Specificity of Procaine and Ester Hydrolysis by Human, Minipig, and Rat Skin and Liver

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ABSTRACT:

The capacity of human, minipig, and rat skin and liver subcellular fractions to hydrolyze the anesthetic ester procaine was compared with carboxylesterase substrates 4-methylumbelliferyl-acetate, phenylvalerate, and para-nitrophenylacetate and the arylesterase substrate phenylacetate. Rates of procaine hydrolysis by minipig and human skin microsomal and cytosolic fractions were similar, with rat displaying higher activity. Loperamide inhibited procaine hydrolysis by human skin, suggesting involvement of human carboxylesterase hCE2. The esterase activity and inhibition profiles in the skin were similar for minipig and human, whereas rat had a higher capacity to metabolize esters and a different inhibition profile. Minipig and human liver and skin esterase activity was inhibited principally by paraoxon and bis-nitrophenyl phosphate, classical carboxylesterase inhibitors. Rat skin and liver esterase activity was inhibited additionally by phenylmethylsulfonyl fluoride and the arylesterase inhibitor mercuric chloride, indicating a different esterase profile. These results have highlighted the potential of skin to hydrolyze procaine following topical application, which possibly limits its pharmacological effect. Skin from minipig used as an animal model for assessing transdermal drug preparations had similar capacity to hydrolyze esters to human skin.

Understanding the nature of skin xenobiotic metabolizing enzymes is important in assessing the fate of absorbed xenobiotics and drugs applied to the skin. Following absorption through the stratum corneum, most metabolism in the skin occurs in the basal keratinocytes of the epidermis (Hotchkiss, 1992). Despite the importance of esterases in the hydrolysis of topically applied drugs and prodrugs, investigation of skin esterases has been limited.

Esterases are members of the hydrolase family of enzymes that primarily hydrolyze endogenous and exogenous esters, with substrate specificity overlapping with lipases (Williams, 1985; Mentlein et al., 1988). They are ubiquitously expressed in mammalian liver, blood, and extrahepatic tissues, including skin, kidney, intestines, testes, brain, central nervous system, and lung (Satoh and Hosokawa, 1998). Carboxylesterases (EC 3.1.1.1) are present in both the endoplasmic reticulum and cytosol (McCracken et al., 1993). The nature of microsomal esterases in human liver and gut have been most extensively studied (Huang et al., 1996) because of their role in hydrolyzing orally ingested drugs. Satoh and Hosokawa (2006) have attempted to rationalize the classification of carboxylesterases (CESs)[1]. Three human CES isoforms, hCE1, hCE2, and hCE3, have been identified in microsomes, with hCE1 highly expressed in liver but not the gut, and hCE2 expressed extrahepatically, especially in the gut and at lower

levels in the liver (Sanghani et al., 2004; Imai et al., 2006; Satoh and Hosokawa, 2006). Carboxylesterases are also found in the cytosol, but they have not been classified. Cloning of hCE1, hCE2, and hCE3 has allowed ranking of the specificity for hydrolysis of drugs; procaine, aspirin and irinotecan had specificity for hCE2 (Wu et al., 2004) and methylphenidate and meperidine had specificity for hCE1 (Sun et al., 2004). hCE3, which is mainly expressed in the brain and gut, had low affinity for these substrates (Sanghani et al., 2004). The rat expresses up to six carboxylesterases that are classified as CES1 type and or CES2 type (Sanghani et al., 2004). Two carboxylesterases have been identified in the pig, and they have been classified as CES1 and CES (Satoh and Hosokawa, 2006). David et al. (1998) reported a pig intestinal carboxylesterase that showed high similarity with that of rat and human intestinal carboxylesterases.

Clark et al. (1993) and Hewitt et al. (2000) showed that esters applied to the skin surface were hydrolyzed during dermal absorption using skin in vitro in a diffusion cell. Ester drugs and chemicals applied to the skin surface undergo hydrolysis if they are substrates for the carboxylesterase isoforms expressed in the skin. This might influence the efficacy of drugs such as procaine that are used as a local anesthetic both transdermally and subcutaneously. It was hypothesized that differing esterase isozyme profiles and affinity of skin esterases would influence systemic availability. Development of an ester prodrug increases lipophilicity and promotes passage through the stratum corneum. If absorbed prodrugs are converted to the active

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ABBREVIATIONS: CES, carboxylesterase; hCE1, human carboxylesterase-1; hCE2, human carboxylesterase-2; hCE3, human carboxylesterase-3; HPLC, high-performance liquid chromatography; MUA, 4-methylumbelliferyl acetate; PV, phenyl valerate; PA, phenyl acetate; NPA, para-nitrophenyl acetate; BNPP, bis-nitrophenylphosphate; DMSO, dimethyl sulfoxide; PMSF phenylmethylsulfonyl fluoride; Bw284c51, bis-(4-allyl dimethyl ammonium phenyl)-pentane-3-one-dibromide; IsoOMPA, tetraisopropyl-pyrophosphoramide.
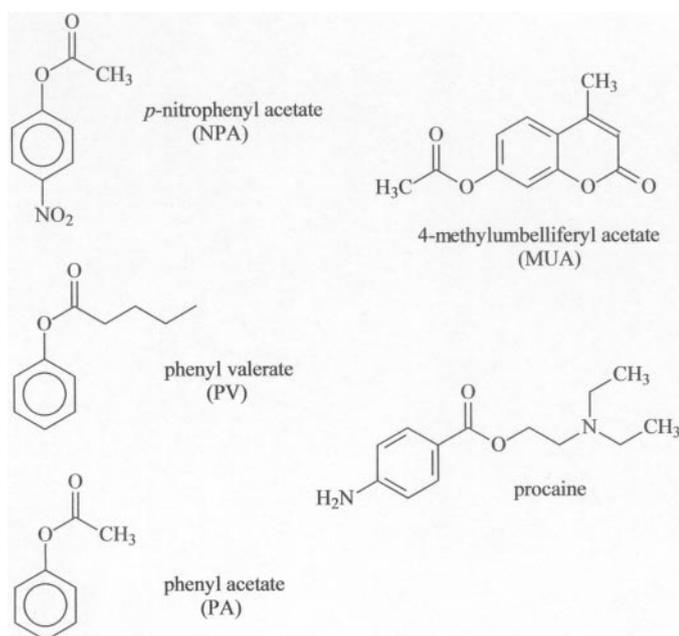
FIG. 1. Structures of substrates.

form of the drug by dermal esterases, this form passes directly into the systemic circulation (Liederer and Borchardt, 2006). To allow successful prodrug development for dermal absorption, the esterase isoforms in human skin need to be identified and their substrate specificity needs to be defined. The primary focus of this study was to investigate the hydrolysis of the local anesthetic procaine, specific for hCE2, by the skin of human, minipig, and rat. The hydrolysis of procaine was compared with less specific esters. The secondary aim was to assess the suitability of minipig as a model for studying local hydrolysis of dermally applied drugs.

