IMMUNOHISTOCHEMICAL LOCALIZATION OF THE ACYLASES THAT CATALYZE THE DEACETYLATION OF N-ACETYLCYLSTEINE AND HALOALKENE-DERIVED MERCAPTURATES

VINITA UTTAMSINGH,1 RAYMOND B. BAGGS, DARIA M. KRENITSKY, AND M. W. ANDERS

Departments of Pharmacology and Physiology (V.U., D.M.K., M.W.A.) and Laboratory Animal Medicine (R.B.B.), University of Rochester Medical Center, Rochester, New York

(Received September 27, 1999; accepted February 17, 2000)

This paper is available online at http://www.dmd.org

ABSTRACT:

Acylases catalyze the hydrolysis of a range of S-substituted N-acetylcysteines. The hydrolysis of N-acetylcysteine is catalyzed by cytosolic acylase I, and activity is present in human endothelial cells and rat lung, intestinal, and liver homogenates. Many haloalkenes are metabolized to mercapturates, which also undergo acylase-catalyzed hydrolysis. The acylases that catalyze the deacetylation of haloalkenes and several haloalkene-derived mercapturates have been recently identified: acylase I catalyzes the deacetylation of N-acetylcysteine and some haloalkene-derived mercapturates whereas an acylase purified from rat kidney cytosol catalyzes the deacetylation of a distinct set of substrates, including several haloalkene-derived mercapturates. The objective of these studies was to examine the tissue and subcellular localization of acylase I and purified rat kidney acylase. Immunoblotting showed the presence of immunoreactive acylase I and purified rat kidney acylase in rat kidney, liver, lung, and brain. Both acylases were identified by immunohistochemistry in several rat organs, including kidney, liver, lung, brain, stomach, intestines, adrenals, pancreas, and testis, indicating that acylase activity is widespread in rat tissues.

Acylases catalyze the deacetylation of N-acetyl-α-amino acids to give fatty acids and amino acids as products. Acylases are cytosolic enzymes whose concentrations are higher in the kidney than in other tissues. Several acylases have been identified: acylase I (EC 3.5.1.14, N-acylamino acid amidohydrolase); aspartoacylase or acylase II (EC 3.5.1.15, N-acetyl-L-aspartate amidohydrolase); acyllysine deacylase (EC 3.1.5.17, N-acetyl-L-lysine amidohydrolase); and acylase III, which catalyzes preferentially the deacetylation of N-acetyl aromatic amino acids (Anders and Dekant, 1994). The acylases may participate in the catabolism of terminal N-acylpeptides or in the salvage of N-acetylated amino acids (Anders and Dekant, 1994). Acylases also play an important role in the metabolism of the prodrug N-acetylcysteine (NAC)2 (Uttamsingh et al., 1998) and in bioactivation of haloalkenes to nephrotoxic intermediates (Commandeur et al., 1988, 1989).

Several nephrotoxic haloalkenes are metabolized to the corresponding mercapturates (S-substituted NAC conjugates) (Anders and Dekant, 1998). Mercapturates are generally more water-soluble than the parent xenobiotics and are excreted in the urine. In the kidney, the mercapturates may also be deacetylated by acylases to afford cysteine S-conjugates, which undergo bioactivation by cysteine conjugate β-lyase (β-lyase) to form cytotoxic metabolites (Dekant et al., 1994; Anders and Dekant, 1998). β-Lyase is a pyridoxal phosphate-dependent enzyme that requires substrates with a free amino group; hence, mercapturates require deacetylation before β-lyase-catalyzed formation of reactive intermediates can occur.

NAC is used in clinical practice for the treatment of congestive and obstructive lung disorders (Webb, 1962; Reas, 1963) and acetaminophen toxicity (Lauterburg et al., 1983; De Flora et al., 1995). NAC may act directly as an antioxidant (Aruoma et al., 1989) or may serve as an L-cysteine prodrug and, thereby, support glutathione synthesis (Lauterburg et al., 1983). NAC is deacetylated in isolated hepatocytes and supports glutathione synthesis in these cells (Thor et al., 1979). Human liver homogenates and endothelial cells deacetylate NAC as do rat intestines and homogenates of rat lung, and liver (Sheffner et al., 1966; Sjödin et al., 1989).

