INVOLVEMENT OF LIVER CARBOXYLESTERASES IN THE IN VITRO METABOLISM OF LIDOCAINE

STEFAN E. H. ALEXSON, MARGARETA DICZFALUSY, MAGNUS HALLDIN, AND STELLAN SWEDMARK

Division of Clinical Chemistry, Karolinska Institute, Huddinge University Hospital, Stockholm (S.E.H.A., M.D.), and Preclinical Development (M.H.) and Research DMPK (S.S.), AstraZeneca R&D, Södertälje, Sweden

(Received December 5, 2001; accepted February 22, 2002)

This article is available online at http://dmd.aspetjournals.org

Abstract:
Although lidocaine has been used clinically for more than half a century, the metabolism has still not been fully elucidated. In the present study we have addressed the involvement of hydroxylations, deethylations, and ester hydrolysis in the metabolism of lidocaine to 2,6-xylidine. Using microsomes isolated from male rat liver, we found that lidocaine is mainly metabolized by deethylation to N-N-ethylglycyl)-2,6-xylidine, and N-(N-ethylglycyl)-2,6-xylidine is mainly metabolized to N-glycyl-2,6-xylidine, also by deethylation. However, 2,6-xylidine can be formed both from lidocaine and N-(N-ethylglycyl)-2,6-xylidine, but not from N-glycyl-2,6-xylidine, in an NADPH-independent reaction, suggesting that the amido bond in these compounds can be directly hydrolyzed by esterases. To test this hypothesis, we incubated lidocaine, N-(N-ethylglycyl)-2,6-xylidine, and N-glycyl-2,6-xylidine with purified liver carboxylesterases. Rat liver microsomal carboxylesterase ES-10, but not carboxylesterase ES-4, hydrolyzed lidocaine and N-(N-ethylglycyl)-2,6-xylidine to 2,6-xylidine, identifying this esterase as a candidate enzyme in the metabolism of lidocaine.

Lidocaine (LIDO) is a commonly used drug that has been on the market for over half a century and is a widely used amide-type local anesthetic. LIDO is also used as an antiarrhythmic drug, mainly in patients with acute myocardial infarction as an attempt to prevent ventricular fibrillation. LIDO is eliminated primarily by hepatic metabolism, but still its metabolism has not been completely elucidated due to the complexity of the metabolism that involves formation of several intermediates and metabolites. Coutts et al. (1987) showed that only one major metabolite, 3-hydroxy-N-(N-ethylglycyl)-2,6-xylidine (3-OH-MEGX), was excreted in the male Sprague-Dawley (S-D) rat after intraperitoneal administration of LIDO (at a dose of 40 mg/kg), whereas N-(N-ethylglycyl)-2,6-xylidine (MEGX), 2,6-xylidine (XYL), N-glycyl-2,6-xylidine (GX), 4-hydroxy-2,6-xylidine (4-OH-XYL), and 3-OH-N-glycyl-2,6-xylidine (3-OH-GX) were found as minor metabolites. 3-OH-lidocaine (3-OH-LIDO), 3-OH-XYL, and 4-OH-xylylcaine (4-OH-LIDO) were found only in trace amounts. Keenaghan and Boyes (1972) observed a different pattern in urine after oral administration (at a dose of 10 mg/kg) to female S-D rats. They found that about 85% of the administered dose was excreted in the urine and that 3-OH-MEGX and 3-OH-MEGX were the major metabolites, whereas 4-OH-XYL was excreted to a lesser extent. MEGX, GX, and XYL were all found to be minor metabolites. The discrepancies between these two studies may be explained by the use of different genders of rats, the dose and route of administration, age of animals, or different techniques used for identification of metabolites.

Studies on the in vitro metabolism of LIDO, using liver microsomes isolated from male S-D rats, showed that the metabolism is rather different from the metabolism in vivo. Significantly, MEGX was the more prominent metabolite formed (Oda et al., 1989). In addition, 3-OH-LIDO and methylhydroxylidocaine (Me-OH-LIDO) were formed in vitro, the latter metabolite having been first described by Kawai et al. (1986). However, no GX was detected. In addition, it was shown that formation of MEGX and Me-OH-LIDO could be induced by administration of phenobarbital to S-D rats. Masubuchi et al. (1991) compared the metabolism of LIDO in isolated hepatic microsomes from Dark Agouti and Wistar rats of both genders. They found that Dark Agouti rats (an animal model for poor human debrisoquine metabolizers, which are deficient in CYP2D6) do not 3-hydroxylate LIDO or MEGX. Furthermore, some metabolites that are not found in vivo can be detected in vitro, e.g., an N-oxide of LIDO (Patterson et al., 1986).

