V.　研究成果の刊行に関する一覧表

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書籍

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VI.　研究成果の刊行物・別刷

4

CARBOXYLESTERASES: OVERVIEW, STRUCTURE, FUNCTION, AND POLYMORPHISM

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4.1 INTRODUCTION

Mammalian carboxylesterases (CarbEs, EC 3.1.1.1) comprise a multigene family whose gene products are localized in the endoplasmic reticulum (ER) of many tissues. These enzymes efficiently catalyze the hydrolysis of a variety of ester- and amide-containing chemicals as well as pesticides 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 (Furihata et al. 2004a, 2004b, 2005; Hosokawa 1990; Hosokawa and Satoh 1996; Hosokawa et al. 2001; Maki et al. 1991; Mentlein and Heymann 1984; Mentlein et al. 1980). We have reviewed the characteristics of CarbEs in relation to the metabolism of xenobiotics (Hosokawa et al. 2007; Satoh and Hosokawa 1995, 1998, 2006). Multiple isozymes of hepatic microsomal CarbE exist in various animal species (Hosokawa et al. 1987, 1990, 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 mammal species (Brzezinski et al. 1994, 1997; Ellinghaus et al. 1998; Hosokawa 1990; Kroetz et al. 1993; Kusano et al. 1996; Langmann et al. 1997a, 1997b; Morgan et al. 1994; Yan et al. 1994, 1995a, 1995b, 1995c). 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 (Hosokawa et al. 2007; Satoh and Hosokawa 1998, 2006). and the majority of CarbEs that have been identified belong to the CES1 or CES2 family. It has also been shown that striking species differences exist (Hosokawa et al. 1990, 1994; Prueksaritanont et al. 1996). For example, Inoue et al. (1979a) showed that esterase activity in the dog intestine is very weak and produced no appreciable active band in a disc 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; Imai et al. 2006; Inoue et al. 1979b; Mansbach and Nevin 1998;

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Prueksaritanont et al. 1996; Satoh and Hosokawa 2006) and found to produce a few active bands in an electrophoretic assay. Since toxicokinetic data for pesticides obtained from preclinical experiments using various animals are generally used as references for human studies, it is important to clarify the biochemical properties of each CarbE isozyme, such as substrate specificity, tissue distribution, and transcriptional regulation.

CarbEs show ubiquitous tissue expression profiles with the highest levels of CarbE activity present in liver microsomes in many mammals (Derbel et al. 1996; Furihata et al. 2004a; Hattori et al. 1992; Hosokawa and Satoh 1993; Hosokawa et al. 1984, 1995; Lehner et al. 1999; Maki et al. 1991; Mentlein and Heymann 1984; Mentlein et al. 1980; Watanabe et al. 1993). 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 I and phase II reactions. CarbEs are categorized as phase I drug metabolizing enzymes that can hydrolyze a variety of ester-containing drugs and prodrugs, such as angiotensin-converting enzyme inhibitors (temocapril, cilazapril, quinapril, and imidapril; Furihata et al. 2004a; Geshi et al. 2005; Mori et al. 1999; Takai et al. 1997), antitumor drugs (CPT-11 and Capecitabin; Danks et al. 1998; Guichard et al. 1998; Humerickhouse et al. 2000; Kojima et al. 1998; Potter et al. 1998; Sanghani et al. 2004; Satoh et al. 1994; Tabata et al. 2004), and narcotics (cocaine, heroin, and meperidine; Brzezinski et al. 1997; Kamendulis et al. 1996; Zhang et al. 1999). In this regard, it is thought that CarbEs are one of the major determinants for toxicokinetics of drugs (Fig. 4.1). Actually, it has been shown that the 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 antitumor drug (Ohtsuka et al. 2003; Pindel et al. 1997; Sanghani et al. 2003; Zhang et al. 2002).