Materials and Methods

Materials. All chemicals used were purchased from Sigma Chemical (Poole, Dorset, UK). Chemical structures for procaine and carboxylesterase substrates 4-methylumbelliferyl-acetate (MUA), phenylvalerate (PV), and *para*-nitrophenylacetate (NPA) and the arylesterase substrate phenylacetate (PA) are shown in Fig. 1.

Tissue Preparation. Human skin was obtained following breast reduction surgery of healthy female individuals. Patients gave informed consent, and ethical approval was obtained from Durham University Hospital (Newcastle upon Tyne, UK). Human liver was from the liver bank of the Toxicology Unit, University of Newcastle upon Tyne (Newcastle upon Tyne, UK). Tissues were stored at $-70°C$. Four 28-day-old Male Wistar rats were killed by cervical dislocation, clipped of fur, and dorsal skin and livers were stored at $-70°C$. Liver and skin from three male minipigs (9 ± 1.1 kg) were obtained from Pfizer, Inc. (New York, NY), and they were stored at $-70°C$.

Human, minipig, and rat skin were cut with a dermatome to 350 μm to obtain the epidermis and minimal thickness of upper dermis. Tissue was weighed, minced, pulverized with liquid nitrogen with a mortar and pestle, and added to 1 ml of ice-cold KCl/phosphate buffer (150 mM KCl and 0.1 M K_2HPO_4, pH 7.4) per 100 mg of tissue. Tissue was homogenized with an Ultra-Turrax homogenizer (Ultra-Turrax T25; IKA Werke GmbH & Co. KG, Staufen, Germany) with 3×10-s bursts. The homogenate was centrifuged for 10 min at 750g followed by ultracentrifugation of the supernatant for 10 min at 10,000g to remove mitochondria, nuclei, and cell debris. Further centrifugation of the supernatant for 70 min at 100,000g separated the microsomes from the cytosol. Cytosol was retained and stored at $-70°C$. The microsomal pellet was resuspended with phosphate buffer and centrifuged at 100,000g for 70 min, and then the pellet resuspended in glycerol buffer (10% glycerol, 0.05 M Tris, 0.1 mM KCl, and 250 mM sucrose, pH7.4) and stored at $-70°C$ for analysis.

Liver samples were minced and added to 1 ml of ice-cold KCl/phosphate buffer per 100 mg of tissue. Liver was homogenized ($4°C$) with 2×10-s bursts with an Ultra-Turrax homogenizer. The same procedure for homogenization and centrifugation was repeated as for the skin. Microsomal and cytosolic samples were stored at $-70°C$ until required.

Enzyme Assays. Due to the small size of available tissue samples, it was not possible to determine the full kinetic profiles for hydrolysis of the substrates used. Therefore, substrate concentrations reported in the published literature that gave hydrolysis rates approaching the maximal rate were used.

Procaine Hydrolysis. Procaine (100 μM) (Wu et al., 2004) in 100 mM phosphate buffer, pH 8.0, was incubated with microsomal or cytosolic protein (200 μg of skin or 10 μg of liver) in a reaction volume of 250 μl for 30 min at 37°C. Procainamide (50 μM) was used as an internal standard, and the reaction was stopped by 250 μl of acetonitrile/methanol/phosphoric acid (80:19.2:0.8). Less protein was required for minipig liver microsomes to maintain a linear reaction. The reaction mixture was centrifuged for 10 min at 3000g, and the supernatant removed to HPLC vials for analysis (10 μl on column). The hydrolysis product *para*-amino benzoic acid was quantified using an HPLC (Prostar; Varian, Inc., Palo Alto, CA), with a C18 Gemini microbore column (5 μm, 250 $\times$ 2 mm; Phenomenex, Torrance, CA) maintained at 40°C, and detection at 280 nm. Separation was achieved using a flow rate of 0.4 ml/min and gradient from 100% phosphate buffer (25 mM; pH 2.6) changing linearly to 15% acetonitrile, 25% methanol, and 60% phosphate buffer over 3 min maintained for 10 min and returned to 100% phosphate buffer (25 mM; pH 2.6) and re-equilibrated for 10 min. Retention times were procainamide at 6.1 min, procaine at 6.8 min, and *p*-aminobenzoic acid at 7.4 min (Fig. 2). A calibration curve of peak area ratio (metabolite versus internal standard) against concentration was constructed for *para*-amino benzoic acid. Lower limit of detection was 1 ng on column. The substrate was at sufficient concentration to achieve maximum rate of hydrolysis, and the reaction was linear with time over the assay period (data not shown).

4-Methylumbelliferyl Acetate Hydrolysis. Hydrolysis of MUA to 4-methylumbelliferone was determined in a 96-well blackened fluorescence plate. Each well contained 1 μg of microsomal protein or 10 μg of cytosolic protein, 1 mM MUA, and 1.2 mg/ml bovine serum albumin in 100 mM phosphate buffer, pH 7.4, in a final volume of 200 μl. Reaction was started by addition of 1 mM MUA, and fluorescence was recorded for 15 min using an excitation wavelength of 355 nm and an emission wavelength of 460 nm. Spontaneous hydrolysis of MUA was measured in wells in the absence of protein under the same conditions. A standard curve of 4-methylumbelliferone versus fluorescence at 355/460 nm in the presence of bovine serum albumin was used to calculate product formed during the reaction. MUA at 1 mM was at a sufficient concentration for the maximum rate of hydrolysis (Wadkins et al., 2005). The rate of hydrolysis was linear with time.

