Arylacetamide Deacetylase Is a Determinant Enzyme for the Difference in Hydrolase Activities of Phenacetin and Acetaminophen

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ABSTRACT:

Phenacetin was withdrawn from the market because it caused renal failure in some patients. Many reports indicated that the nephrotoxicity of phenacetin is associated with the hydrolyzed metabolite, p-phenetidine. Acetaminophen (APAP), the major metabolite of phenacetin, is also hydrolyzed to p-aminophenol, which is a nephrotoxicant. However, APAP is safely prescribed if used in normal therapeutic doses. This background prompted us to investigate the difference between phenacetin and APAP hydrolase activities in human liver. In this study, we found that phenacetin is efficiently hydrolyzed in human liver microsomes (HLM) [Clintmax 1.08 ± 0.02 ml/(min · mg)], whereas APAP is hardly hydrolyzed [0.02 ± 0.00 ml/(min · mg)]. To identify the esterase involved in their hydrolysis, the activities were measured using recombinant human carboxylesterase (CES) 1A1, CES2, and arylacetamide deacetylase (AADAC). Among these, AADAC showed a K\text{m} value (1.82 ± 0.02 mM) similar to that of HLM (3.30 ± 0.16 mM) and the highest activity [V\text{max} 6.03 ± 0.14 nmol/(min · mg)]. In contrast, APAP was poorly hydrolyzed by the three esterases. The large contribution of AADAC to phenacetin hydrolysis was demonstrated by the prediction with a relative activity factor. In addition, the phenacetin hydrolase activity by AADAC was activated by flutamide (5-fold) as well as that in HLM (4-fold), and the activity in HLM was potently inhibited by eserine, a strong inhibitor of AADAC. In conclusion, we found that AADAC is the principal enzyme responsible for the phenacetin hydrolysis, and the difference of hydrolase activity between phenacetin and APAP is largely due to the substrate specificity of AADAC.

Introduction

Phenacetin [N-(4-ethoxyphenyl)acetamide] had been widely used as an analgesic antipyretic drug. Although phenacetin was developed as a prodrug of acetaminophen (APAP), it was withdrawn from the market because it was found to cause renal failure in some patients (Sicardi et al., 1991; Gago-Dominguez et al., 1999). Phenacetin is primarily metabolized to APAP through deethylation by CY1A2, but it was also metabolized to p-phenetidine through deacetylation by esterase in humans (Butler et al., 1989; Kudo et al., 2000). p-Phenetidine is further metabolized to N-hydroxyphenetidine, and this metabolite is considered to cause renal failure (Fig. 1) (Shudo et al., 1978; Vaught et al., 1981; Wirth et al., 1982). Studies on carcinogenic arylamines have implicated the metabolic conversion of N-hydroxylamines to nitroso derivatives as a critical pathway to the covalent binding to DNA (Hein et al., 1987; McManus, 1989). Thus, identification of the enzymes responsible for the phenacetin hydrolysis pathway is considered to be important to elucidate the phenacetin-induced renal failures.

APAP is well known to be biotransformed to quinoneimine or some other radical species, leading to hepatotoxicity (Moldes et al., 1982; Dahlin et al., 1984). Moreover, p-aminophenol (PAP), the hydrolyzed metabolite of APAP, was reported to act as a nephrotoxicant in F344 rats, and inhibition of acetaminophen deacetylation by bis-(p-nitrophenyl) phosphate greatly diminished the nephrotoxicity by preventing the formation of PAP, suggesting that the nephrotoxicity of acetaminophen is due, at least in part, to PAP (Newton et al., 1982, 1985). However, in humans, there are no reports that the renal toxicity caused by APAP was associated with the production of PAP.