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

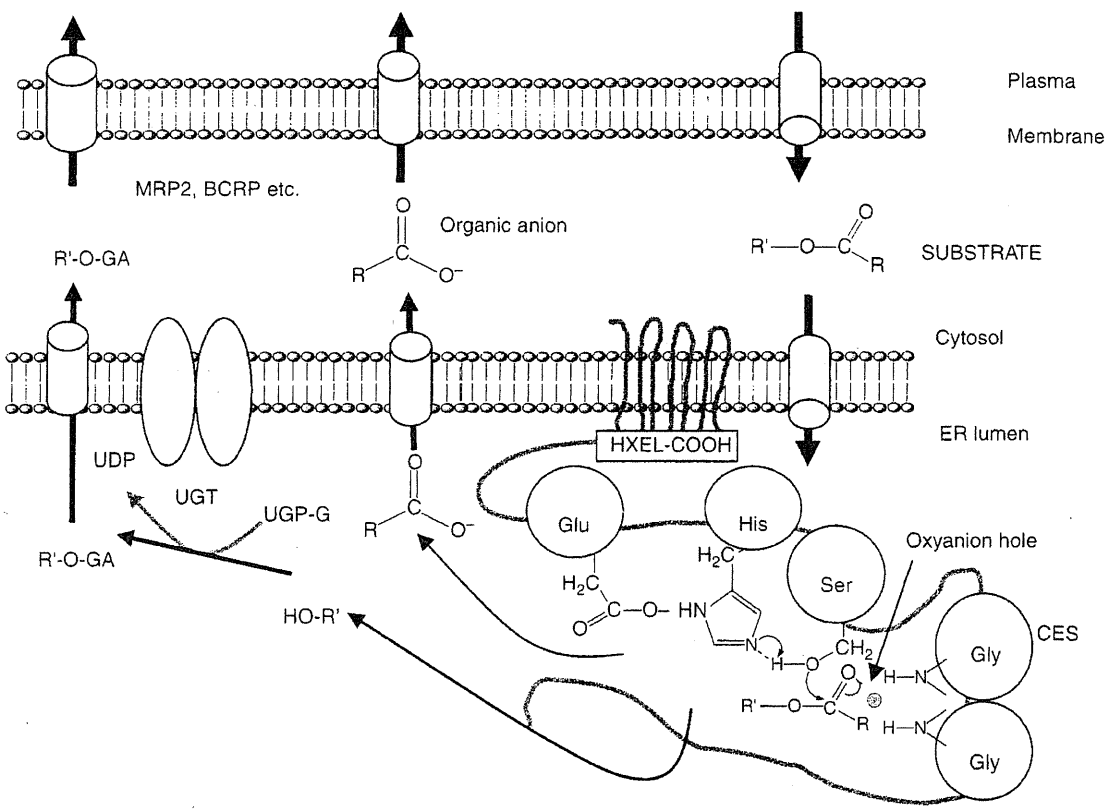

Figure 4.1 CarbE-UGT interaction in the luminal side of the ER membrane and CarbE- transporter interaction in the cell. Two hydrolyzed products from ester-substrate are formed by CarbE; alcohol or phenol, which are substrates for UGT, and organic anions, which are substrates for organic anion transporter such as multidrug resistance-associated protein 2 (MRP2) or breast cancer resistance protein (BCRP).

(BCRP), were co-expressed in liver and small intestine, the hydrolysis activity in the liver and small intestine contributes to drug metabolism and drug transport with phase II drug metabolizing enzyme or drug transporter. The CarbEs and the UGT family, 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 I and II reactions (Fig. 4.2). Products hydrolyzed by CarbEs, such as SN-38 from CPT-11, are also good substrates for UGT. Thus, we speculated that CarbE-UGT interaction in