Phenylvalerate and Phenylacetate Hydrolysis. Hydrolysis of PV (carboxylesterase substrate) or PA (aryl esterase substrate) to phenol was determined in a 96-well plate by a modification of the method used by Mutch (1998). To each well was added 1 μg of microsomal protein or 10 μg of cytosolic protein, 2.5 mM 4-aminoantipyrine, and 5 mM potassium ferricyanide in 200 μl of 50 mM Tris buffer, pH 8.0, at 37°C. The reaction was started by addition of PV or PA at 3 mM, and increase in absorption followed at 510 nm for 15 min. Spontaneous hydrolysis was measured in the absence of protein. A phenol standard curve (range 0.3–2 μg/well) was prepared in parallel to determine phenol formation. Phenol liberated by hydrolysis was converted to the colored ferricyanide complex. Substrate was at 3 mM (Mutch, 1998), and this concentration was sufficient to give linearity and maximum rate of reaction over the assay period.

***p*-Nitrophenyl Acetate Hydrolysis.** Hydrolysis of NPA (carboxylesterase and arylesterase substrate) to *p*-nitrophenol was determined using a 96-well plate method. Microsomal protein (1 μg) or 10 μg of cytosolic protein in 200 μl of 50 mM Tris buffer, pH 8.0, at 37°C was added to each well of a 96-well plate, and 0.5 mM NPA was added to start the reaction. Absorption was measured at 406 nm for 6 min. Spontaneous hydrolysis of NPA was measured in the absence of protein. A standard curve of *p*-nitrophenol (0.1–1 μg/well) was used to quantitate formation of product. Substrate concentration was 0.5 mM (Prusakiewicz et al., 2006), and it was sufficient to achieve maximum rate of reaction and linearity with time over the assay period.

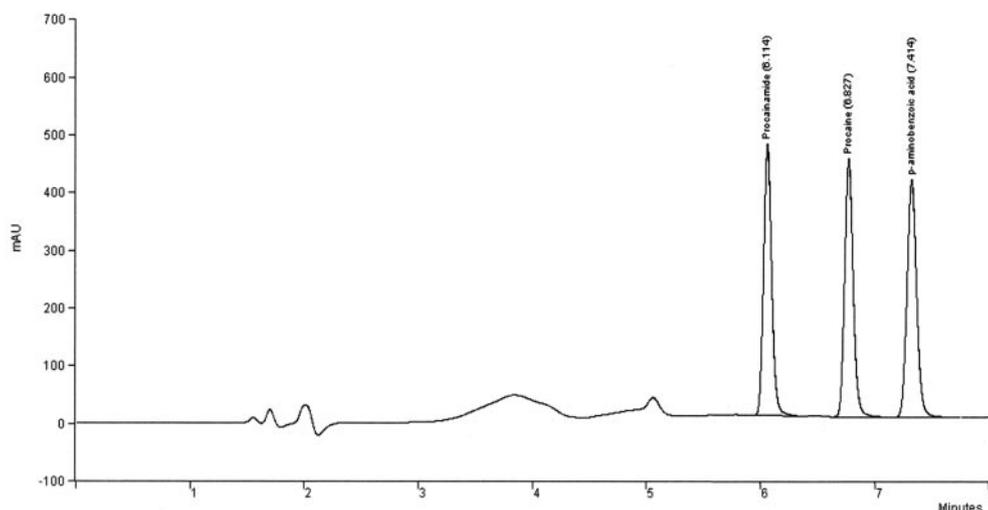**Inhibition Assays.** The carboxylesterase inhibitors bis-nitrophenylphos-

Fig. 2. HPLC chromatogram showing separation and retention times of procainamide (6.1 min), procaine (6.8 min), and *p*-amino benzoic acid (7.4 min) with 200 ng of each compound injected on column.

phate (BNPP; stock solution of 1 mM made up in DMSO) (Yoshigae et al., 1999), paraoxon (stock solution of 1 mM made up in ethanol) (Imai et al., 2003), and phenylmethylsulfonyl fluoride (PMSF; stock solution of 1 mM made up in DMSO) (Yoshigae et al., 1999) were added at a final concentration of 1 μM. Additionally, to determine the presence of cholinesterases, the acetyl/butyryl cholinesterase inhibitor eserine (stock solution of 1 mM made up in DMSO) (Hewitt et al., 2000), the specific acetylcholinesterase inhibitor Bw284c51 (stock solution of 1 mM made up in water), and the specific butyrylcholinesterase inhibitor tetraisopropyl-pyrophosphoramide (IsoOMPA; stock solution of 1 mM made up in ethanol) were used at 1 μM. Bw284c51 and IsoOMPA were shown to completely inhibit acetylcholinesterase and cholinesterase activity at 1 μM (data not shown). To determine whether arylesterase (paraoxonase) contributed to the hydrolysis of ester substrates, mercuric chloride (stock solution of 1 mM made up in water), which completely inhibited arylesterase activity, was added at a final concentration 1 μM (McCracken et al., 1993). All inhibitors were added to microsomes and cytosol fractions from human, minipig, and rat skin and liver in the presence of procaine, MUA, PV, PA, or NPA. Loperamide, an inhibitor of hCE2, was added to incubations with procaine and phenylvalerate at 20 μM (stock solution of 20 mM made up in DMSO), the concentration used by Quinney et al. (2005). All inhibitors were added to incubations for 5 min, and they were maintained at 37°C before addition of substrate. Solvent vehicles alone, at the concentrations used, had no effect on esterase activity.

Protein Assay. Protein concentration in microsomal and cytosolic fractions was measured using bicinchoninic acid according to the method of Smith et al. (1985) and by using 200 mg/ml bovine serum albumin as the protein standard (Sigma Chemical).

