharit *et al.,* 2002) and human CarbE (Wong and Schotz, 2002; Bencharit *et al.,* 2003a). CarbE has a three-dimensional α,β-hydrolase-fold structure, which is a structural feature of all lipases (Wong and Schotz, 2002). In general, the structure of CarbE may be viewed as comprising a central catalytic domain surrounded by α-β and regulatory domains (Bencharit *et al.,* 2002; Bencharit *et al.,* 2003a; Bencharit *et al.,* 2003b). In essence, the α,β-hydrolase-fold consists of a central β-sheet surrounded by a variable number of α-helices and accommodates a catalytic triad composed of Ser, His and a carboxylic acid. This suggests that the catalytic function of these proteins is conserved across species.

The catalytic triad is located at the bottom from about 25 Å deep active site, approximately in the center of the molecule and comprises a large flexible pocket on one side of Ser_{203} and a small rigid pocket on the opposite side (Bencharit *et al.,* 2003a). The orientation and location of the active site provide an ideal hydrophobic environment for the hydrolysis of a wide variety of hydrophobic substrates (Bencharit *et al.,* 2003a). The small rigid active site pocket is adjacent to the oxyanion hole formed by $Gly_{123\text{-}124}$ and is lined by several hydrophobic residues (Bencharit *et al.,* 2003a).

Short acyl chains would be easily accommodated within the small rigid pocket. The larger flexible active site pocket is lined by several non-polar residues and could accommodate larger or polycyclic molecules such as cholesterol. The large pocket is adjacent to a side door secondary pore that would permit small molecules (substrates and reaction products) to enter and exit the active site (Bencharit *et al.,* 2003a). Longer acyl chains may be oriented for catalysis in such a way that they extend through the side door. Indeed, the presence of a hydrophobic residue at position 423 in mice CES1B2 and 425 in humans CES1A1 is necessary for efficient hydrolysis of hydrophobic substrates, as mutation of Met present in position 423 of the related rat lung CarbE (CES1B4) to Ile increased the CarbE activity towards a more hydrophobic substrate without affecting activity towards short-chain esters (Wallace *et al.,* 1999).

Most CarbE isozymes are glycol-proteins, and the carbohydrate chain is required for the enzyme activity of CarbEs (Hosokawa, 1990; Kroetz *et al.,* 1993; Satoh and Hosokawa, 1998; Bencharit *et al.,* 2003a; Imai, 2006; Hosokawa *et al.,* 2007). Human CES2A1 contains a glycosylation site at two different positions (Asn_{103} and Asn_{267}), while CES1A1 contains only one glycosylation site at Asn_{79}. This glycosylation site is modified by a carbohydrate chain with first N-acetylglucosamine and terminal sialic acid and appears to be involved in the stabilization of the CES1A1 trimer by packing into the adjacent monomer in its crystal structure (Bencharit *et al.,* 2003a).

According to the X-ray crystal structure of human CES1, this residue lines the flexible pocket adjacent to the side door (Bencharit *et al.,* 2003a). Given the wide range of

substrates that CarbEs are known to hydrolyze, the large flexible pocket confers the ability to hydrolyze many structurally distinct compounds, whereas the rigid pocket is much more selective with regard to the substrates that may be accommodated.

GENE STRUCTURE AND REGULATION OF CarbE ISOZYMES

Both the murine (Hosokawa *et al.,* 2007) and human (Shibata *et al.,* 1993; Langmann *et al.,* 1997b) CES1 genes span about 30 kb and contain 14 small exons. Recently, sequencing of the mouse and human genomes have been completed, enabling detailed sequence comparisons. Previously published sequences of individual exons, splice junctions, size of the introns and restriction sites within the murine and human CarbE genes are consistent with their respective genes sequenced by the mouse and human genome projects. Therefore, the organization of the CarbE gene is evolutionarily conserved in mice and humans. Previous studies have mapped the human CarbE gene to chromosome 16 at 16q13-q22.1 (Zschunke *et al.,* 1991; Kroetz *et al.,* 1993). This region is syntenic to a region of mouse chromosome 8 at 8C5. The murine CarbE Es22 (Ovnic *et al.,* 1991a) and Es-N (Ovnic *et al.,* 1991b) have been previously mapped to chromosome 8. The completion of the mouse genome sequencing project unambiguously demonstrated that the murine CarbE gene was located on the minus strand of chromosome 8 at 8C5 in a cluster of six CarbE genes that spans 260.6 kb in total.