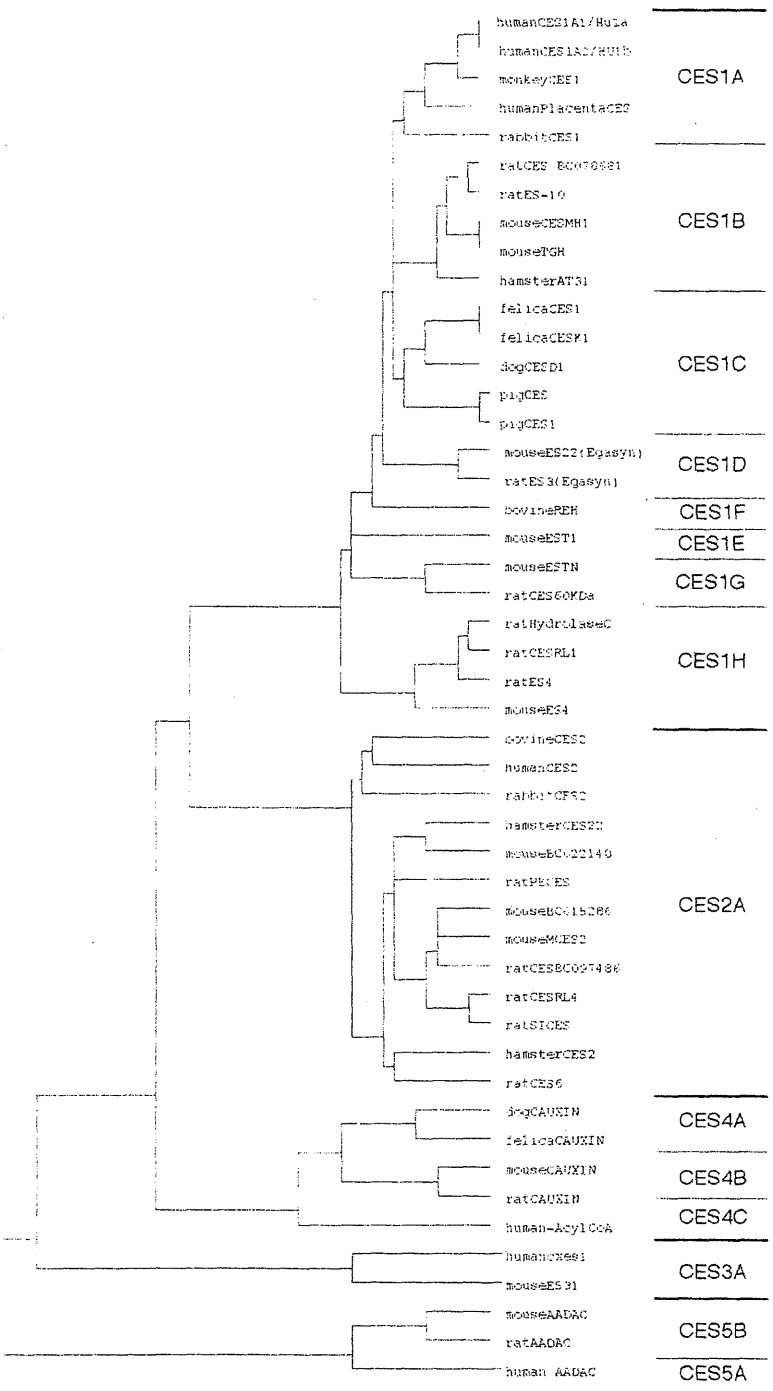

Figure 4.2 Phylogenic tree and nomenclature of CarbE families. CarbE isozymes are classified into five families, CES1, CES2, CES3, CES4, and CES5. Each family is also divided into subfamilies.

the luminal side of the ER membrane is important for drug metabolism. As shown in Figure 4.1, 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 MRP2 or BCRP. In this regard, we thought that CarbE is a major drug metabolizing enzyme for enzyme–enzyme interaction or enzyme–transporter interaction.

According to the classification of esterases by Aldridge (1993), the serine superfamily of esterase, that is, acetylcholinesterase (AChE), butyrylcholinesterase (BuChE), and CarbE, fall into the B-esterase group. It is becoming increasingly clear that esterases tend to have broad and overlapping substrate specificity toward amides and esters. A single esterolytic reaction is frequently mediated by several kinds of enzyme. Recent studies on esterases as well as other enzymes, such as AChE, BuChE, cholesterol esterase, triacylglycerol lipase, and CarbE, involved in xenobiotic metabolism have provided evidence of multiple forms.

