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DISTRIBUTION MAPS OF D-DOPACHROME TAUTOMERASE IN THE MOUSE BRAIN

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Abstract—D-Dopachrome tautomerase is an enzyme related by amino acid sequence and catalytic activity to macrophage migration inhibitory factor. Both of these small molecules are pro-inflammatory cytokines mediating broad innate immune responses. Although it is well established that the gene product of D-dopachrome tautomerase is widely expressed in liver and kidney cells, no study has mapped the distribution pattern of this tautomeric enzyme in the mammalian nervous system. Here, we address this void by characterizing the cellular localization of D-dopachrome tautomerase in the adult mouse brain. Two well-characterized polyclonal antibodies were used for Western blotting and immunohistochemical localization of the endogenous tautomeric enzyme. Our results show that D-dopachrome tautomerase is present throughout the brain parenchyma with a large fraction of heterogeneous interneurons harboring a stable and robust expression of the enzyme. These data point to a potential involvement of D-dopachrome tautomerase activity in the mature mouse brain, and suggest some functional and evolutionary relationship between innate immunity and tautomerization of D-dopachrome in mammalian species.

Key words: cortex, cytokine, hippocampus, macrophage migration inhibitory factor, neurotrophic factor, tautomerase activity.

INTRODUCTION

D-Dopachrome tautomerase (D-DT) is an eukaryotic, ~13 kDa cytoplasmic enzyme that converts 2-carboxy-2, 3-dihydroindole-5,6-quinone (D-dopachrome) into 5, 6-dihydroxyindole (Odh et al., 1993; Sugimoto et al., 1999). Its tautomerase activity is shared with the cytokine macrophage migration inhibitory factor (MIF) which is critically involved in a broad spectrum of systemic inflammatory diseases (e.g., septic shock, arthritis and glomerulonephritis) and immune signaling cascades (Coleman et al., 2008; Merk et al., 2011). This catalytic similarity is based upon the remarkable intron–exon structural features and amino acid sequence homologies that exist between D-DT and MIF (Esumi et al., 1998; Nishihira et al., 1998). This observation raises the possibility that D-DT may also be involved in regulating pro-inflammatory signaling events during disease progression or malignancy. Indeed, recent studies show D-DT levels correlating with disease severity in sepsis and blister fluid formation in human skin cells (Sonesson et al., 2003; Merk et al., 2011). Thus, D-DT is a MIF-like cytokine with important regulatory functions in innate immunity (Xin et al., 2010).

D-DT is constitutively and abundantly expressed in different mammalian tissues including, the brain, heart, liver, lung kidney, spleen, testis and ovary as demonstrated by Western blotting techniques (Merk et al., 2011). Northern blot analysis also shows D-DT mRNA species in the liver and to a lesser extent, heart and spleen (Nishihira et al., 1998). The fact that D-DT is found in the mouse brain suggests that this enzyme may have cell-specific regulation of innate immunity or neurotrophic function in the nervous system. If this is the case, we must first expand our knowledge of the distribution pattern of D-DT-containing neurons within the adult nervous system and document whether its distribution and expression encompass phenotypically diverse populations of neurons. To accomplish this experimental goal, we have carried out a detailed Western blot and immunohistochemical localization of D-DT in the mouse brain.

EXPERIMENTAL PROCEDURES

Animals

This study was performed on C57BL/6J adult mice (35–45 g) of both sexes (n = 5–6/gender). All animals were raised in our (NYCOM/NYIT) facility under standard laboratory conditions with free access to food and water and group-housed, 3–4 per cage under a 12-h light:dark cycle (lights on 07:00 h). All experiments were performed during the lights on period and were conducted in accordance with the NIH Guide for the Care

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Abbreviations: D-DT, D-dopachrome tautomerase; EDTA, ethylenediamine tetraacetic acid; EGTA, ethylene glycol tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MIF, macrophage migration inhibitory factor.

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and Use of Laboratory Animals and with approval from the NYIT/NYCOM IACUC. All efforts were made to minimize animal stress and to reduce the number of rodents used for this study.

Western blotting: dissection of tissue

For Western blot analysis, rodent hippocampus, hypothalamus, pre-frontal cortex, cerebellum and striatum were dissected and flash-frozen in liquid nitrogen after mice had been sacrificed by rapid decapitation. In addition, we also collected whole adrenal tissue from the same set of animals. Tissues were homogenized in a low-salt lysing buffer (20 mM Tris–HCl, 150 mM NaCl, 1 mM MgCl, 5 MM EDTA, 2 mM EGTA, 1% NP40, and 5% deoxycholate) with protein inhibitors (leupeptin, PMSF, aprotinin, and pepstatin A) included in the buffer solution. The extracts were then centrifuged at 10,000 rpm for 10 min, and the supernatant measured for protein content. Aliquots were then mixed with equal amounts of loading buffer containing 200 mM Tris, 8% SDS, 0.4% bromophenol blue, 40% glycerol, and 5% 2-mercaptoethanol. Samples were then heated and immediately loaded onto 8% SDS–polyacrylamide gels, electrophoresed, and transferred to nitrocellulose membranes.