Esterases contribute to the hydrolysis of 10% of clinical therapeutic drugs including ester, amide, and thioester bonds (Williams et al., 2004). In particular, human carboxylesterase (CES), a major serine esterase, contributes to the hydrolysis of a majority of drugs and xenobiotics. The CES isoforms responsible for drug metabolism are CES1A1 (protein accession number: NP_001020365.1) and CES2 (NP_003860.2), but it was reported that these enzymes are not involved in phenacetin hydrolysis (Takai et al., 1997). As a candidate

ABBREVIATIONS: APAP, acetaminophen; PAP, p-aminophenol; CES, carboxylesterase; AADAC, arylacetamide deacetylase; HLM, human liver microsomes; PNPA, p-nitrophenyl acetate; CPT-11, irinotecan hydrochloride; SN-38, 7-ethyl-10-hydroxycamptotecin; DMSO, dimethyl sulfoxide; HPLC, high-performance liquid chromatography; RAF, relative activity factor.
enzyme, human arylacetamide deacetylase (AADAC) (NP_001077.2) could be considered. Ross and Crow (2007) reported that when SDS-polyacrylamide gel electrophoresis gel of human liver microsomes (HLM) was treated with a fluorophosphate probe that reacts specifically with serine hydroxylase enzymes, three enzymes, AADAC, CES1A1, and CES2, could be detected. Thus, AADAC, as well as CES enzymes, is a major serine hydroxylase in HLM. AADAC was identified as the enzyme responsible for deacetylation of the carcinogen, 2-acetylaminofluorene (Probst et al., 1991), and we first demonstrated that AADAC is a principal enzyme in the hydrolysis of fluoxetine, an antidepressant drug (Watanabe et al., 2009). In this study, we hypothesized that phenacetin is hydrolyzed by AADAC and investigated the difference in the catalytic efficiencies between phenacetin and APAP hydrolysis in HLM and AADAC.

Materials and Methods

Chemicals and Reagents. Imidapril hydrochloride and imidaprilat were kindly supplied by Mitsubishi Tanabe Pharma Corporation (Tokyo, Japan). Flutamide, 5-amino-2-nitrobenzotrifluoride, phenacetin, APAP, and PAP were purchased from Wako Pure Chemicals (Osaka, Japan). p-Nitrophenyl acetate (PNPA) and p-nitrophenidine were purchased from Sigma-Aldrich (St. Louis, MO). Iminotecan hydrochloride (CPT-11) and 7-ethyl-10-hydroxycamptothecin (SN-38) were purchased from Toronto Research Chemicals Inc. (North York, ON, Canada). HLM (pooled, n = 50) were purchased from BD Gentest (Woburn, MA). Primers were commercially synthesized at Hokkaido System Sciences (Sapporo, Japan). All other chemicals used in this study were of analytical or the highest quality commercially available.

Expression of Human AADAC in Sf21 Cells. Expression of human AADAC using a Bac-to-Bac Baculovirus Expression System (Invitrogen, Carlsbad, CA) was performed according to the manufacturer’s protocol. In brief, human AADAC cDNA was prepared by a reverse transcription-polymerase chain reaction technique using total RNA from human liver (Stratagene, La Jolla, CA) with the following primer sets: sense primer (5′-TAGAGACCAGAAAGCGGGA-3′) and antisense primer (5′-GCTACTGTTTCTACTATATTCTC-3′). The polymerase chain reaction product was first subcloned into pTARGET Mammalian Expression Vector (Promega, Madison, WI). The AADAC cDNA in the pTARGET vector was then transferred into the pFastBac1 vector using the appropriate restriction enzymes. The pFastBac1 vector containing AADAC cDNA was transformed into DH10Bac competent cells, followed by transposition of the inserts into bacmid DNA. The sequence of the AADAC cDNA was determined using a Thermo Sequenase Cy5.5 Dye Terminator Cycle Sequencing kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK) with a Long-Read Tower DNA sequence (GE Healthcare). In this study, the nucleotide sequence of AADAC was referred to as NM_0010862. Nonrecombinant bacmid DNA (mock) was also prepared by the same procedures.