The acylase-catalyzed deacetylation of several xenobiotic-derived mercapturates and NAC has been investigated. Acylase I catalyzes the deacetylation of NAC, N-acetyl-S-(1,1,2,2-tetrafluoroethyl)-L-cysteine, N-acetyl-S-(2-chloro-1,1,2-trifluoroethyl)-L-cysteine, N-acetyl-S-(2-bromo-1,1,2-trifluoroethyl)-L-cysteine, and several S-alkyl NACs (Uttamsingh et al., 1998). Another acylase (purified from rat kidney) selectively catalyzes the deacetylation of several substrates, including N-acetyl-S-(1,2-dichlorovinyl)-L-cysteine, N-acetyl-S(2,2-dibromo-1,1-difluoroethyl)-L-cysteine, N-acetyl-S-(1,2,3,4,4-pentachlorobutadienyl)-L-cysteine, and N-acetyl-S-benzyl-L-cysteine (Uttamsingh and Anders, 1999).

The tissue and subcellular localization of the acylases has not been thoroughly studied. In the pig kidney, acylase I is found in the distal tubule (Löffler et al., 1982), and aspartoacylase is localized in the...
Materials and Methods

Tissue Preparations. Male, Fischer 344 rats (175–200 g; Charles River Breeding Laboratories, Inc., Wilmington, MA) were anesthetized with ether and then sacrificed. Organs were removed, fixed in 10% (v/v) neutralized formalin (J. T. Baker, Phillipsburg, NJ), and embedded in paraffin. Two adjacent serial sections (5 μm) were cut and mounted on each Superfrost Plus Slide (VWR Scientific Products, Bridgeport, NJ) to provide a negative control for each antibody-treated section.

Antibody Preparation. Acylase I (0.5 mg, grade III; Sigma Chemical Co., St. Louis, MO) and purified rat kidney acylase (0.2 mg), prepared as described previously by ammonium sulfate precipitation and column chromatography (Uttamsingh and Anders, 1999), were loaded on a 12% SDS polyacrylamide gel, separated from impurities by gel electrophoresis, and detected with Coomassie Blue stain. The respective protein bands (molecular mass = 46 kDa for acylase I, and molecular mass = 34 kDa for purified rat kidney acylase) were excised, and the gel slices were sent to Charles River PharmServices (Southbridge, MA) for the production of rabbit antisera. The polyclonal antibodies and preimmune sera obtained were stored at −80°C until used.

Western Blotting. Protein samples (50–100 μg) were loaded on a 12% SDS polyacrylamide gel and separated by gel electrophoresis with a Protein mini-gel apparatus (Bio-Rad Laboratories, Richmond, CA). Proteins were transferred to nitrocellulose membranes with a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad Laboratories). Membranes were treated with blocking solution (3% gelatin in Tris-buffered saline), primary antibody (1:1000 dilution), and alkaline-phosphatase-linked goat anti-rabbit secondary antibody provided with the Immuno-Blot Assay Kit (Bio-Rad Laboratories). Membranes were also treated with primary antibodies that had been preabsorbed with acylase I or purified rat kidney acylase. Reagents and protocols provided with the kit were used for the detection of immunoreactive bands.