Statistical Analysis. Enzyme activities were expressed as micromoles of product per minute per milligram of protein (mean $\pm$ S.E.M.) and micromoles per minute per gram of tissue (mean $\pm$ S.E.M.). Activities were compared by analysis of variance followed by Dunnett's post hoc test using Prism (Graph-Pad Inc., San Diego, CA). Activities in the presence of inhibitors were expressed as a percentage of the control.

Results

Enzyme Assays. Procaine was hydrolyzed to *p*-aminobenzoic acid by skin microsomes and cytosol from human, minipig, and rat at similar rates when expressed in terms of milligrams of protein (Table 1). Procaine hydrolysis by minipig liver microsomes was about 10-fold higher than rat and about 70-fold higher than human ($P < 0.001$). For liver cytosolic fractions, procaine hydrolysis by minipig was about 40 times higher than rat and 370 times higher than human ($P < 0.001$) (Table 1). When expressed per gram of tissue, skin cytosolic hydrolysis of procaine was significantly higher than microsomal fractions for all species ($P < 0.01$) (Table 1). Rates of procaine hydrolysis

were slower (picomoles per minute) than all other ester substrates (micromoles per minute rates).

MUA, a nonspecific carboxylesterase substrate (Barker and Clothier, 1997), was hydrolyzed by skin microsomes and cytosol from minipig and human, but no hydrolysis was detected with rat skin fractions (Table 2). Rates of MUA hydrolysis by skin fractions were lower than by liver fractions ($P < 0.01$). Twenty and 26 times higher hydrolysis rates for MUA were seen with minipig liver microsomes compared with rat or human liver microsomes, respectively ($P < 0.001$). For liver cytosol, MUA hydrolysis by minipig was only 3 times higher than rat and 4 times higher than human ($P < 0.001$). However, when cytosolic hydrolysis was expressed per gram wet weight tissue, hydrolysis of MUA by rat was 4 times higher than minipig and 16 times higher than human ($P < 0.001$).

Skin cytosolic PV hydrolysis was higher than microsomal when expressed per milligram of protein for all species, but not significantly different (Table 3). Minipig liver microsomal and cytosolic fractions showed the highest rate of PV hydrolysis ($P < 0.001$). Rat skin cytosol hydrolysis of PA was similar to human cytosolic activity, and although minipig cytosolic PA hydrolysis was higher, the difference was not significant (Table 4). PA hydrolysis was lower with liver cytosol than microsomes for all species, although only significantly for human and rat ($P < 0.001$).

Rat skin had the highest hydrolase activity with NPA as substrate ($P < 0.001$) (Table 5). Human skin had the lowest microsomal NPA activity, although not significantly lower than minipig ($P > 0.05$). Minipig had the lowest cytosolic NPA activity, but only significantly lower than rat ($P < 0.001$). Rates of NPA hydrolysis by skin cytosolic fractions were greater than with the skin microsomal fraction for all species, in contrast to the liver, which showed higher microsomal activity toward NPA than cytosol.

The rate of NPA hydrolysis by rat and human liver microsomes and cytosol was more rapid than all other substrates ($P < 0.01$). Human liver microsomes showed the highest microsomal NPA hydrolysis, and rat liver cytosol showed the highest cytosolic NPA hydrolysis rates. For minipig liver microsomes, the substrate with the highest rate of hydrolysis was MUA, being significantly higher than procaine, PA, and NPA ($P < 0.01$), but not PV. The activities were expressed in relation to the wet weight of tissue as well as protein to define the relative contributions of microsomes and cytosol within the liver or skin. The cytosolic and microsomal protein contents of minipig, human, and rat liver and skin microsomal and cytosolic fractions are detailed in Table 6. However, it is difficult and perhaps inappropriate

TABLE 1

Rates of procaine (100 μ M) hydrolysis in liver and skin microsomal and cytosolic fractions expressed as picomoles per minute per milligram of protein (picomoles per minute per gram of wet weight tissue in italics)

Data are mean ± S.E.M.; n = 3 for minipig and human, n = 4 for rat.

	Liver			Skin		
	Minipig	Human	Rat	Minipig	Human	Rat
Microsomes	486 ± 93	6.99 ± 2.56	52.9 ± 5.6	1.89 ± 0.29	1.79 ± 0.51	2.33 ± 0.28
	5703 ± 1085	*69.9 ± 25.6*	*524 ± 56*	*2.38 ± 0.37*	*4.79 ± 1.37*	*9.24 ± 1.12*
Cytosol	145 ± 5	0.39 ± 0.15	3.49 ± 0.31	2.09 ± 0.31	1.35 ± 0.35	2.56 ± 0.20
	15,110 ± 498	*39 ± 15*	*439 ± 39*	*32.9 ± 4.81*	*43.1 ± 11.3*	*90.3 ± 6.9*

TABLE 2

Rates of 1 mM 4-methylumbelliferyl acetate hydrolysis in liver and skin microsomal and cytosolic fractions expressed as micromoles per minute per milligram of protein (micromoles per minute per gram of wet weight tissue in italics)

Data are mean ± S.E.M.; n = 3 for minipig, n = 4 for human and rat.

	Liver			Skin		
	Minipig	Human	Rat	Minipig	Human	Rat
Microsomes	15.1 ± 2.8	0.57 ± 0.05	0.77 ± 0.06	0.16 ± 0.04	0.02 ± 0.01	N.D.
	177 ± 33	*5.69 ± 0.45*	*74.0 ± 6.80*	*0.20 ± 0.05*	*0.06 ± 0.02*	
Cytosol	0.25 ± 0.01	0.06 ± 0.01	0.08 ± 0.01	0.15 ± 0.03	0.01 ± 0.001	N.D.
	25.9 ± 0.52	*6.20 ± 1.10*	*98.2 ± 4.1*	*2.36 ± 0.50*	*0.255 ± 0.032*	

N.D., not detectable.

TABLE 3

Rates of 3 mM phenyl valerate hydrolysis in liver and skin microsomal and cytosolic fractions expressed as micromoles per minute per milligram of protein (micromoles per minute per gram of wet weight tissue in italics)

Data are mean ± S.E.M., n = 4 for rat and human, n = 3 for minipig.