It seems almost impossible to classify these CarbE isozymes based on their substrate specificity along the lines of the International Union of Biochemistry (IUB) classification because the individual hydrolases exhibit properties of CarbE, lipase, or both. Mentlein et al. (1984) proposed classification of these hydrolases as unidentified CarbEs (EC 3.1.99.1 to 3.1.99.x). Based on amino acid sequence alignment of the encoding genes, we tried to classify CarbE isozymes into five families, CES1, CES2, CES3, CES4, and CES5 (Fig. 4.2; Satoh and Hosokawa 1998, 2006). The CES1 family includes the major forms of CarbE isozymes (more than 60% homology of human CES1A1).

Most of the CES1 family, except CES1G, is mainly expressed in liver. The CES1A subfamily includes the major forms of human, monkey, and rabbit CarbE, and the CES1B includes the major isoforms of rat, mouse, and hamster CarbE, and CES1C includes the major isoforms of dog, cat, and pig CarbE (Furihata et al. 2004a; Hosokawa and Satoh 1996; Hosokawa et al. 2001; Mori et al. 1999; Potter et al. 1998; Ovnic et al. 1991b; Robbi and Beaufay 1987; Yan et al. 1995c; Zschunke et al. 1991). The CES 1H subfamily includes CarbE RL1 (CES1H4), mouse ES 4 (Ces1H1), and hydrolase B (CES1H3) and C (CES1H2), which catalyze long-chain acyl-CoA hydrolysis (Hosokawa and Satoh 1996; Hosokawa et al. 1987; Robbi et al. 1996; Yan et al. 1994, 1995c). Members of the CES 1G family are not retained in ER; CarbE isozymes are secreted to blood from liver (Ovnic et al. 1991b; Yan et al. 1995b). It is interesting that the CES1G family isozymes are found only in rats and mice, and are not found in humans. Although a high level of hydrolase activity was detected in the blood of rats and mice, no such activity is detected in the blood of humans.

On the other hand, the CES2 family includes human intestinal CarbE (CES2A1; Humerickhouse et al. 2000; Imai et al. 2006; Schwer et al. 1997; Shi et al. 2008; Taketani et al. 2007; Yang et al. 2007), rCES2 (CES2A10; Furihata et al. 2005), rat intestinal CarbE, mCES2 (Ces2A6; Furihata et al. 2003), rabbit form2 (Ozols 1989), and hamster AT51 (CES2A11; Sone et al. 1994), which are mainly expressed in small intestine. The CES3 family 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, 2006b). The CES5 family includes 46.5-kDa CarbE isozymes (Probst et al. 1991), which have a different structure from the structures of isozymes in other CarbE families. A 46.5-kDa ES 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. 4.2).

It is well recognized that carboxylesterase is much more susceptible to antichloinesterase pesticides such as organophosphates (OP) than AChE. In addition, CarbEs are deeply involved in detoxification of ester-containing OPs as well as pharmaceuticals. In this chapter, we describe the molecular characteristics of CarbE isozymes, the different structure-activity relationship of substrates with each CarbE families, genomic structure and regulation of CarbE genes, and genetic polymorphism of CarbE genes. The most current information is important to understand the detoxification and metabolism of pharmaceuticals and pesticides.

4.2 STRUCTURE AND CATALYTIC MECHANISM OF CarbE ISOZYMES

It has been shown that several proteins of the ER lumen have a common carboxy-terminal 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 (1988) and Ozols (1989) have established the primary structures of two microsomal esterases purified from rabbit liver and designated them 60-kDa esterase forms 1 and 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). 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. (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 (1994) isolated a cDNA clone of another rat liver CES1D2 (ES-3) 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 could not be bound to KDEL-receptor, and these isozymes are secreted to blood (Ovnic et al. 1991b).