These six CarbE genes are presumed to be originated from repeated gene duplications of a common ancestral gene that encoded a CarbE (Shibata *et al.,* 1993), and subsequent evolutionary divergence may occur.

Recently, we have identified a mouse liver microsomal acylcarnitine hydrolase, mCES2, as a member of the CES 2 family (Furihata *et al.,* 2003). It has been revealed that this enzyme is significantly induced by di(2-ethylhexyl) phthalate (DEHP) and shows medium- and long-chain acylcarnitine hydrolase activity (Furihata *et al.,* 2003). In addition, we have found that mCES2 is expressed in various tissues with higher levels of expression in the liver, kidney and small intestine. It was shown that three transcription factors, specificity protein (Sp) 1, Sp3 and upstream stimulatory factor 1, could bind to the promoter region of the *mCES2* gene, leading to a synergistic transactivation of the promoter (Furihata *et al.,* 2004b). Although this mechanism may explain the ubiquitous tissue expression profiles of mCES2, it is unlikely to contribute to the higher levels of mCES2 expression in the liver, kidney and small intestine.

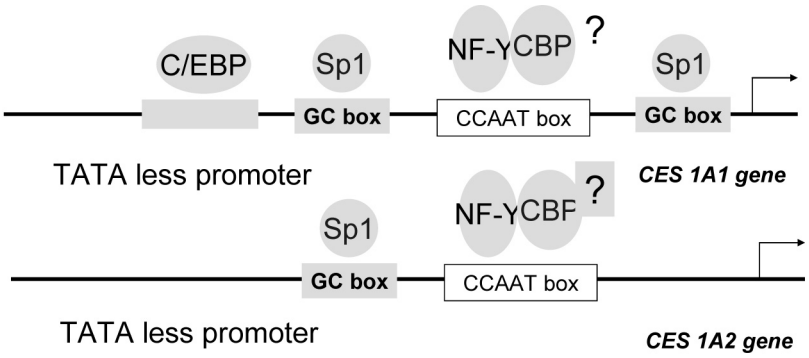
Therefore, it is thought that there exists another mechanism controlling this tissue-specific transcription of the mCES2 gene (Furihata *et al.,* 2004b). More recently, we have shown that hepatocyte nuclear factor-4 alpha (HNF-4α) can strongly enhance mCES2 gene transcription and that the involvement of HNF-4α accounts for the high expression level of mCES2 in the liver (Furihata *et al.,* 2006). These findings are notable when physiological roles of mCES2 are studied, since HNF-4α is involved in various hepatic functions, such as glucose and cholesterol metabolism and drug metabolism. In addition, we found that bile acid can repress mCES2 gene transcription by repressing HNF-4α-mediated transactivation (Furihata *et al.,* 2006).

We have isolated and characterized two genes encoding the human CES1A1 (AB119997) and CES1A2 (AB119998), and we also cloned and sequenced the 5' flanking region of each gene in order to elucidate the structure of the promoter (Hosokawa *et al.,* 2008)(Fig. 3).

It is noteworthy that both the CES1A1 and CES1A2 genes are located on chromosome 16q13-q22 with a tail-to-tail structure. A comparison of the nucleotide sequences of CES1A1 and CES 1A2 genes revealed about 98% homology in 30 Kbp. There are only six nucleotide differences resulting in four amino acid differences in the open reading frame, and all of the differences exist in exon 1.

Gene duplication has generally been viewed as a necessary source of material for the origin of evolutionary novelties, and duplicate genes evolve new functions. The majority of gene duplicates are silenced within a few million years, with the small number of survivors subsequently being subjected to strong purifying selection. Although duplicate genes may only rarely evolve new functions, the stochastic silencing of such genes may play a significant role in the passive origin of new species. Since exon 1 of the CES1 gene encodes a signal peptide region, intracellular localization of the CES1 gene product was preliminary investigated using a signal peptide/EYFP-ER chimera protein-expressing system. It was interesting that the CES1A1 signal peptide/EYFP-ER chimera protein was localized to the endoplasmic reticulum, whereas the CES1A2 signal peptide/EYFP-ER chimera protein was distributed in the endoplasmic reticulum and cytosol.