	Liver			Skin		
	Minipig	Human	Rat	Minipig	Human	Rat
Microsomes	11.0 ± 0.5	5.22 ± 0.52	5.67 ± 0.27	0.023 ± 0.002	1.06 ± 0.13	0.403 ± 0.037
	129 ± 4	*51.30 ± 5.61*	*56.2 ± 7.74*	*0.029 ±T0.001*	*2.84 ± 0.35*	*1.60 ± 0.13*
Cytosol	1.04 ± 0.05	0.200 ± 0.090	0.24 ± 0.02	0.041 ± 0.011	0.390 ± 0.120	0.224 ± 0.026
	108 ± 12	*20.0 ± 9.0*	*29.8 ± 3.5*	*0.646 ± 0.165*	*12.4 ± 3.8*	*7.91 ± 0.84*

TABLE 4

Rates of 3 mM phenyl acetate hydrolysis in liver and skin microsomal and cytosolic fractions expressed as micromoles per minute per milligram of protein (micromoles per minute per gram of wet weight tissue in italics)

Data are mean± S.E.M.; n = 3 for minipig, n = 4 for human and rat.

	Liver			Skin		
	Minipig	Human	Rat	Minipig	Human	Rat
Microsomes	3.49 ± 0.13	3.15 ± 0.71	6.61 ± 0.43	0.023 ± 0.004	0.145 ± 0.012	0.183 ± 0.012
	41.1 ± 1.09	*31.5 ± 7.1*	*65.4 ± 12.5*	*0.029 ± 0.003*	*0.286 ± 0.034*	*0.727 ± 0.042*
Cytosol	0.364 ± 0.013	0.082 ± 0.019	0.356 ± 0.035	0.062 ± 0.022	0.111 ± 0.010	0.115 ± 0.006
	37.8 ± 3.38	*8.20 ± 1.90*	*44.7 ± 5.35*	*0.977 ± 0.330*	*2.58 ± 0.31*	*4.06 ± 0.19*

to make direct comparisons between liver and dermatomed skin (containing epidermis and some dermis) on the basis of wet weight, because the enzyme activity in skin is predominantly from the basal layer of the epidermis, whereas it is more widely distributed in the hepatocytes in liver tissue.

Effect of Inhibitors on Hydrolysis of Procaine. Procaine hydrolysis was completely inhibited by BNPP and paraoxon at 1 μM, confirming the involvement of carboxylesterases (data not shown). Loperamide inhibited procaine hydrolysis by skin and liver microsomal and cytosolic fractions from human, minipig, and rat to varying degrees (Fig. 3). Procaine hydrolysis by human liver microsomes and cytosol was completely inhibited by loperamide, whereas 4.0% activity remained with minipig liver microsomes and 20.6% with rat microsomes, and with similar effects on the cytosolic fractions. Pro-

caine hydrolysis by skin of all species was less sensitive to inhibition by loperamide than the liver. Procaine hydrolysis by rat skin cytosol was inhibited by loperamide more than by microsomes (41.7 and 65.3% activity remaining, respectively). Procaine hydrolysis by minipig skin was the least inhibited by loperamide with 69.6% activity remaining for microsomal and 77.9% for cytosolic fractions. Of the three species, procaine hydrolysis by human skin showed the greatest sensitivity to loperamide, with a reduction of hydrolysis down to 26.9%.

Effects of Inhibitors on Hydrolysis of MUA, PV, PA, and PNA. A concentration of 1 μM was selected for comparative studies looking at sensitivity of hydrolysis of substrates to an inhibitor. The results for minipig, human, and rat are shown in Figs. 4 to 6. Paraoxon at 1 μM, inhibited NPA hydrolysis by microsomal and cytosolic liver and skin

TABLE 5

Rates of 0.5 mM p-nitrophenyl acetate hydrolysis in liver and skin microsomal and cytosolic fractions expressed as micromoles per minute per milligram (micromoles per minute per gram of wet weight tissue in italics)

Data are mean ± S.E.M.; n = 3 for minipig, n = 4 for human and rat.

	Liver			Skin		
	Minipig	Human	Rat	Minipig	Human	Rat
Microsomes	4.07 ± 0.33	18.2 ± 1.2	9.85 ± 0.44	0.061 ± 0.013	0.036 ± 0.009	0.380 ± 0.040
	47.8 ± 2.7	*182 ± 11*	*97.5 ± 12.7*	*0.076 ± 0.009*	*0.069 ± 0.015*	*1.51 ± 0.14*
Cytosol	0.412 ± 0.009	1.68 ± 0.32	2.25 ± 0.14	0.095 ± 0.014	0.128 ± 0.008	0.770 ± 0.050
	42.8 ± 2.34	*168 ± 32*	*282 ± 21*	*1.49 ± 0.21*	*2.95 ± 0.29*	*27.2 ± 1.61*

TABLE 6

Recovery of subcellular protein from liver and skin of minipig, human, and rat

Data are mean ± S.E.M.; n = 3 for minipig, n = 4 for human and rat.

	Recovered Subcellular Protein		
	Minipig	Human	Rat
		mg/g tissue	
Liver microsomes	11.8 ± 1.26	10[a] ± —	9.84 ± 1.03
Liver cytosol	104 ± 7.09	100[a] ± —	126 ± 1.62
Skin microsomes	1.40 ± 0.17	1.93 ± 0.11	4.02 ± 0.15
Skin cytosol	16.1 ± 0.74	22.9 ± 1.39	35.3 ± 0.77

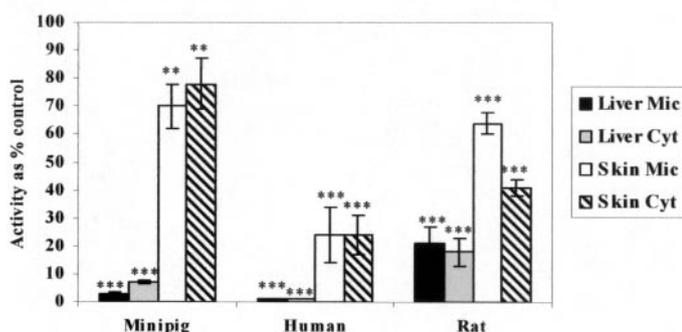[a] Recovery data for samples not determined; data provided as an approximation based on previous studies.