Immunohistochemistry. Slides mounted with serial paraffin-embedded tissue sections were heated for 1 h at 60°C, paraffin was removed with Pro-par clearant (Analtech Ltd., Battle Creek, MI), hydrated with an ethanol gradient (100–30%), and then washed with water. Endogenous peroxidases in tissue sections were quenched with 3% H2O2 in methanol for 5 min; the slides were then washed with water. Antigen retrieval was performed by incubating the slides in 10 mM citric acid (pH 6.0) for 10 min in a microwave oven (3 min at power level 8, and 7 min at power level 2) in covered plastic Coplin jars. Slides were cooled for a minimum of 30 min and then washed sequentially with 10 mM citric acid (pH 6.0), water, and PBS-X [1.1 mM monobasic potassium phosphate, 154 mM sodium chloride, and 3 mM dibasic sodium phosphate heptahydrate (pH 7.4) containing 0.5% Triton X-100]. Slides were blocked with 20% Avidin D (Avidin/Biotin Blocking Kit; Vector Laboratories, Burlingame, CA) and normal goat serum (1% for acylase I, or 5% for purified rat kidney acylase; Sigma Chemical Co.) in PBS-X in a humid chamber for 30 min at room temperature. The slides were then washed two times for 5 min with PBS-X. On each slide, one tissue section was incubated with 20% biotin solution (Avidin/Biotin Blocking Kit; Vector Laboratories), normal goat serum (1% for acylase I, and 5% for purified rat kidney acylase; Sigma Chemical Co.) and primary antibody (1:500 dilution for acylase I or 1:250 dilution for purified rat kidney acylase) in PBS-X. The other tissue section on each slide was used as a negative control and was treated similarly, but without the primary antibody. Slides were incubated overnight at 4°C in a humid chamber. Cross-contamination of solutions used to treat the two tissue sections on a slide was prevented with a hydrophobic pen (Pap Pen; Polysciences, Inc., Warrington, PA). After approximately 20 h, slides were warmed to room temperature for a minimum of 30 min and washed twice with PBS-X. Slides were then incubated with 1:200 biotinylated goat anti-rabbit IgG antibody (Vectastain Elite ABC Kit; Vector Laboratories), and preimmune sera obtained were stored at −80°C until used.

Results and Discussion

This study describes the tissue and cellular localization of acylase I and a purified rat kidney acylase as determined by immunohistochemistry. Acylase I and purified rat kidney acylase antigens were detected in differing amounts in the organs studied and were present in the cytoplasm of various cell types.

Immunoblotting studies with rat kidney, liver, lung, and brain homogenates with the anti-rabbit antibodies generated against the two acylases yielded single bands in the respective immunoblots, indicating selectivity for the two acylases. The immunoreactive bands displayed mobilities consistent with known subunit molecular masses of the two acylases (~46 kDa for acylase I and ~34 kDa for purified rat kidney acylase) (Fig. 1, A and B). Immunoreactive bands were not
observed on blots treated with antibodies that had been preabsorbed with the respective pure acylases.

In the kidney, acylase I and purified rat kidney acylase antigens were detected in the glomeruli and in the proximal and distal convoluted tubules (Fig. 2, A and B). The podocytes showed mild to moderate staining with acylase I antibodies, whereas with purified rat kidney acylase antibodies, slight staining of the endothelial cells was observed in the same region. The proximal convoluted tubules demonstrated moderate staining of the cytoplasm with no basal to apical differentiation for acylase I antibodies; staining with purified rat kidney acylase antibodies was mild. The brush border was less intensely stained than the cytoplasm of the proximal convoluted tubules with antibodies raised against both acylases. In the distal convoluted tubule, staining of the cytoplasm was observed with acylase I antibodies with no apical to basal differentiation. With purified rat kidney acylase antibodies, the distal convoluted tubules showed moderate staining of the cytoplasm and intense staining of the apical border of the epithelial cells. Note the abundant nonspecific staining adjacent to the erythrocytes with the purified rat kidney acylase antibodies (B).

Sections were incubated with polyclonal anti-acylase I antibodies (A), with polyclonal anti-purified rat kidney acylase antibodies (B), or without primary antibody (C and D) and stained as described in Materials and Methods. P, proximal convoluted tubules; D, distal convoluted tubules; G, glomeruli. Original magnification: 400×; bar = 50 µm. A, proximal and distal convoluted tubules showed moderate staining of the cytoplasm with no basal to apical differentiation for acylase I. B, proximal convoluted tubules showed mild staining of the cytoplasm with no basal to apical differentiation for the purified rat kidney acylase. The distal convoluted tubules showed moderate staining of the cytoplasm and intense staining of the apical border of the epithelial cells. Note the abundant nonspecific staining adjacent to the erythrocytes with the purified rat kidney acylase antibodies (B).