Spodoptera frugiperda Sf21 cells (Invitrogen) were grown in SF-900 II SFM containing 10% fetal bovine serum at 27°C. The recombinant and mock bacmid DNAs were separately transfected into Sf21 cells with Cellfectin Reagent (Invitrogen), and the virus was harvested by collecting the cell culture medium at 72 h after transfection. Cells were routinely harvested 72 h after infection, washed twice with phosphate-buffered saline, and stored at 80°C until use. Cell homogenates were prepared by suspension in TGE buffer [10 mM Tris-HCl buffer (pH 7.4), 20% glycerol, and 1 mM EDTA (pH 7.4)] and by disruption by freeze-thawing three times according to the method reported by Ren et al. (2000). Then the suspensions were homogenized with a Teflon-glass homogenizer for 10 strokes. The enzyme preparations were stored at ~80°C until use. The AADAC expression was confirmed with Western blotting according to the previous report (Watanabe et al., 2009). The protein concentrations were determined according to Bradford (1976).

Phenacetin Hydrolysis Activity. Phenacetin hydrolysis activity was determined as follows. A typical incubation mixture (final volume of 0.2 ml) contained 100 mM potassium phosphate buffer (pH 7.4) and various enzyme sources (HLM and Sf21 cell homogenates expressing esterases) and incubation time (~60 min). Phenacetin was dissolved in dimethyl sulfoxide (DMSO), and the final concentration of DMSO in the incubation mixture was 1.0%. The reaction was initiated by the addition of 0.05 to 4 mM phenacetin after a 2-min preincubation at 37°C. After the 20-min incubation at 37°C, the reaction was terminated by the addition of 10 µl of ice-cold 60% perchloric acid. After removal of the protein by centrifugation at 9500g for 5 min, a 50-µl portion of the supernatant was subjected to HPLC. The HPLC analysis was performed using an L-7100 pump (Hitachi, Tokyo, Japan), an L-7200 autosampler (Hitachi), an L-7405 UV detector (Hitachi), and a D-2500 chromato-integrator (Hitachi) equipped with a Mightyrs RP-18 GP column (5-µm particle size, 4.6 mm i.d. × 150 mm; Kanto Chemical, Tokyo, Japan). The eluent was monitored at 232 nm with a noise-base clean Uni-3 (Union, Gunma, Japan), which can reduce the noise by integrating the output and increase the signal 3-fold by differentiating the output and 5-fold by further amplification with an internal amplifier, resulting in a maximum 15-fold amplification of the signal. The mobile phase was 10% acetonitrile containing 25 mM potassium dihydrogen phosphate. The flow rate was 1.0 ml/min. The column temperature was 35°C. The quantification of p-phenetidine was performed by comparing the HPLC peak height with that of an authentic standard. For kinetic analyses of phenacetin hydrolysis activity, the parameters were estimated from the fitted curves using a computer program (KaleidaGraph; Synergy Software, Reading, PA) designed for nonlinear regression analysis.

To examine the esterase responsible for phenacetin hydrolysis, the stimulatory effect of flutamide on the phenacetin hydrolase activity was investigated. Flutamide was added to the reaction mixture described above at concentrations of up to 500 µM. Flutamide was dissolved in DMSO, and the final concentration of DMSO was 1.5% in all incubations. The phenacetin concentration was 1 mM. The control incubations were conducted without flutamide.

Moreover, inhibition analysis of phenacetin hydrolysis activity was performed using eserine. The concentration of eserine ranged from 1 to 10 µM. The phenacetin concentration was 1 mM. Eserine was dissolved in distilled water, and the final concentration of DMSO in the incubation mixture was 1.0%.