FIG. 3. Effect of the inhibitor loperamide (20 μM) on the hydrolysis of 100 μM procaine with minipig, human, and rat liver and skin microsomal and cytosolic fractions. Control had solvent vehicle only. Activity expressed as a percentage of control (mean ± S.E.M.; n = 4 for rat, n = 3 for human and minipig). *, P < 0.05; **, P < 0.01; and ***, P < 0.001).

fractions for all species (P < 0.001). Paraoxon inhibited hydrolysis of all substrates by minipig and human microsomes (P < 0.01), but it was less effective with cytosolic fractions, not significantly inhibiting minipig skin microsomal PV, PA, or MUA hydrolysis or minipig skin cytosolic PA hydrolysis. In human skin, paraoxon inhibited hydrolysis of all substrates (P < 0.001) except for cytosolic PA hydrolysis. In rat liver, paraoxon did not significantly inhibit microsomal PV hydrolysis or cytosolic PV or MUA hydrolysis. In rat skin cytosolic fractions, paraoxon did not significantly inhibit PV hydrolysis.

BNPP at 1 μM significantly inhibited hydrolysis of all ester substrates by liver fractions from all species (P < 0.01). However, BNPP did not inhibit minipig skin hydrolysis of any substrate except PV by the cytosolic fraction (P < 0.001). With human skin BNPP inhibited microsomal hydrolysis of all substrates and only cytosolic PV hydrolysis. In rat, BNPP significantly inhibited hydrolysis of all substrates by skin fractions (P < 0.01) except for skin cytosolic PA hydrolysis.

PMSF at 1 μM was not an effective inhibitor of ester hydrolysis by human or minipig liver, and it only inhibited minipig liver microsomal

NPA and PV hydrolysis, liver cytosolic PV hydrolysis and skin microsomal NPA hydrolysis (P < 0.001). In human, only liver and skin microsomal NPA was inhibited (P < 0.001). In rat, PMSF significantly inhibited liver microsomal and cytosolic NPA hydrolysis (P < 0.05) and PA and MUA hydrolysis (P < 0.001). Hydrolysis by rat skin was inhibited similarly except for microsomal NPA hydrolysis, which was not inhibited.

Mercuric chloride at 1 μM was also a poor inhibitor of minipig and human liver and skin ester hydrolysis. Only minipig liver microsomal NPA hydrolysis and human liver microsomal NPA and PA hydrolysis were inhibited (P < 0.001). In rat, mercuric chloride inhibited liver microsomal and cytosolic NPA, PA, and MUA hydrolysis (P < 0.01), skin microsomal NPA and PA hydrolysis, and skin cytosolic NPA and PV hydrolysis (P < 0.001).

Eserine and Bw284c51, inhibitors of acetylcholinesterase, and IsoOMPA, a cholinesterase inhibitor, at 1 μM had little effect on hydrolysis of any of the substrates by liver microsomal or cytosolic fractions of any species tested (data not shown).

Discussion

Carboxylesterase activity with PV, PNA, and procaine as substrate, expressed in terms of milligrams of protein for skin microsomes and cytosol, was lower than liver for human, minipig, and rat. The interspecies profile of activity in the skin was not predicted by that in the liver, suggesting differing expression of the carboxylesterase isozymes. Procaine, specific for hCE2 in the gut and recombinant hCE2 enzyme, was most efficiently hydrolyzed by minipig liver and least by human liver. Skin hydrolysis of procaine was greatest with rat but at similar levels for minipig and human. The activity in human skin suggested expression of hCE2, and the equivalent isoforms in minipig and rat skin remain to be identified. Hydrolysis of hCE2-specific substrates has not previously been studied with skin, but Zhu et al. (2005) showed that hCE2 protein was highly expressed in human skin, whereas hCE1 was weakly expressed or not detectable. The capacity of skin microsomes and cytosol to metabolize procaine suggests that skin has similarities to other extrahepatic tissues such as in the gut (Sanghani et al., 2003) and lung (Mutch et al., 2007). Procaine administered transdermally would be a substrate for these esterase isoforms locally in the skin, limiting local anesthetic effects and contributing to its low potency. Loperamide has been shown to specifically inhibit hCE2 in gut and liver (Quinney et al., 2005). This study has shown that loperamide specifically inhibited procaine hydrolysis in human skin microsomes and in cytosol by 73% and in rat skin microsomes and cytosol by 25 and 60% respectively, but that it had less effect on minipig skin, with hydrolysis reduced by only 30% for microsomes and 20% for cytosol. By comparison, loperamide had little effect on the hydrolysis of the less specific substrates. Lack of hydrolysis of MUA by rat skin
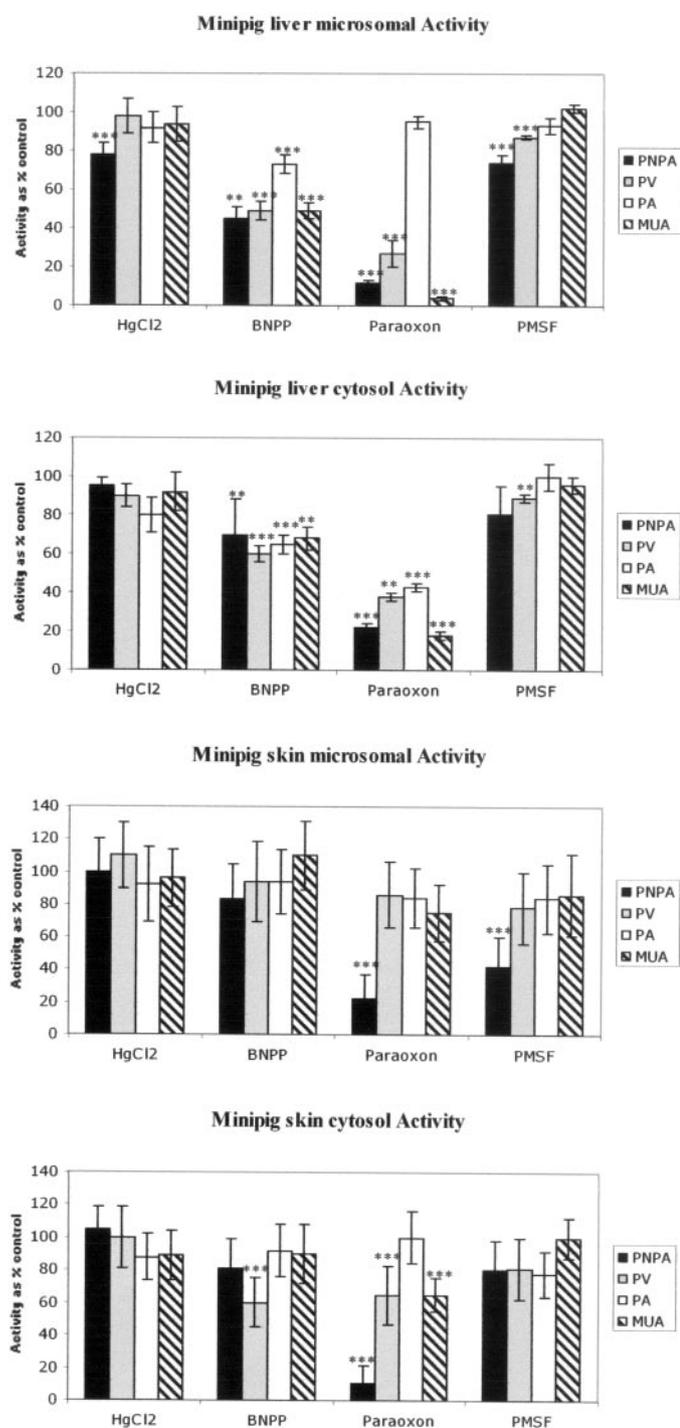
FIG. 4. Effect of the inhibitors (1 μM) HgCl$_2$, BNPP, paraoxon, and PMSF on the hydrolysis of the substrates PNPA (0.5 mM), PV (3 mM), PA (3 mM), and MUA (1 mM) with minipig liver and skin microsomal and cytosolic fractions. Controls were treated with solvent vehicle only. Activity is expressed as a percentage of control (mean $\pm$ S.E.M.; $n = 3$). *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