Acylase I and purified rat kidney acylase catalyze the hydrolysis of the mercapturates derived from several haloalkenes that are selectively nephrotoxic (Uttamsingh et al., 1998; Uttamsingh and Anders, 1999). The selective nephrotoxicity of these haloalkenes is associated with the ability of the kidney to accumulate the mercapturates, which undergo acylase-catalyzed hydrolysis to give cysteine S-conjugates that are bioactivated by β-lyases. The S4 segment of the renal proximal tubules is the most severely affected region in rats exposed to the cytotoxic and nephrotoxic haloalkenes (Ishmael et al., 1982; Dohn et al., 1985). Immunohistochemical analyses of β-lyase showed that the enzyme is uniformly distributed in all three segments of the proximal tubules (Jones et al., 1988; MacFarlane et al., 1989; Kim et al., 1997). These experiments indicate that the acylases are also uniformly distributed in the proximal and distal convoluted tubules. Thus, the localization of the acylases may not be responsible for the observed regional toxicity of the haloalkenes, and other factors, including the localization of other enzymes involved in the mercapturate pathway, may be important determinants of the segment-specific nephrotoxicity of the haloalkenes.

Hepatocytes showed moderate cytoplasmic acylase I antigen (Fig. 3, A and B). The staining within the cytoplasm was irregular, and the bile ducts were also stained. With purified rat kidney acylase antibodies, the staining was less intense but more punctate within hepa-
tocytes compared with antibodies to acylase I, but was diffusely spread throughout the lobule (Fig. 3B). The bile duct epithelium was more intensely stained than the hepatocytes and its cytoplasmic distribution was diffuse. Immunostaining was not observed in sections incubated without primary antibody (Fig. 3, C and D).

Liver cytosol catalyzes the deacetylation of NAC and the haloalkene-derived mercapturates, (Sheffner et al., 1966; Suzuki and Tateishi, 1981; Sjödin et al., 1989; Commandeur et al., 1991) although activities of the acylases are lower compared with kidney cytosolic fractions. The immunohistochemical detection of acylase I in hepatocytes is consistent with the observation that administration of NAC for the treatment of acetaminophen toxicity provides protection by increasing glutathione concentrations (Lauterburg et al., 1983). The observed increase in glutathione concentrations indicates that NAC is deacetylated to yield L-cysteine, which is used for the biosynthesis of glutathione. L-Cysteine is the limiting substrate for the biosynthesis of glutathione, and under conditions of stress, when the intracellular glutathione stores are depleted, a supply of L-cysteine is essential to stimulate glutathione biosynthesis (Anderson and Meister, 1987).

Acylase I and purified rat kidney acylase antigens were detected in the gray matter in the brain. The white matter was devoid of staining. Both acylases were detected in the cortex, although staining with purified rat kidney acylase antibodies was milder than with acylase I antibodies. Antibodies to acylase I demonstrated relatively homogeneous labeling of cell bodies of neurons in the pyramidal cell layer, including CA1, CA2, and CA3 (Fig. 4A). A less specific pattern of labeling was observed in the dentate gyrus, which was not abolished by incubation with preimmune serum (Fig. 4C). In contrast, studies with antibodies to purified rat kidney acylase demonstrated faint immunolabeling of CA1, CA2, and CA3 (Fig. 4B), which was abolished when tissue sections were incubated with preimmune serum (Fig. 4D). Again, a less specific pattern of labeling for purified rat kidney acylase antigens was observed in the dentate gyrus, which was not abolished by incubation with preimmune serum (Fig. 4, B and D).

These findings indicate that multiple forms of acylase are expressed in hippocampal pyramidal neurons. Future experiments to investigate whether these acylases have a functional role in protecting pyramidal neurons from excitotoxic and oxidative insults, including hypoxia-ischemia, glucose deprivation, inflammation, and conditions that predispose toward epileptogenesis, are warranted. It will also be of interest to investigate whether expression of these acylases is developmentally regulated.

Acylase I antigen was present in the cytoplasm of neuronal soma of Purkinje cells and in the molecular layer in the cerebellum (Fig. 4E). Staining of the molecular layer was mild and difficult to visualize due to the density of closely packed neuronal nuclei, whereas the Purkinje cells were intensely stained. With purified rat kidney acylase antibodies, little identifiable staining was observed in the molecular layer and in the Purkinje cells (Fig. 4F). Immunostaining was not observed in sections incubated without primary antibody (Fig. 4, G and H).