CarbE has a signal peptide of 17 to 22 amino acid residues of *N*-terminal amino acid, including hydrophobic amino acid. In the CES1 family, exon1 encodes a signal peptide (Ovnic et al. 1991a; Shibata et al. 1993). In the CES1 family, a bulky aromatic residue (Trp) followed by a small neutral residue (Gly) directly precedes the cleavage site (von Heijne 1983). CarbE has 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. (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* acetylcholinesterase and *Geotrichum candidum* lipase. According to these authors, Ser_{203}, Glu_{336}, and His_{450} form a catalytic triad, and Gly_{124}–Gly_{125} may be part of an oxyanion hole. 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 Ser_{203} (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. 4.3). The mechanism of CarbE could thus be divided into the following steps.

1. The enzyme-substrate complex forms, 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} stabilize the negatively charged oxygen (O^-). This configuration, in which negatively charged carboxyl oxygen is hydrogen bonded to two N—H groups, is called an 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 mechanism facilitated by the low barrier hydrogen bonds 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. 4.3). 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, the deacylation step, which is essentially the reverse of the acylation step, occurs, with a water molecule substituting for the alcohol group of the original substrate (Fig. 4.3).

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 (Bencharit et al. 2003a; Wong and Schotz 2002). CarbE has a three-dimensional α,β-hydrolase-fold structure, which is a structural feature of all lipases (Wong and Schotz