In humans, about 80% of a single dose of LIDO can be detected as excreted metabolites, with 4-OH-XYL being the major metabolite after acid hydrolysis (Keenaghan and Boyes 1972; Tam et al., 1987). The primary route of metabolism is via N-deethylation to MEGX followed by a second N-deethylation to GX and hydrolysis to XYL. In humans, 3-hydroxylation apparently represents a minor pathway of metabolism. From studies on the in vitro metabolism of LIDO, using human liver microsomes, it was shown that deethylation is a major metabolic pathway, whereas hydroxylation seems to be less extensive, which is different from the metabolism in rat (Hermansson et al., 1980).

The metabolism of LIDO in rat apparently involves mainly hydroxylation and deethylation reactions, suggesting that NADPH-de-
pendent P450 enzymes play important roles in the metabolism of LIDO. However, the formation of OH-XYL/XYL implies that esterases may be involved in hydrolyzing the amide bond in LIDO. Liver microsomes contain a number of esterases that can hydrolyze carboxylesterases, thioesters, and amide bonds of a variety of natural and xenobiotic substrates (Mentlein et al., 1980, 1984; Mentlein and Heymann, 1984). These esterases may also hydrolyze several pharmaceutical drugs (for reviews, see Leinweber, 1988; Satoh and Hosokawa, 1998). The major issue for this report was therefore to elucidate whether LIDO and/or its metabolites are 4-hydroxylated prior to the formation of 4-OH-XYL, or whether nonhydroxylated LIDO and/or its nonhydroxylated metabolites are being hydrolyzed to XYL and subsequently hydroxylated in the 4-position. This is an important issue since 4-OH-XYL is a substantial metabolite in the female S-D rat (Keenaghan and Boyes, 1972) and the major metabolite in humans (Tam et al., 1987) after administration of LIDO. We report here that carboxylesterases are involved in hydrolysis of LIDO and MEGX, which can occur prior to hydroxylation.

Experimental Procedures

Materials. LIDO, MEGX, GX, XYL, 3-OH-MEGX, and 4-OH-xyldine were synthesized at AstraZeneca (Södertälje, Sweden). 1-Octanesulfonic acid and bis-(nitrophenyl) phosphate (BNPP) were obtained from Sigma-Aldrich (St. Louis, MO). All solvents were of HPLC grade, and all other chemicals were of reagent-grade quality.

Preparation of Rat Liver Microsomes. Male S-D rats were obtained from B & K Universal AB (Sollentuna, Sweden) and kept under normal animal house conditions with food (R36; Lactamin, Vadstena, Sweden) and water ad libitum. Rats were euthanized by CO2 treatment followed by cervical dislocation. Livers were excised and homogenized in 0.25 M sucrose, 10 mM Tris/HCl, 1 mM EDTA, 0.1% ethanol, pH 7.4. Microsomes were prepared as described earlier (Wilcke and Alexson, 1994; Diczfalusy et al., 1999b). Protein was determined according to Bradford (1976).

Metabolism of LIDO, MEGX, and GX in Isolated Rat Liver Microsomes. Rat liver microsomes (0.5 mg) were incubated with LIDO, or metabolites of lidocaine (MEGX and GX), at a final concentration of 0.5 mM and additions as indicated. The incubation medium contained 100 mM potassium phosphate buffer (pH 7.4), and the total volume was 250 μl. Incubations were carried out for 60 min at 37°C, after which the samples were extracted by addition of 0.5 ml ice-cold methanol. The samples were centrifuged at 490g for 10 min and filtered through a 0.45 μm filter prior to HPLC analysis.

Incubation of LIDO and MEGX, 3-OH-MEGX, and GX with Purified Carboxylesterases. Rabbit liver esterase (E-9636) and porcine liver esterase (ES-4 and ES-10) were purified as described earlier (Diczfalusy et al., 1999). These esterases may be involved in hydrolyzing the amide bond in LIDO. Liver microsomes contain a number of esterases that can hydrolyze carboxylesterases, thioesters, and amide bonds of a variety of natural and xenobiotic substrates (Mentlein et al., 1980, 1984; Mentlein and Heymann, 1984). These esterases may also hydrolyze several pharmaceutical drugs (for reviews, see Leinweber, 1988; Satoh and Hosokawa, 1998). The major issue for this report was therefore to elucidate whether LIDO and/or its metabolites are 4-hydroxylated prior to the formation of 4-OH-XYL, or whether nonhydroxylated LIDO and/or its nonhydroxylated metabolites are being hydrolyzed to XYL and subsequently hydroxylated in the 4-position. This is an important issue since 4-OH-XYL is a substantial metabolite in the female S-D rat (Keenaghan and Boyes, 1972) and the major metabolite in humans (Tam et al., 1987) after administration of LIDO. We report here that carboxylesterases are involved in hydrolysis of LIDO and MEGX, which can occur prior to hydroxylation.