Acetaminophen Hydrolysis Activity. The APAP hydrolysis activity was determined as follows. A typical incubation mixture (final volume of 0.2 ml) contained 100 mM potassium phosphate buffer (pH 7.4), and various enzyme sources (HLM and Sf21 cell homogenates expressing esterases): 1.0 mg/ml human microsomal protein and Sf21 cell homogenates expressing esterases) and incubation time (~120 min). APAP was dissolved in DMSO, and the final concentration of DMSO in the incubation mixture was 1.0%. The reaction was initiated by the addition of 0.5 to 25 mM APAP after a 2-min preincubation at 37°C. After the 60-min incubation at 37°C, the reaction was terminated by the addition of 100 µl of ice-cold methanol. After removal of the protein by centrifugation at 9500g for 5 min, a 50-µl portion of the supernatant was subjected to HPLC. The HPLC analysis was performed as described above. The eluent was
monitored at 312 nm with a noise-base clean Uni-3. The mobile phase was 2% methanol containing 50 mM ammonium acetate. The flow rate was 1.0 ml/min The column temperature was 35°C. The quantification of PAP was performed by comparing the HPLC peak height with that of an authentic standard. The kinetic parameters were estimated as described above.

Other Hydrolase Activities. PNPA, flutamide, imidapril, and CPT-11 hydrolyase activities were determined as described previously (Takahashi et al., 2009; Watanabe et al., 2009; Maruichi et al., 2010). The substrate concentrations of PNPA, flutamide, imidapril, and CPT-11 were 200, 500, 100, and 2 μM, respectively.

Contribution of AADAC, CES1A1, and CES2 to Phenacetin Hydrolase Activity in HLM. The percent contributions of AADAC, CES1A1, and CES2 to phenacetin hydrolase activity were estimated by applying the relative activity factor (RAF) as the ratio of activity values. The RAF values for AADAC (RAF_AADAC) were determined as the ratios of the flutamide hydrolase activity in HLM to the values for recombinant AADAC. The RAF values for CES1A1 (RAF_CES1A1) were determined as the ratios of the imidapril hydrolase activity in HLM to the values for recombinant CES1A1. The RAF values for CES2 (RAF_CES2) were determined as the ratios of the CPT-11 hydrolase activity in HLM to the values for recombinant CES2. With use of RAF, the phenacetin hydrolase activities by AADAC, CES1A1, and CES2 in human liver microsomes (V_AADAC, V_CES1A1, and V_CES2) were determined as the ratios of the flutamide hydrolysis, both hydrolase activities were measured in HLM.

Comparisons of two and several groups were made with an unpaired, two-tailed Student’s t test and a nonparametric analysis of variance, respectively. P < 0.05 was considered statistically significant.

Results

Kinetic Analyses of Phenacetin and APAP Hydrolase Activities in HLM. To examine the catalytic efficiencies of phenacetin and APAP hydrolysis in HLM, both hydrolase activities were measured in pooled HLM (Fig. 2; Table 1). The maximum concentrations were 4 mM phenacetin and 25 mM APAP in the incubation mixture. For phenacetin hydrolysis, the $K_{m}$ and $V_{max}$ values in HLM were 3.30 ± 0.16 mM and 3.58 ± 0.20 nmol/(min · mg), respectively, resulting in an intrinsic clearance (CLint) value of 1.08 ± 0.02 μl/(min · mg) protein (Fig. 2A). On the other hand, for the APAP hydrolysis, the maximum substrate concentration (25 mM) was not sufficiently high to determine the $K_{m}$ values because the solubility of APAP limited the substrate concentration that could be achieved in the incubation mixture (Fig. 2B). Therefore, the CLint value was calculated with the initial slope of the plots of velocity versus the substrate concentration. The CLint value of the APAP hydrolysis activity in HLM was 0.02 ± 0.00 μl/(min · mg) protein, which was a value significantly ($P < 0.001$) lower than that of the phenacetin hydrolysis activity. Therefore, the efficiency of phenacetin hydrolysis in HLM was much greater than that of APAP hydrolysis.