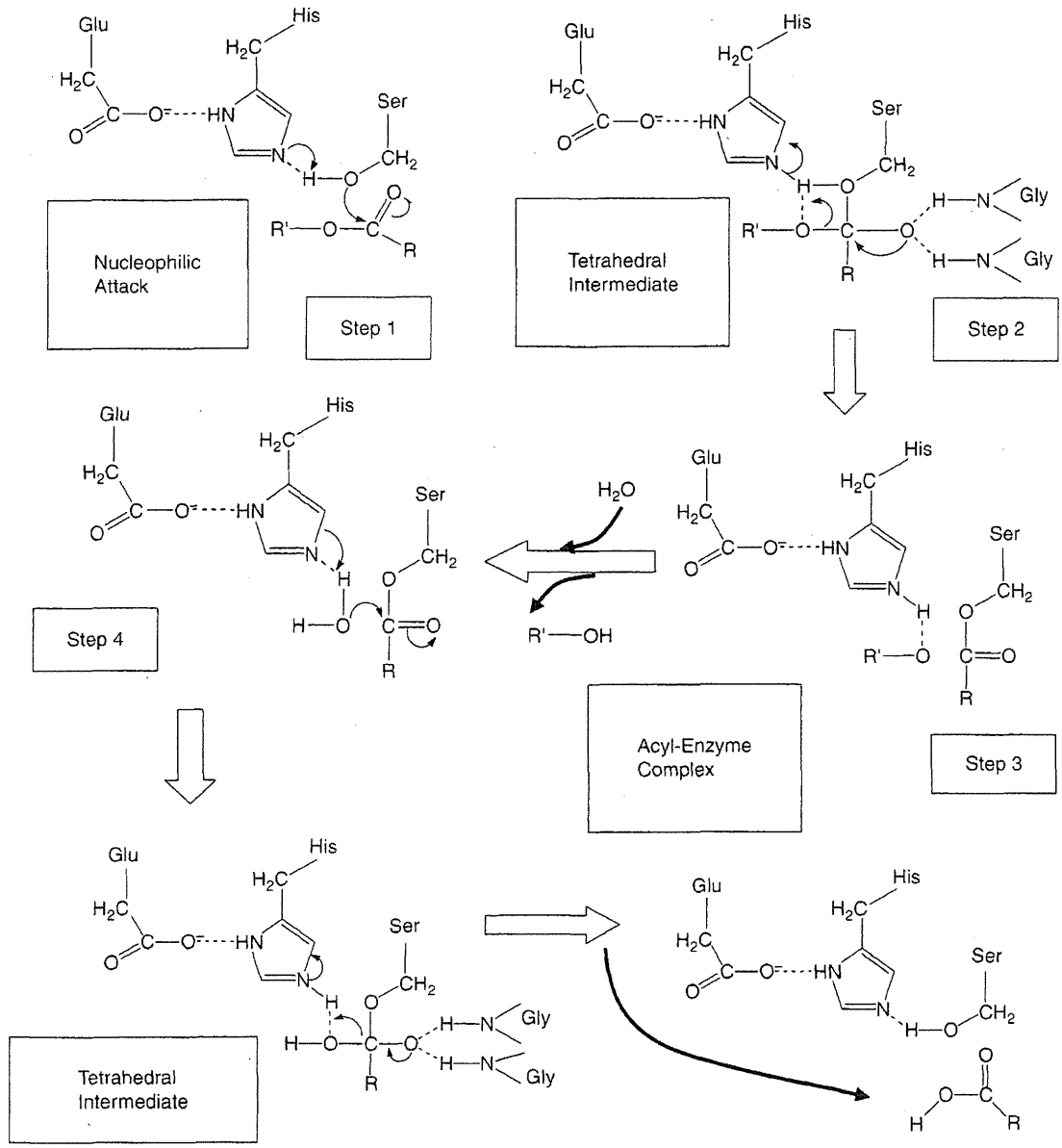

Figure 4.3 Proposed mechanism for the action of CarbE. Conformation of the Ser-His-Glu catalytic triad in CarbE.

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, 2003a, 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 provides 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 $Gly_{123-124}$ 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 nonpolar 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 (Bencharit et al. 2003a; Hosokawa 1990; Hosokawa et al. 2007; Imai 2006; Kroetz et al. 1993; Satoh and Hosokawa 1998). 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.

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 (Furihata et al. 2004a; Mori et al. 1999; Ose et al. 2009; Pindel et al. 1997; Satoh et al. 2002; Shi et al. 2006; Takai et al. 1997). Interestingly, 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 anticholinergic drug oxybutynin with large alcohol substitutes are substrates for CES2 but not CES1. Procainamide also with large alcohol substituents is thought to be 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 CPR-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 AcChE (Christopher et al. 1999; Lynch et al. 1997; Mattes et al. 1996). CPT-11 is a relatively potent and selective inhibitor of human AcChE that has properties similar to 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, reverse transcription (RT)-PCR and real time PCR analysis. As shown in Table 4.1, human CES1A was highly expressed in liver and lung, human CES2A was highly expressed in small intestine and kidney. Knowledge of these substrate structure-activity relationships and the tissue distribution of CarbE isozymes is critical for predicting the metabolism and the pharmacokinetics and pharmacodynamics of pesticides.

4.3 GENE STRUCTURE AND REGULATION OF CarbE ISOZYMES

Both the murine (Hosokawa et al. 2007) and human (Langmann et al. 1997b; Shibata et al. 1993) *CES1* genes span about 30 kb and contain 14 small exons. Recently, sequencing of the mouse and human genomes has 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. In previous studies, the human CarbE gene had been mapped to chromosome 16 at 16q13–q22.1 (Kroetz et al. 1993; Zschunke et al. 1991). 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

TABLE 4.1 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	+	+++	+++	−

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

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 have originated from repeated gene duplications of a common ancestral gene that encoded a CarbE (Shibata et al. 1993), and subsequent evolutionary divergence occurred.

We have identified a mouse liver microsomal acylcarnitine hydrolase, mCES2, as a member of the CES2 family (Furihata et al. 2003). It has been revealed that this enzyme is significantly induced by di(2-ethylhexyl)phthalate 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 also 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). 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 CES1A2 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 existed 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 investigated 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. 4.4; Hosokawa et al. 2008).

More recently, Fukami et al. (2008) reported that the sequences of the *CES1A2* gene downstream and upstream of intron 1 are identical with those of the *CES1A1* and *CES1A3* genes, respectively. A *CES1A1* variant of exon 1 that 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. 4.4). 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. 1995), it was thought that these results provide information on individual variation of human CES1.