HPLC Analysis. HPLC analyses were carried out on a 150 × 4.6 mm Genesis C4 column, 4 μm (Jones Chromatography, Inc., Lakewood, CO), equipped with a Genesis C8 precolumn (1 cm × 10 mm) connected to a Kontron (Newport Beach, CA) chromatographic system consisting of pump model 420, autosampler model 460, and a UV detector model 432. Elution was performed at a flow rate of 1 ml/min with 18 or 20% acetonitrile in Millipore-grade water containing 0.1% phosphoric acid and 0.1% 1-octanesulfonic acid (ion-pair reagent). Analytes were monitored by UV detection at 210 nm. The different metabolites were identified by comparison to authentic standards. The data sampling and the peak integrations were performed using chromatography software Chromelone (Dionex Corp., Sunnyvale, CA).

MS Analysis. LC/MS analyses were carried out using a C8 column (Waters Symmetry, 1.0 × 150 mm) for the LC separation. A gradient was applied in which the concentration of acetonitrile was ramped at a rate of vol 1.2%/min from 3 to 60%. The mass spectrometer was a Quattro II (Micromass Ltd., Manchester, UK) equipped with an electrospray source (crossflow counter-electrode). Full-scan LC/MS was applied, with alternate scans involving cone voltages of 30 and 60 V.

Results

Metabolism of LIDO by Rat Liver Microsomes. To study the in vitro metabolism of LIDO, we incubated LIDO, or various metabolites of LIDO, with microsomes isolated from male rat liver in the presence or absence of added NADPH. The samples were extracted and analyzed by HPLC. Addition of ion-pair reagent to the mobile phase improved the chromatographic behavior and allowed separation and quantitation of the various metabolites (Fig. 1). The metabolism of LIDO was strongly dependent on addition of NADPH, and at 4 mM NADPH, more than 60% of LIDO was converted into more polar metabolites (Fig. 2, A and B). The main metabolites were MEGX (45% of total) and OH-LIDO (about 7% and identified by MS analysis). The metabolism of LIDO to OH-MEGX, GX, and OH-XYL was also NADPH-dependent, although these metabolites were formed in low amounts (less than 2%). However, incubation of LIDO in the absence of NADPH resulted in the formation of small amounts of XYL (about 0.5% of total), and this formation was independent of addition of NADPH (Fig. 2B).

These results show that the metabolism of LIDO is mainly catalyzed by NADPH-dependent P450 enzymes. The data also show an apparent difference in NADPH concentration dependence in the formation of the primary metabolites MEGX and OH-LIDO as compared with the formation of the secondary metabolites OH-MEGX, GX, and OH-XYL. Interestingly, XYL was formed in a mainly NADPH-independent manner, suggesting that LIDO may be hydrolyzed by an esterase to form XYL.

Metabolism of MEGX by Rat Liver Microsomes. Incubation of MEGX with isolated rat liver microsomes in the absence of NADPH resulted in formation of small amounts of XYL (about 1–1.5%). Addition of increasing NADPH concentrations up to 2 mM slightly decreased the amount of XYL, whereas the formation of GX and, to a lesser extent, OH-MEGX increased (Fig. 3). These data again
suggest that XYL may be formed in an NADPH-independent manner, possibly involving carboxylesterase(s).

**Metabolism of GX by Rat Liver Microsomes.** Incubation of GX with microsomes in the absence of NADPH did not result in formation of any known metabolites. However, addition of NADPH increased the formation of XYL and OH-XYL slightly (Fig. 4). Interestingly, the formation of XYL from GX appears to be NADPH-dependent, in contrast to the formation of XYL from LIDO or MEGX. Thus, formation of XYL and OH-XYL from GX does not seem to involve esterases, but appears to be due to an NADPH-dependent deamidation reaction.