On the other hand, CES1A2 mRNA was found to be expressed only in human adult liver, although CES1A1 is expressed in human adult liver and fetal liver (Hosokawa *et al.,* 2008). These results suggested that CES1A1 and CES1A2 have different intracellular localizations and different expression profiles in liver differentiation. We inves-



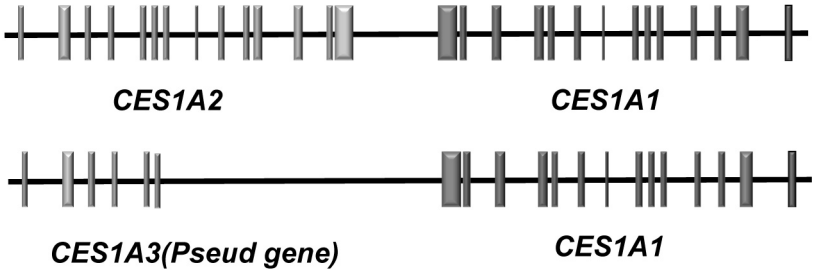


Fig. 3. Structure of the 5' flanking region of CES 1A1 and CES 1A2 genes. Sp1 and C/EBPα could bind to each responsive element of the CES1A1 promoter but that Sp1 and C/EBP could not bind to the 5' flanking region of the CES1A2 promoter. NF-Y, nuclear factor Y, CBF, CCAAT-binding factor.

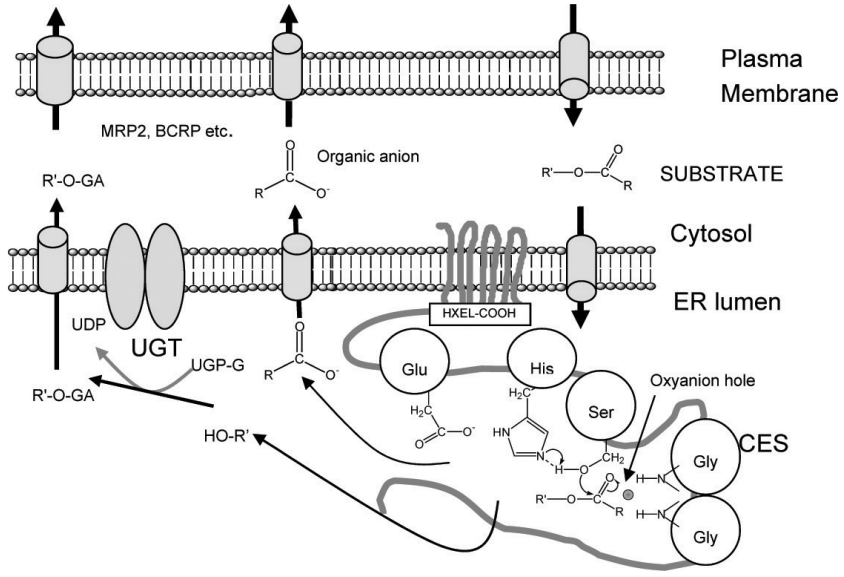


Fig. 4. CarbE-UGT interaction in the luminal side of the ER membrane and CarbE- transporter interaction in the cell. Two hydrolyzed products from ester-substrate are formed by CarbE; alcohol or phenol, which are substrates for UGT, and organic anions, which are substrates for organic anion transporter such as multidrug resistance-associated protein 2 (MRP2) or breast cancer resistance protein (BCRP).

tigated the transcriptional regulation of these two CarbE genes. Reporter gene assays and electrophoretic mobility shift assays demonstrated that Sp1 and C/EBPα could bind to each responsive element of the CES1A1 promoter but that Sp1 and C/EBP could not bind to the responsive element of the CES1A2 promoter (Fig. 4) (Hosokawa *et al.,* 2008).