4.4 GENETIC POLYMORPHISM

Geshi et al. (2005) first reported that a single nucleotide polymorphism (SNP), −816A/C of the *CES1A2* gene, is associated with the responsiveness to an angiotensin-converting enzyme (ACE) inhibitor, imidapril, whose activity is achieved by the CES1 isozyme. Recently we re-sequenced the *CES1A2* promoter region (~1 kB) in 100 Japanese hypertensive patients. Altogether 10 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 ($D' = 1.00$; $r^2 = 0.97$; 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 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 45 human livers. Altogether, six 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 the *CES1A1* gene. The +71A/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 +71A/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

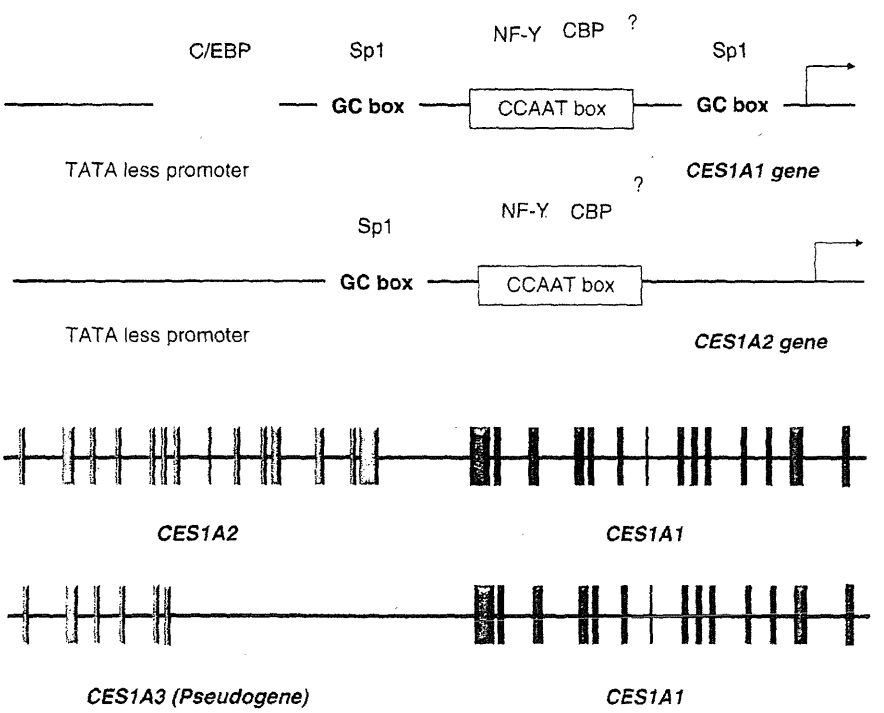

Figure 4.4 Structure of the 5′ flanking region of CES1A1 and CES1A2 genes. Sp1 and C/EBPα could bind to each responsive element of the CES1A1 promoter but not to the 5′ flanking region of the CES1A2 promoter. NF-Y, nuclear factor Y; CBF, CCAAT-binding factor.

at least in part for the interindividual difference of CarbE activity in human live microsomes. These polymorphism of *CarbE genes* may be a good candidate for studying pharmacogenetics for the detoxification of drugs and pesticides.

4.5 CONCLUSIONS

Multiple CarbE 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 increased when two or more drugs compete for hydrolysis by the same CarbE isozyme.

Exposure to pesticides or to lipophilic drugs can result in induction of CarbE activity. Several drug-metabolizing enzymes, such as CYP, UGT, and SULT, have been studied extensively to clarify the substrate specificity using molecular cloning and cell expression systems. The structure and substrate specificity of CarbE isozymes and tissue-specific expression profile of CarbE isozymes were also described in this chapter. 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 on CEs in this chapter is useful to understand the multiplicity and substrate specificity of the CarbE family involved in detoxification and toxicity of anticholinesterase pesticides such as OPs.

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