The role of acylases in neuronal function of the cerebellum is unknown, but future experiments that focus on the ability of cerebellar neurons to regulate expression of acylase I in the conditions of oxidative stress would be of interest. Acylase activity has been detected in brain homogenates. Aspartoacylase is present in high concentrations in the brain and is believed to play an important role in
Fig. 4. Immunohistochemical detection of acylase I and purified rat kidney acylase antigens in rat brain.

Sections were incubated with polyclonal anti-acylase I antibodies (A, E), with polyclonal anti-purified rat kidney acylase antibodies (B, F), or without primary antibody (C, D, G, H) and stained as described in Materials and Methods. Original magnification for A through D: 40×; bar = 500 μm; original magnification for E through H, 400×; bar = 50 μm. Note the distinct neuronal staining (arrow) for acylase I (A) and for purified rat kidney acylase (B) antigens in the hippocampus. In the cerebellum, the Purkinje cells (arrow) stained intensely for acylase I (C); with purified rat kidney acylase antibodies (D), there was little staining of the Purkinje cells (arrow).
brain metabolism (Matalon et al., 1988). Bovine brain aspartoacylase has been purified to homogeneity, and immunohistochemical studies show that aspartoacylase is present in the white matter, although the concentrations of N-acetyl-L-aspartate are higher in the gray matter in the brain than in the white matter (Kaul et al., 1991). The detection of acylase I and purified rat kidney acylase antigens in the brain indicates the ability of the brain to deacetylate NAC and to bioactivate mercapturates. Recent studies show that neuron- and astroglia-rich primary cultures from the brains of neonatal rats express uptake systems for NAC and are able to support glutathione synthesis from extracellular NAC (Kranich et al., 1998; Dringen and Hamprecht, 1999), indicating that NAC undergoes an acylase-dependent deacetylation.

Several enzymes involved in the mercapturate pathway, including glutathione S-transferases, γ-glutamyl transeptidase, cysteine S-conjugate N-acetyltransferase, and β-lyase, are present in the brain. The glutathione-dependent bioactivation of dichloroacetylene is thought to be involved in the dichloroacetylene-induced neurotoxicity (Patel et al., 1994). Dichloroacetylene-derived mercapturates may undergo acylase-catalyzed hydrolysis to S-(1,2-dichlorovinyl)-L-cysteine and contribute to the observed neurotoxicity of dichloroacetylene. Also, the observed accumulation of 5-(N-acetyl-L-cystein-S-yl)-α-methyl-dopamine in the brain after i.c.v. administration of 5-(glutathion-S-yl)-α-methylidopamine is thought to contribute to 3,4-±(methylenedioxy)amphetamine and 3,4-±(methylenedioxy)metamphetamine-mediated neurotoxicity (Miller et al., 1995). These studies, along with the detection of acylases in the brain, indicate that the acylases may contribute to xenobiotic-induced neurotoxicities.

In the lung, the acylase I antigen staining was more intense than with purified rat kidney acylase antigen (Fig. 5, A and B). With acylase I antibodies, the basilar portion of the bronchiolar epithelium was somewhat more intensely stained than the apical portion (Fig. 5A). The muscularis of the arterioles was diffusely stained. In the alveoli, the type-II epithelial cells were more intensely stained than the muscularis, as was a superficial covering of the type-I cells. With purified rat kidney acylase antibodies, no distinct partitioning of staining in the bronchiolar epithelium was observed (Fig. 5B). The muscularis of the arterioles and the alveolar epithelial cells were faintly stained. Immunostaining was not observed in sections incubated with preimmune serum (Fig. 5, C and D).

The administration of NAC for the treatment of pulmonary disorders leads to an increase in glutathione concentrations, indicating that NAC is deacetylated by lung cells (Bridgeman et al., 1994). Also, NAC supplied in the perfusion medium to isolated perfused rat lung supports glutathione synthesis (Berggren et al., 1984) and increases glutathione concentrations in lung cells (De Flora et al., 1995). NAC is deacetylated by acylase I, hence, the detection of acylase I antigen in lung cells supports the finding that the cells of the lung deacetylate NAC.