FIG. 5. Effect of the inhibitors (1 μM) HgCl$_2$, BNPP, paraoxon, and PMSF on the hydrolysis of the substrates PNPA (0.5 mM), PV (3 mM), PA (3 mM), and MUA (1 mM) with human liver and skin microsomal and cytosolic fractions. Controls were treated with solvent vehicle only. Activity expressed as a percentage of control (mean $\pm$ S.E.M.; $n = 3$). *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

indicated that esterases had low affinity for MUA but that they were able to hydrolyze PV and NPA.

Studies in which a range of ester substrates were incubated with liver microsomes have previously shown that the sizes of the alcohol and acyl leaving groups influence the specificity for hCE1 and hCE2. hCE1 metabolized molecules with a small alcohol group and large acyl group (e.g., methyl cocaine); conversely, hCE2 metabolized substrates with a small acyl and large alcohol group (e.g., procaine).
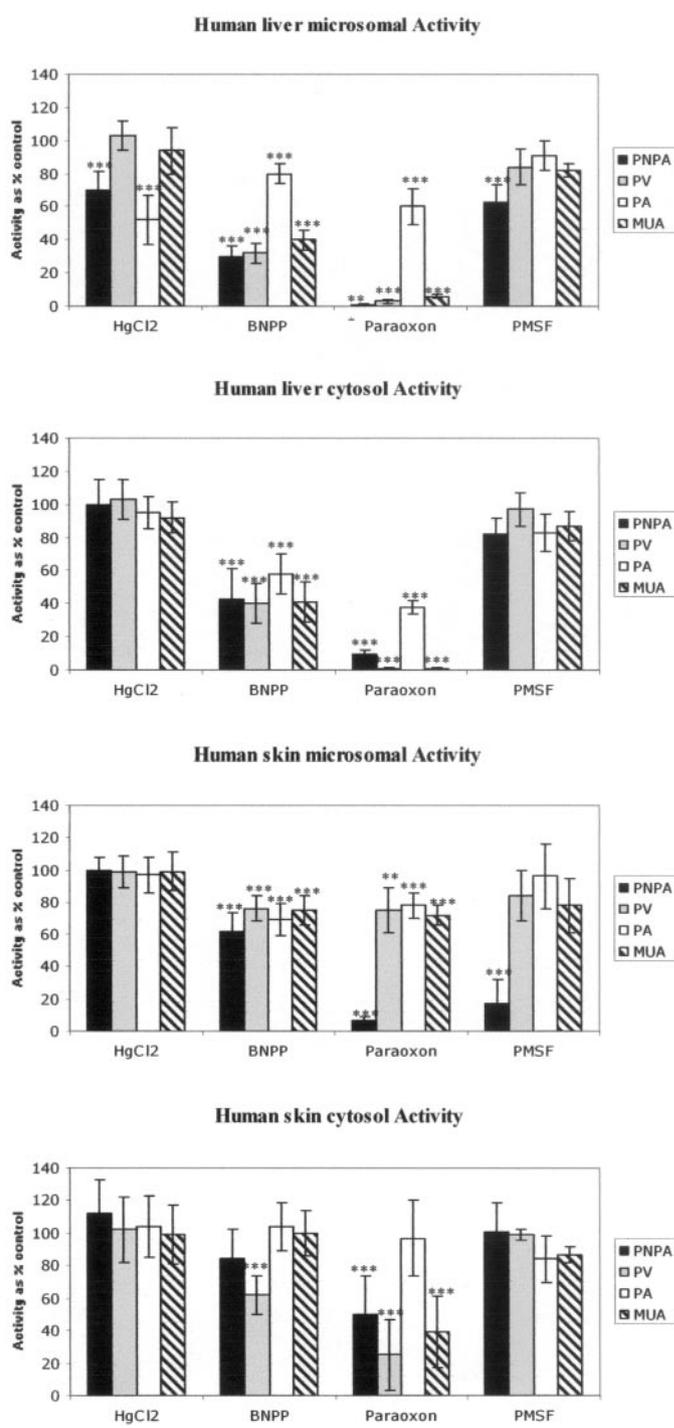
For homologous series of esters with increasing sizes of acyl group, the specificity changed from hCE2 to hCE1 (Yoshigae et al., 1999; Imai et al., 2006; Satoh and Hosokawa, 2006). Inspection of the formulae (Fig. 1) of the substrates used indicate that PV and NPA have small alcohol and acyl groups and that they may be nonspecific substrates for carboxylesterase isoforms. MUA has a large umbelliferyl alcohol group contributing to a different activity profile from the other substrates, possibly having some affinity for hCE2. Procaine
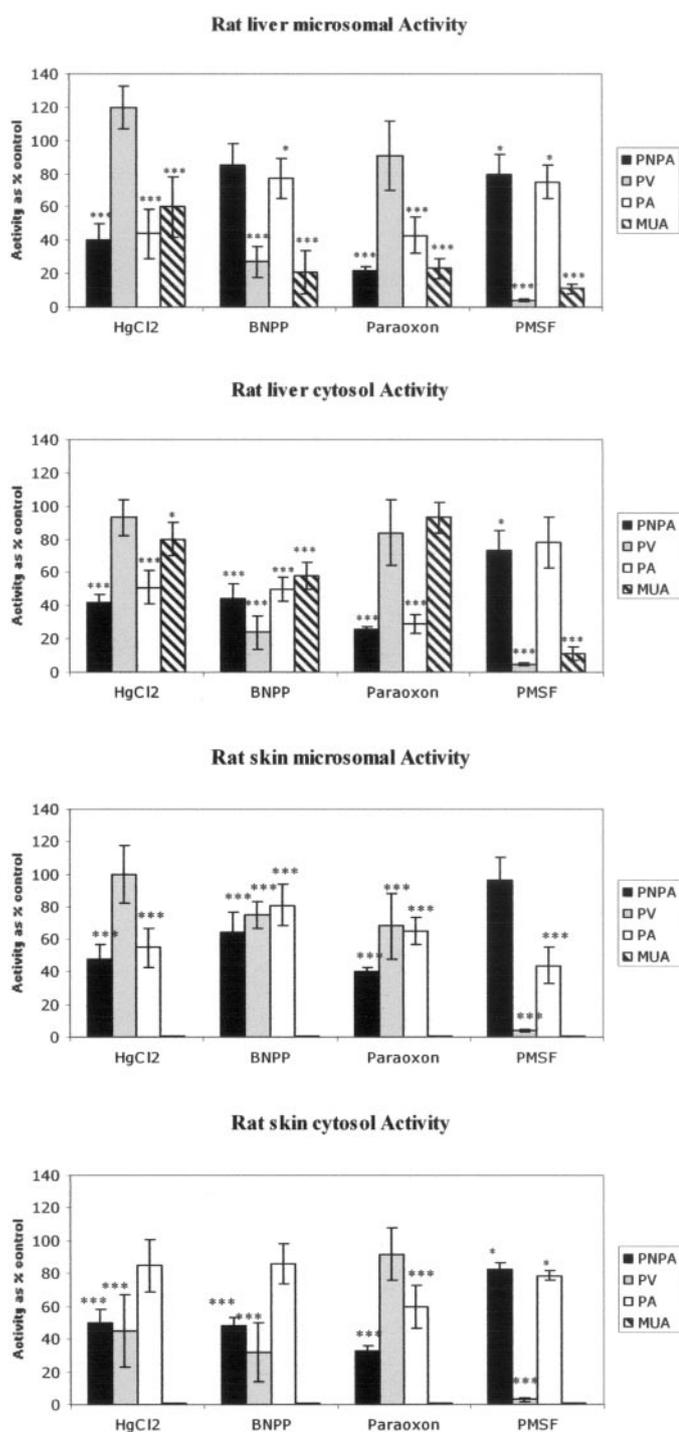
FIG. 6. Effect of the inhibitors (1 μM) HgCl$_2$, BNPP, paraoxon, and PMSF on the hydrolysis of the substrates PNPA (0.5 mM), PV (3 mM), PA (3 mM), and MUA (1 mM) with rat liver and skin microsomal and cytosolic fractions. Activity expressed as a percentage of control (mean $\pm$ S.E.M.; $n = 4$). *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

has a large alcohol group, and this would be consistent with preferential hydrolysis by hCE2. PA, which has previously been shown to have a greater affinity for arylesterase (cysteine active site), was also hydrolyzed by skin carboxylesterases.