PNPA, Flutamide, Imidapril, and CPT-11 Hydrolase Activities by Recombinant Human AADAC, CES1A1, and CES2. We previously constructed the baculovirus expression systems of CES1A1 and CES2 (T. Fukami, S. Takahashi, N. Nakagawa, T. Maruichi, M. Nakajima, and T. Yokoi, submitted for publication). To confirm the substrate specificities of the homogenates of Sf21 cells expressing human AADAC, CES1A1, and CES2, the hydrolase activities of PNPA and substrates specific for each esterase were measured. The hydrolase activities of PNPA, a general esterase substrate, at a concentration of 200 μM by AADAC, CES1A1, and CES2 were 453.6 ± 32.2, 867.6 ± 21.1, and 364.7 ± 17.7 nmol/(min · mg) protein, respectively. Moreover, flutamide, imidapril, and CPT-11, which are typical substrates for AADAC, CES1A1, and CES2, respectively, were also measured (Takai et al., 1997; Humercihouse et al., 2000; Watanabe et al., 2009). The flutamide hydrolase activity at a concentration of 500 μM was detected by AADAC [0.68 ± 0.03 nmol/(min · mg) protein] but not by CES1A1 and CES2. The imidapril hydrolase activity at a concentration of 100 μM was detected only by CES1A1 [1.73 ± 0.05 nmol/(min · mg) protein]. The CPT-11 hydrolase activity at a concentration of 2 μM was highly detected by CES2 [2.92 ± 0.09 nmol/(min · mg) protein] and slightly detected by AADAC [0.04 ± 0.00 nmol/(min · mg) protein]. These results indicate that the recombinant enzymes show specific catalytic functions of each isoform.

Kinetic Analyses of Phenacetin and APAP Hydrolase Activities by Recombinant AADAC, CES1A1, and CES2. To identify the esterase responsible for phenacetin and APAP hydrolysis in HLM, both hydrolase activities were measured using recombinant AADAC, CES1A1, and CES2 (Fig. 3; Table 2). As shown in Fig. 3A, for phenacetin, the $K_{m}$ and $V_{max}$ values by recombinant AADAC were 1.82 ± 0.02 mM and 6.03 ± 0.14 nmol/(min · mg) protein, respectively, resulting in a CLint value of 3.32 ± 0.05 μl/(min · mg) protein. On the other hand, CES1A1 and CES2 showed lower $K_{m}$ values (0.27 ± 0.01 and 0.30 ± 0.02 mM, respectively) than AADAC but showed lower $V_{max}$ values [0.29 ± 0.02 and 0.38 ± 0.03 nmol/(min · mg) protein, respectively], with the result that the CLint value by AADAC was significantly ($P < 0.001$) higher than those by CES1A1.
and CES2 [1.09 ± 0.01 and 1.25 ± 0.01 μl/(min ⋅ mg) protein, respectively]. Thus, all of the enzymes could catalyze the phenacetin hydrolysis, but AADAC showed a $K_m$ value similar to that in HLM (3.30 ± 0.16 mM) and the highest $V_{max}$ value among the three enzymes. For APAP hydrolysis, the maximum substrate concentration (25 mM) was not sufficiently high to determine the $K_m$ values of CES1A1 and CES2. In AADAC, the $K_m$ and $V_{max}$ values could be calculated, but the calculated $K_m$ value (32.2 ± 1.3 mM) was unreliable because the value was higher than the maximum substrate concentration (25 mM). Therefore, the $V_{max}$ values were calculated with the initial slope of the plots of velocity versus the substrate concentration. The values by AADAC, CES1A1, and CES2 were 0.07 ± 0.02, 0.01 ± 0.00, and 0.02 ± 0.00 μmol/(min ⋅ mg) protein, respectively. Thus, the phenacetin and APAP hydrolytic activities were detected by all enzymes, but their $V_{max}$ values of APAP hydrolysis were much lower than those of phenacetin hydrolysis.