**Metabolism of LIDO, MEGX, 3-OH-MEGX, and GX by Pig, Rabbit, and Rat Carboxylesterases.** Based on the findings that LIDO can be metabolized to XYL in an NADPH-independent reaction, we set out to examine whether carboxylesterases may be involved in the metabolism of LIDO to XYL. First, MEGX was incubated with commercial preparations of pig and rabbit esterases and the incubations were analyzed by HPLC. The pig esterase showed clear activity to hydrolyze MEGX to XYL, whereas the rabbit esterase showed barely detectable activity (results not shown). We next incubated purified rat carboxylesterase ES-10 or ES-4 (which represent the major rat liver microsomal carboxylesterases), with LIDO, MEGX, or GX. The results showed that ES-10 hydrolyzed LIDO and MEGX to XYL, whereas ES-4 did not hydrolyze MEGX (Table 1). However, purified ES-10 did not hydrolyze GX, consistent with the NADPH-dependent formation of XYL from GX using isolated microsomes as discussed above. Addition of the carboxylesterase inhibitor BNPP completely blocked the hydrolysis of LIDO and MEGX by ES-10.

**Discussion**

The metabolism of LIDO has been thoroughly studied since the drug entered the market more than 50 years ago. However, the metabolism has been shown to be complex, and different results have been obtained from in vitro and in vivo studies (Coutts et al., 1987; Oda et al., 1989). In the present study we have focused on one important aspect regarding the metabolism of LIDO in the rat: the
pattern of metabolites of LIDO suggests that LIDO and/or the first deethylated product MEGX is hydroxylated in the 3-position followed by excretion, and that excreted XYL is hydroxylated in the 4-position, suggesting different routes of formation. The question, therefore, is whether the formation of 4-OH-XYL is formed by direct hydroxylation of LIDO, MEGX, or GX and subsequently hydrolyzed to OH-XYL, or whether nonhydroxylated LIDO, MEGX, or GX is first hydroxylated, giving rise to XYL, and XYL subsequently hydroxylated. Another issue of interest is which enzymes might be responsible for the hydrolysis to form XYL.

In the present study, we therefore examined the route of metabolism of LIDO by incubating LIDO and various metabolites of LIDO with isolated rat liver microsomes in the presence or absence of NADPH. Our results show that inclusion of NADPH was essential for the formation of MEGX and GX as well as for the formation of the hydroxylated metabolites OH-LIDO, OH-MEGX, and OH-XYL (Fig. 2, A and B). Hence, there seems to be a strict requirement of cytochrome P450 enzymes for these reactions. Notably, P450 enzymes are required not only for hydroxylation of LIDO and MEGX, but also for deethylation of LIDO to MEGX and a second deethylation of MEGX to form GX. In addition, MEGX was clearly the major metabolite formed (about 80% of the identified metabolites) when including 4 mM NADPH in the incubations. Thus, OH-LIDO and OH-MEGX are minor metabolites, demonstrating that hydroxylation is not a major pathway compared with N-dealkylation of LIDO to MEGX. However, when excluding NADPH from the incubations, we found that low amounts of XYL were still formed (about 1% of total metabolites), indicating that enzymes other than the P450s are also involved. Notably, the formation of XYL was independent of NADPH, suggesting a possible involvement of esterase(s) for its formation. The formed XYL is subsequently hydroxylated to OH-XYL in the presence of NADPH, probably mainly in the 4-position.

Incubation of MEGX with rat liver microsomes in the absence of NADPH resulted in the formation of only XYL, again indicating the involvement of an esterase in the hydrolysis of MEGX. However, when incubating MEGX in the presence of NADPH, GX is the major metabolite, followed by OH-MEGX, and only minute amounts of XYL (Fig. 3). Thus, as for LIDO, hydroxylation of MEGX and the subsequent metabolite GX is not the main pathway of metabolism, but rather N-dealkylations of LIDO to MEGX and, further, to GX. Interestingly, when GX was incubated with rat liver microsomes in the absence of NADPH, no XYL was formed. However, addition of NADPH to these incubations resulted in formation of only small amounts of XYL and OH-XYL (Fig. 4). Thus, it appears that GX is not being a good substrate for hydroxylations, but that XYL is formed from GX in a P450-catalyzed reaction.