More recently Fukami et al. (Fukami *et al.,* 2008) reported that the sequences of downstream and upstream of intron of CES1A2 gene are identical with those of CES1A1 and CES1A3 genes, respectively. A CES1A1 variant of which exon 1 is converted with that of the CES1A3 gene (the transcript is CES1A2) has recently been identified. They find that the CES1A2 gene is a variant of the CES1A3 pseudogene (Fig. 3). The expression level of CES1A1 mRNA is much higher than that of CES1A2 mRNA in the liver (Hosokawa *et al.,* 2008). Since CES1A1 is highly variable in the individual liver (Hosokawa *et al.,*

Table I. Tissue-specific expression profile of CES1 and CES2 isozymes in mammals and humans

Species	Isozyme	Liver	Small intestine	Kidney	Lung
Mouse	CES1	+ + +	−	+ + +	+ + +
	CES2	+ + +	+ + +	+ + +	−
Rat	CES1	+ + +	−	+ + +	+ + +
	CES2	−	+ + +	−	−
Hamster	CES1	+ + +	−	+ + +	NT
	CES2	+ + +	+ + +	−	NT
Guinea pig	CES1	+ + +	+ + +	+ +	NT
	CES2	−	+	−	NT
Beagle dog	CES1	+ + +	−	NT	+ + +
	CES2	+ +	−	NT	+
Monkey	CES1	+ + +	+ +	−	NT
	CES2	+	+ + +	+	NT
Human	CES1	+ + +	−	+	+ + +
	CES2	+	+ + +	+ + +	−

−: undetectable, +: weakly expressed, + +: moderately expressed, + + +: strongly expressed, NT: not tested.

1995), it was thought that these results provide information on individual variation of human CES1.

As shown in Table I, human CES1 and CES2 were highly expressed in liver and lung, and small intestine and kidney, respectively. Knowledge of these substrate structure-activity relationships and the tissue distribution of CarbE isozymes is critical for predicting the metabolism and the pharmacokinetics and pharamcodynamics of pesticides.

POSSIBLE ROLE OF CarbE ISOZYMES IN DRUG METABOLISM AND PHARMACOKINETICS

Drug metabolism

Drug metabolizing enzymes that are present predominantly in the liver are involved in biotransformation of both endogenous and exogenous compounds to polar products to facilitate their elimination. These reactions are categorized into phase 1 and phase 2 reactions.

CarbEs show ubiquitous tissue expression profiles with the highest levels of CarbE activity present in liver microsomes in many mammals (Mentlein *et al.,* 1980; Hosokawa *et al.,* 1984; Mentlein and Heymann, 1984; Maki *et al.,* 1991; Hattori *et al.,* 1992; Hosokawa and Satoh, 1993; Watanabe *et al.,* 1993; Hosokawa *et al.,* 1995; Derbel *et al.,* 1996; Lehner *et al.,* 1999; Furihata *et al.,* 2004a). CarbEs are categorized as phase 1 drug metabolizing enzymes that can hydrolyze a variety of ester-containing drugs and prodrugs. These include angiotensin-converting enzyme (ACE) inhibitors (temocapril, cilazapril, quinapril, and imidapril) (Takai *et al.,* 1997; Mori *et al.,* 1999; Furihata *et al.,* 2004a; Geshi *et al.,* 2005), anti-tumor drugs (CPT-11 and capecitabin) (Satoh *et al.,* 1994; Danks *et al.,* 1998; Guichard *et al.,* 1998; Kojima *et al.,* 1998; Potter *et al.,* 1998; Humerickhouse *et al.,* 2000; Sanghani *et al.,* 2004; Tabata *et al.,* 2004), and narcotics (cocaine, heroin and meperidine) (Kamendulis *et al.,* 1996; Brzezinski *et al.,* 1997; Zhang *et al.,* 1999). Thus, CarbEs are one of the most important enzymes involved in prodrug activation notably with respect to tissue distribution, up-regulation in tumor cells and turnover rates.