Gel electrophoresis and Western blotting

For Western blot analysis, 5 μg of total protein from brain tissue and whole adrenals were separated on 4%-20% HEPES acrylamide gels (Pierce, Rockford, IL, USA) and transferred to nitrocellulose-supported membranes. To confirm transfer of proteins, Ponceau-S staining (0.1% [w/v] Ponceau-S in 5% [v/v] acetic acid) was used prior to film development. Membranes were blocked with 5% milk and incubated with primary antibodies overnight at 4 °C. Primary antibodies were dissolved in 5% milk in Tris-buffered saline with 0.1% Tween-20. After primary antibody incubation, membranes were incubated with a horseradish peroxidase-coupled secondary antibody (HRP-conjugated goat IgG; 31430; Pierce, Rockford, IL, USA) for 1 h at room temperature and processed with the ECL Plus chemiluminescence system (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA). Luminescence signals were recorded on a clear blue X-ray film (Pierce, Rockford, IL, USA), scanned and evaluated for appropriate signal.

Immunohistochemical procedures and immunofluorescence microscopy

Adult mouse brain material was frozen on dry ice, mounted on a sliding microtome and cut into 50-μm coronal sections. To unmask target epitopes, free-floating brain sections were first washed with 0.5 M potassium phosphate buffer saline (KPBS) and then incubated in a sodium citrate buffer solution (10 mM; pH 9.0) for 30 min in a water-bath at 80 °C (Jiao et al., 1999; Torres et al., 2004). Following the above antigen retrieval phase, brain sections were incubated for 48 h with D-DT antibodies as listed in Table 1. After labeling, the sections were washed in PBS, incubated with 2% normal goat serum in 1% BSA and incubated with a goat anti-rabbit Alexa Fluor 594-conjugated antibody (1:100; Molecular Probes, Eugene, OR, USA; Cat. #A11012) or goat anti-rabbit Rhodamine Red-X-conjugated antibody (1:200; Jackson Immuno-Research Laboratories, Inc., West Grove, PA, USA; Cat. #111-295-144). For control procedures, primary antibodies were omitted or substituted with normal goat or rabbit serum. The immunolabeled sections were then cover-slipped with Vectashield mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Inc., Burlingame, CA, USA). Fluorescence images were captured into a computer using an Olympus BX53 fluorescence microscope equipped with Olympus DP72 digital camera. Final figures were prepared using Adobe Photoshop software (Adobe Systems, Inc., San Jose, CA, USA). To help identify relevant anatomical structures, additional mouse brain sections were counterstained with Neutral Red.

Antibodies characterization

Two antibodies were used for Western blotting and immunohistochemistry. One of the antisera (T-14: sc-86407) was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) whereas the second antiserum was produced in the Department of Internal Medicine, Yale University School of Medicine, New Haven, CT (''Yale'' antibody). The D-DT T-14 antibody is a rabbit polyclonal antibody raised against a peptide sequence mapping to human D-DT (gene locus: D-DT 22q11.23). The second antibody (i.e., ''Yale'' antibody) is also a rabbit polyclonal antibody raised against both mouse and human D-DT (Table 1). This particular antibody has been extensively characterized by immunoblotting and its specificity has been documented in prior investigations (Merk et al., 2011).

Data collection and analysis

We mapped only neurons that contained D-DT immunoreactivity at a frequency of 200-μm through the rostrocaudal extent of the olfactory bulb (Bregma 2.40 mm) through the cerebellum (Bregma −6.90 mm) as detailed in The Comparative Cytoarchitectonic Atlas of the C57BL/6 and 129/Sv Mouse Brains (Hof et al., 2000). As we did not find differences between the genders, we grouped male and female brains for the quantitative analysis. Total number of immunopositive neurons was counted using the dissector principle as previously described by our group (Torres et al., 2004) on a one to six series of 50-μm thick coronal sections. Distribution of D-DT cells was computed from brain sections observed under fluorescence microscopy at magnifications of 10×, 20×, 40× and 60× objectives. Images were obtained from an Olympus BX41 microscope and then processed with a computer-based digital image analysis technique. Data for cell population estimates are reported as means ± SEM from the right side of each brain.

RESULTS

Western blot analysis

Specificity of the D-DT antibody (T-14) was validated by Western blotting of mouse brain and adrenal homogenates. D-DT (T-14) is an affinity-purified rabbit polyclonal antibody raised against a peptide (50 amino

Table 1. Primary antibodies used in the study

Antibody	Immunogen	Manufacturer information	Dilution used
T-14	Affinity-purified D-DT	Rabbit polyclonal (sc-86407)	1:100–1000[a]
''Yale''	Recombinant mouse/human D-DT	Rabbit polyclonal	1:100–1000[b]

[a] Western blots 1:1000; immunohistochemistry 1:1000.
[b] Western blots 1:1000; immunohistochemistry 1:1000.