Incubation of LIDO and MEGX in the absence of NADPH resulted in the formation of small amounts of XYL, and addition of increasing concentrations of NADPH, if anything, slightly decreased the formation of XYL, suggesting that both LIDO and MEGX could be substrates for esterase(s). Rat liver microsomes contain several carboxylesterases, and the group of Rolf Mentlein isolated five of the most prominent carboxylesterases that were separated based on isoelectric point (Mentlein et al., 1980). These enzymes were named hydroxylases pl 5.2, pl 5.6, pl 6.0, pl 6.2, and pl 6.4 based on their differences in isoelectric points. These hydroxylases all have different substrate specificities, and in addition to various endogenous lipids, they can also hydrolyze a variety of xenobiotics and pharmaceutical drugs (for reviews, see Leinweber, 1988; Satoh and Hosokawa, 1998). Hydroxylases pl 6.0 and pl 6.4 are also referred to as esterases ES-10 and ES-4, and, more recently, as CES 1A2 and CES 1B (Satoh and Hosokawa, 1998), respectively. We have previously purified and characterized the ES-4 and ES-10 esterases with respect to acyl-CoA thioesterase activity and possible involvement in formation of fatty acid ethyl esters (Alexson et al., 1993; Diczfalusy et al., 1999a,b). These two carboxylesterases are located in the lumen of the endoplasmic reticulum, and they have previously been shown to hydrolyze a large variety of natural as well as xenobiotic esters. Our present data show that ES-10 hydrolyzes MEGX and LIDO, and that addition of the carboxylesterase inhibitor BNPP (Mentlein et al., 1984, 1985) completely blocked this hydrolysis. However, purified ES-10 appeared to hydrolyze 3-OH-MEGX to some extent, although this activity seemed insensitive to BNPP, suggesting the possibility of some nonspecific reaction. ES-10 did not hydrolyze GX, suggesting a structural requirement of the hydrophobic side chain of LIDO and MEGX for activity. Such a structural specificity has previously been demonstrated for these carboxylesterases inasmuch as ES-10 readily hydrolyzes butanilicaine, whereas ES-4 or hydrobrolase pl 5.6 does not (Hosokawa et al., 1987).

The apparent specificity of purified ES-10 to hydrolyze LIDO and MEGX, but not GX, agrees well with the results from incubations with microsomes showing that XYL is formed from LIDO and MEGX (NADPH-independent formation) but not from GX. The ES-10 and ES-4 esterases are highly expressed in rat liver, and they have been estimated to comprise about 1.5 and 0.5% of the microsomal protein, respectively (Morgan et al., 1994). The ES-10 esterase is likely to be highly expressed also in humans, whereas the ES-4 esterase is not (Diczfalusy et al., 2001). Therefore, the ES-10 carboxylesterase is a strong candidate esterase enzyme to be involved in the metabolism of LIDO in both rat and human.

Keenaghan and Boyes (1972) demonstrated that 3-OH-LIDO (31.2%) and 3-OH-MEGX (36.9%) were major metabolites excreted of orally administered LIDO in female S-D rats, but also substantial amounts of 4-OH-XYL (12.4%) were excreted. Although MEGX (0.7%), GX (2.1%), XYL (1.5%), and LIDO (0.2%) were identified as minor metabolites, no 3-OH-XYL was detected. These findings further support a concept that LIDO may be metabolized by two pathways in the rat, one involving 3-hydroxylations of LIDO and MEGX followed by excretion, and another pathway involving direct esterase-catalyzed hydrolysis, or deethylation of LIDO to MEGX followed by esterase-catalyzed hydrolysis, to form XYL, which may subsequently be 4-hydroxylated and excreted as outlined in Fig. 5. These data also indicate that the latter metabolic scheme may be found in humans since Tam et al. (1987) have demonstrated that 4-OH-XYL is the
major metabolite being excreted in human urine, comprising about 80% of intravenously administered LIDO.

Furthermore, this concept is supported by a study by Martin et al. (1997). This group has studied a compound with a structure very similar to that of LIDO, the anticonvulsant 5-methyl-3-isoxazolecarboxamide, that also contains an amide bond. This compound was also hydrolyzed in an NADPH-independent manner, and the hydrolysis was inhibited by BNPP. However, these authors did not identify any responsible amidase/esterase for the hydrolysis of their compound.

In summary, we have demonstrated here that N-dealkylation is the main pathway in the in vitro rat hepatic microsomal metabolism of LIDO, rather than aromatic hydroxylation. Furthermore, our data suggest that liver microsomal carboxylesterases can hydrolyze LIDO and MEGX, suggesting that 4-OH-XYL excreted in S-D rat urine is derived from hydrolyzed LIDO and/or MEGX via esterases to form XYL. XYL can subsequently be hydroxylated in an NADPH-dependent manner, most likely by P450, to 4-OH-XYL.

Acknowledgments. We thank Hans von Euler Chelpin for identification of 4-OH-LIDO on LC/MS.