**Contributions of AADAC, CES1A1, and CES2 to Phenacetin Hydrolysis Activity in HLM.** To predict the contributions of AADAC, CES1A1, and CES2 to the phenacetin hydrolysis activity in HLM, RAF values for AADAC, CES1A1, and CES2 were calculated. It was reported that flutamide and imidapril are substrates specific for AADAC and CES1A1, respectively (Takai et al., 1997; Takahashi et al., 2009; Watanabe et al., 2009). On the other hand, it was reported that flutamide and imidapril are substrates specific for AADAC, CES1A1, and CES2 (Kudo et al., 2000). In this study, it was confirmed that the phenacetin hydrolase activity in HLM was reported to be activated by flutamide concentration dependently, and approximately 5-fold higher activity was shown by the addition of 50 μM flutamide (Fig. 5). Thus, it is conceivable that an esterase responsible for the hydrolysis of phenacetin in HLM is activated by flutamide. Among phenacetin hydrolytic activities by esterases investigated in this study, only the activity by AADAC was activated flutamide concentration dependently, and approximately 4-fold higher activity was shown by the addition of 50 μM flutamide. The activities by CES1A1 and CES2 were not activated by flutamide. Thus, these results suggested that the major esterase responsible for the phenacetin hydrolysis in HLM would be AADAC but not CES1A1 and CES2.

**Effects of Eserine on Phenacetin Hydrolysis Activity in HLM and Recombinant AADAC, CES1A1, and CES2.** We previously reported that the flutamide hydrolytic activity of AADAC was potently inhibited by 0.1 to 1 mM eserine (Watanabe et al., 2009). To further clarify that AADAC is mainly involved in the phenacetin hydrolysis in HLM, the inhibitory effect of eserine on the phenacetin hydrolytic activities in HLM and recombinant AADAC, CES1A1, and CES2 was...
investigated (Fig. 6). The phenacetin hydrolase activities in HLM and AADAC were inhibited in an eserine concentration-dependent manner IC₅₀ values of 4.08 ± 0.57 and 2.62 ± 0.13 μM, respectively. In contrast, the activities by CES1A1 and CES2 were not potently inhibited up to 5 μM eserine and were moderately inhibited by 10 μM eserine (residual activity: 57.6 ± 10.0 and 46.2 ± 4.3%, respectively). The similar inhibitory characteristics of HLM and AADAC also supported the fact that AADAC is the principal enzyme for the phenacetin hydrolysis in HLM.

Discussion

Phenacetin had been widely used as an analgesic antipyretic, but it was withdrawn from the market because it was linked to cases of renal failure (Sicardi et al., 1991; Gago-Dominguez et al., 1999). There are many reports that the renal failure caused by phenacetin is associated with N-hydroxyphenetidine, the metabolite through hydrolysis, and the subsequent N-hydroxylation (Shudo et al., 1978; Vaught et al., 1981; Wirth et al., 1982). Thus, it is conceivable that phenacetin hydrolyse played an important role in phenacetin-induced renal failure. In contrast, APAP, a primary metabolite of phenacetin, is well known to be biotransformed to quinoneimine or some other radical species, leading to hepatotoxicity (Moldéus et al., 1982; Dahlin et al., 1984), but APAP itself has been used as a clinical therapeutic drug. PAP, a hydrolyzed metabolite of APAP, was also demonstrated to be a nephrotoxicant in rat (Newton et al., 1982). APAP is known to cause renal failure in humans, although the risk is much lower than that for phenacetin (Buckalew, 1996). However, it is unknown whether PAP is associated with APAP-induced renal failures in humans.

In the present study, we found that the catalytic efficiency of phenacetin hydrolysis in HLM [CLint 1.08 ± 0.02 μl/(min · mg) protein] was significantly (P < 0.001) higher than that of APAP hydrolysis [CLint 0.02 ± 0.00 μl/(min · mg) protein] (Fig. 2). Although the phenacetin and APAP hydrolyse activities were measured using human renal microsomes, both hydrolyse activities were substantially low [0.27 ± 0.02 and 0.05 ± 0.00 nmol/(min · mg) protein at 4 mM phenacetin and 25 mM APAP, respectively] (data not shown). Thus, it was considered that the difference in the catalytic efficiencies between phenacetin and APAP hydrolysis in HLM might affect renal failure in humans.