Recent studies have shown that there are some differences between these families in terms of substrate specificity, tissue distribution, immunological properties, and gene regulation (Hosokawa *et al.,* 2007). Analysis of substrate structure versus catalytic efficiency for the ester or carbamate substrates reveals that the different family of CarbEs recognizes different structural features of the substrate. For example, the preferential substrates for CES1A1, a human CES1 family isozyme, are thought to be compounds esterified by small alcohols, while those for CES2A1, a human CES2 family isozyme, are thought to be compounds esterified by relatively large alcohols. CES1A1, but not CES2A1, hydrolyzed the methyl ester of cocaine and the ethyl esters of temocapril, meperidine, imidapril and oseltamivir (Pindel *et al.,* 1997; Takai *et al.,* 1997; Mori *et al.,* 1999; Satoh *et al.,* 2002; Furihata *et al.,* 2004a; Shi *et al.,* 2006; Ose *et al.,* 2009).

It was interesting that procainamide inhibited the CES1 mediated imidapril hydrolysis (Takahashi *et al.,* 2009). Procainamide is also known as a choline binding pocket specific inhibitor (Jagnahtan and Boopathy, 1998) and has been reported to competitively inhibit human BuChE (Rush *et al.,* 1981). Takai *et al.* (1997) reported that the local anesthetic drug procaine and the anti-cholinergic drug oxybutynin with large alcohol substitutes are substrates for CES2 but not CES1. Procainamide is also a good substrate for CES2. Because the amino acid sequences at the active site were highly conserved among CES1, CES2 and BuChE (Satoh and Hosokawa, 1995), it is reasonable to assume that procainamide inhibits CES1- mediated imidapril hydrolysis.

In contrast to the specificity of CES1 for the methyl ester of cocaine, only CES2 hydrolyzed the benzoyl ester of cocaine (Pindel *et al.,* 1997). The benzoyl ester of cocaine, heroin and CPT-11 bearing a small acyl moiety and a bulky alcohol group are good substrates for the CES2 isozyme. It was interesting that BuChE hydrolyzed the benzoyl ester of cocaine, and also hydrolyzed CPT-11, but not AChE (Mattes *et al.,* 1996; Lynch *et al.,* 1997; Christopher *et al.,* 1999). CPT-11 is a relatively potent and selective inhibitor

of human AChE that has properties of the acute cholinergic toxicity observed in some patients (Dodds and Rivory, 1999).

It has been suggested that although these two CarbE families exhibit broad substrate specificity for ester, carbamate, or amide hydrolysis, these CarbE isozymes do exhibit distinct catalytic efficiencies that correlate with the relative size of the substrate substituents versus that of the enzyme active sites. Tissue specific expression of CES1 and CES2 was examined by northern blots, RT-PCR and real time PCR analysis.

Pharmacokinetics

It is thought that CarbEs are one of the major determinants for pharmacokinetics and pharmacodynamics of drugs and ester prodrugs (Fig. 4). Actually, it has been shown that dog CES1 isozyme was involved in a pulmonary first-pass effect in the disposition of a propranolol ester prodrug (Imai, 2006; Imai *et al.,* 2006). It has also been shown that the expression level of the human CarbE isozyme was correlated with the conversion ratio of CPT-11 to SN-38, the active metabolite, which is thought to be a key step for the chemotherapeutic action of this anti-tumor drug (Pindel *et al.,* 1997; Zhang *et al.,* 2002; Ohtsuka *et al.,* 2003; Sanghani *et al.,* 2003).

Since many drug metabolizing enzymes, such as cytochrome P450 (CYP), CarbE, UDP-glucuronosyltransferase (UGT) and sulfotransferase, and transporters, such as P-glycoprotein (P-GP), multi-drug resistance-associated protein 2 (MRP2) and breast cancer resistance protein (BCRP), were co-expressed in liver and small intestine, the hydrolysis activity in the liver and small intestine is contribute to drug metabolism and drug transport with phase II drug metabolizing enzyme or drug transporter. The CarbEs and the UGT families, the catalytic domains of which are localized in the luminal sides of the endoplasmic reticulum (ER) membrane, are two major enzyme groups responsible for phase 1 and 2 reactions (Fig. 4). The hydrolyzed products of CarbEs are also the substrate for UGT, such as SN-38 from CPT-11. Thus, we thought that CarbE-UGT interaction in the luminal sides of ER membrane is important for drug metabolism. Furthermore, hydrolyzed products of CarbEs consist of two kinds of chemical properties. One is the alcohol or phenol that are substrates for UGT, and the other is organic anions which are substrates for organic anion transporter such as multidrug resistance-associated protein 2 (MRP2) or BCRP. In this regard, we thought CarbEs are one of the major drug-metabolizing enzymes for enzyme-enzyme interaction or enzyme-transporter interaction.