This is the first time that minipig skin esterase activity has been compared with human using procaine as a substrate in comparison with less specific substrates. Prusakiewicz et al. (2006) reported hydrolysis of NPA by minipig skin. Generally, there is limited literature on metabolism of drug esters by minipig skin despite the use of minipigs as an in vivo model for determination of the kinetics of transdermal drug preparations. In this study, we have included results for dorsal minipig skin, the site at which ester prodrugs would generally be applied.

Previously published investigations have concentrated on the identity and activity of microsomal carboxylesterases, and these results indicate the importance of cytosolic esterase activity in the skin and that the inhibition profile was similar to the microsomes. Both microsomal and cytosolic carboxylesterases could potentially hydrolyze an ester drug during percutaneous penetration. A diffusing ester that enters the keratinocyte would first come into contact with cytosolic enzymes before uptake into the lumen of the endoplasmic reticulum, where microsomal esterases are located. These studies have been conducted with subcellular fractions; however, it is very important to study whole tissue to indicate the actual importance of esterases during absorption. Also, interindividual variability in the expression of isoforms in the skin may influence the efficacy of transdermal ester prodrugs as has been suggested for irinotecan hydrolysis by hCE2 in the gut (Marsh et al., 2004).

In conclusion, ester hydrolysis by human skin was similar to minipig, and both were lower than rat. These results have highlighted the potential of skin to hydrolyze procaine that is topically applied, and loperamide inhibition indicated involvement of hCE2. This knowledge will aid in advancing transdermal prodrug development.

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