In the esophagus, acylase I antigen was present predominantly in the polyhedron cornifying layer (data not shown). The germinal layer was relatively unstained, and the superficial, most highly keratinized layer of squamous cells was completely unstained. Only the intermediate zone of actual keratohydration synthesis was stained. The same regions were more intensely stained with purified rat kidney acylase antigens (data not shown). Immunostaining was not observed in sections incubated without primary antibody (data not shown).
Acylase I and purified rat kidney acylase antigens were detected in the chief-cell cytoplasm in the stomach, although intensity of staining was greater with purified rat kidney acylase antibodies than with acylase I antibodies (data not shown). The parietal cell cytoplasm was conspicuous by its lack of staining with antibodies to both acylases. In the small intestine, staining of acylase I antigen was mild and diffuse and concentrated in the apical portion of the enterocytes (Fig. 6A). Purified rat kidney acylase antigen showed more intense staining of enterocytes than that observed with acylase I antigen (Fig. 6B). There was a tendency for apical staining, although significant basilar staining was also observed. Little staining of the goblet cells was observed with acylase I antibodies, and staining with purified rat kidney acylase antibodies was variable. In the large intestine, acylase I antibodies stained the enterocytes diffusely but not as intensely as with purified rat kidney acylase antibodies (data not shown). Immunostaining was not observed in sections incubated without primary antibody (Fig. 6, C and D).

Acylase I staining was most intense in the basilar portion of the exocrine pancreas (data not shown). Acylase I staining was more intense than staining with purified rat kidney acylase, which is present primarily in the apical portion of the exocrine pancreas (data not shown). There was no significant staining of the endocrine pancreas with either acylase I or purified rat kidney acylase antibodies. Immunostaining was not observed in sections incubated without primary antibody (data not shown).

In the adrenals, acylase I antigen was detected in the adrenal cortex (data not shown). The zona fasciculata, zona reticularis, and the medulla were relatively poorly stained. With purified rat kidney acylase antibodies, staining in the adrenal cortex was more intense than with acylase I antibodies (data not shown), but followed the same pattern as with acylase I.

The ventricular myocardium in the heart was diffusely, although poorly, stained with acylase I antibodies (data not shown). With purified rat kidney acylase antibodies, the myocardium was moderately well stained throughout both the left and right ventricle septa (data not shown). Immunostaining was not observed in sections incubated without primary antibody (data not shown).

In the urinary bladder, staining of acylase I antigen was mild and diffuse throughout the transitional epithelium (data not shown). With purified rat kidney acylase antibodies, staining was moderate and diffuse throughout the transitional epithelium (data not shown). In the testes, the interstitial cells were stained moderately (data not shown). Moderate staining of the cytoplasm of all stages of spermatozoal maturation was observed in the seminiferous tubules. With purified rat kidney acylase antibodies, staining of the interstitial cells was less intense than with acylase I antibodies and was present only in the cytoplasm; little staining of the seminiferous tubules was observed (data not shown). Immunostaining was not observed in sections incubated without primary antibody (data not shown).

Acylase activity has been previously detected in the adrenal medulla, pancreas, testes, and heart although the specific activities are lower than in the kidney (Albert and Szewczuk, 1972; Endo, 1980; Miller and Kao, 1989). Rat lung and intestinal homogenates deacetylate NAC, indicating the presence of acylase I (Sjödin et al., 1989).
The presence of acylase I and purified rat kidney acylase antigens in the gut, pancreas, adrenals, heart, urinary bladder, and testis indicates that the acylases are widely distributed. Thus, NAC and haloalkene-derived mercapturates may be deacetylated in several tissues. The deacetylation of NAC by tissues that show the presence of acylase I may be exploited to more effective design produgs of L-cysteine that may provide protection against injury associated with depletion of intracellular glutathione. Also, the presence of the acylases that catalyze the deacetylation of the haloalkene-derived mercapturates in the several tissues studied may contribute to the organ-selective toxicity of these mercapturates.

References