We recently found that AADAC is a principal enzyme for flutamide hydrolysis (Watanabe et al., 2009). Flutamide is similar to phenacetin in chemical structure and molecular weight. Therefore, we considered that AADAC could be responsible for the phenacetin hydrolysis. However, CES is responsible for the hydrolysis of a majority of esterified drugs and xenobiotics, and the mRNA expression levels of CES1A1 and CES2 in human liver are 116- and 8-fold higher than that of AADAC (Watanabe et al., 2009). In this study, we found that the phenacetin hydrolyse activity was detected by AADAC, CES1A1, and CES2. However, the Kₘ value of AADAC (1.82 ± 0.02 mM) was similar to that of HLM (3.30 ± 0.16 mM). An Eadie-Hofstee plot of phenacetin hydrolyse activity in HLM showed a curvature but not a biphasic pattern (Supplemental Fig. 1), although sigmoidicity was not obvious in the Michaelis-Menten plot (Fig. 2A). In addition, the contribution of AADAC (76.2–95.1%) was predicted to be higher than those of CES1A1 and CES2 (Fig. 4). Takai et al. (1997) reported that purified human CES1A and CES2 proteins could not hydrolyze phenacetin, although the phenacetin hydrolyse activities by CES1A1 and CES2 were slightly detected in this study. The reason for the discrepancy is not clear, but it may partly be attributable to differences in the enzyme sources. The APAP hydrolyse activities by AADAC, CES1A1, and CES2 were scarcely detected [CLint values 0.07 ± 0.01, 0.01 ± 0.00, and 0.02 ± 0.00 μl/(min · mg) protein, respectively]. These values were substantially lower than those of the phenacetin hydrolyse activities by AADAC, CES1A1, and CES2 (1.82 ± 0.02, 0.27 ± 0.01, and 0.30 ± 0.02 mM, respectively). Thus, the difference in the catalytic efficiencies between phenacetin and APAP hydrolysis in HLM is due to the substrate specificity of AADAC.

It has been reported that the phenacetin hydrolyse activity in HLM was activated 4-fold by 300 μM flutamide (Kudo et al., 2000). To confirm the involvement of AADAC in the phenacetin hydrolysis in HLM, we examined whether the phenacetin hydrolyse activity by AADAC was activated by flutamide (Fig. 5). The activities in HLM and AADAC were approximately 5- and 4-fold activated by 50 to 500 μM flutamide, respectively, whereas the activities by CES1A1 and CES2 were not activated. This result supported the fact that AADAC is a phenacetin hydrolyase in HLM. This activation effect implies that AADAC has a binding site for flutamide to enhance the hydrolyse activity. However, no activation effect of flutamide on the PNPA hydrolyse activity by AADAC was observed (data not shown). Although we cannot clearly account for the difference, the activation of AADAC enzyme activity by flutamide would be observed using limited kinds of substrate. To further demonstrate that AADAC is the principle enzyme for phenacetin hydrolysis, the inhibitory effect of...
eserine, which is a potent inhibitor of AADAC (Watanabe et al., 2009), on phenacetin hydrolyase activity was examined (Fig. 6). The phenacetin hydrolyase activities in HLM and AADAC were inhibited in an eserine concentration-dependent manner with IC_{50} values of 4.08 ± 0.57 and 2.62 ± 0.13 μM, respectively. In contrast, the activities by CES1A1 and CES2 were poorly inhibited at up to 5 μM eserine and were only moderately inhibited by 10 μM eserine (residual activity: 57.6 ± 10.0 and 46.2 ± 4.3%, respectively). Although eserine is also a potent inhibitor of butyrylcholinesterase, which is expressed in human plasma as well as liver (Iwatsubo, 1965; Li et al., 2005), the phenacetin hydrolyase activity was not detected in human plasma (data not shown). These results also support the major contribution of AADAC to phenacetin hydrolysis in HLM.

In conclusion, we found that human AADAC is principally involved in phenacetin hydrolysis, and the difference in the catalytic efficiencies between phenacetin and APAP hydrolysis in HLM is due to the substrate specificity of AADAC. Thus, human AADAC would play an important role in phenacetin-induced renal failure.

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