GENETIC POLYMORPHISM

Geshi *at al.* first reported that a single nucleotide polymorphism (SNP), -816A/C of the CES1A2 gene associates with the responsiveness to an angiotensin-converting enzyme (ACE) inhibitor, imidapril, whose activity is achieved by CES1 isozyme (Geshi *et al.,* 2005). Recently we re-sequenced the CES1A2 promoter region (-1 kB) in 100 Japanese hypertensive patients. Altogether ten SNPs and one insertion/deletion (I/D) were identified, among which six SNPs and one I/D residing between −47 and −32 were in almost complete linkage disequilibrium (D'=1.00, r2=0.97) (Yoshimura *et al.,* 2008). They consisted of a minor and a major haplotype, the allele frequencies of which were 22% and 74%, respectively. The minor haplotype possessed two putative Sp1 binding sites while the major haplotype did not have any Sp1 binding site. The minor haplotype had a higher transcription and Sp1 binding activities than the major haplotype, *in vitro.*

More recently, we studied the relationship between CES1A1 polymorphisms and CES activity in forty-five human liver tissues (in preparation). Altogether, six single nucleotide polymorphisms (SNPs), −75G/T, −46A/G, −39A/G, −21C/G, −20G/A, −2G/C and one insertion/deletion (I/D), +71A/del were identified in the promoter region of CES1A1 gene. The +71 A/del was significantly associated with the efficacy of conversion of CPT-11 to SN38 and the level of immunoreactive CES1 protein in the liver microsomes. The +71 A/del was not associated with CES1A1 mRNA level in the liver, and an in vitro reporter assay indicated that +71A/del does not affect transcription. These results suggest that CES1A1 +71A/del may account at least in part for the inter-individual difference of CarbE activity in human liver microsomes. These polymorphism of CarbE genes may be a good candidate for studying pharmacogenetics for the detoxification of drugs and chemicals

CONCLUSIONS

Multiple CarbEs play an important role in the hydrolytic biotransformation of a vast number of structurally diverse drugs. These enzymes are major determinants of the pharmacokinetic behavior of most therapeutic agents containing an ester or amide bond. There are several factors that influence CarbE activity, either directly or at the level of enzyme regulation. In the clinical field, drug elimination is decreased and the incidence of drug-drug interactions increases when two or more drugs compete for hydrolysis by the same CarbE isozyme.

Exposure to chemicals or lipophilic drugs can result in induction of CarbE activity. Several drug-metabolizing enzymes, such as CYP, UGT and sulfotransferase have been extensively studied to clarify the substrate specificity using molecular cloning and cell expression systems. Consequently, the novel findings obtained reveal that the substrate specificity of CarbE is, at least in part, explained by the differences in the nucleotide sequences of the individual CarbE isozymes.

It is clear that membrane-bound type CarbE isozymes in microsomes are required to possess the KDEL tetrapeptide motif at the carboxy terminal of the molecule. Mammalian CarbEs have been found to have acyl glycerol, acyl-CoA, and acyl-carnitine hydrolyzing activities *in vitro,* however, physiological roles of CarbE remain unclear. To clarify the substrate specificity of each CarbE isozyme, we have begun to study for searching the substrate recognition site of each isozyme.

In the present review, we described the substrate specificity and tissue-specific expression profile of CarbE isozymes. Therefore, successful design of ester-containing drugs will be greatly improved by further detailed analysis for the mechanism of action and substrate recognition site of CarbE isozymes.

In conclusion, the molecular based information of CarbEs in this review must be useful to understand the multiplicity and substrate specificity of CarbE family associated with efficacy, side effects and toxicity of chemicals.

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