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Immunohistochemical study of arginases 1 and 2 in the olfactory bulbs of the Korean roe deer, *Capreolus pygargus*

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ABSTRACT

Arginases are enzymes of the urea cycle that catalyze the hydrolysis of L-arginine to ornithine and urea. The enzymes are core components of the arginine–ornithine–glutamate–γ-amino butyric acid pathway of the central nervous system. In the present study, we immunohistochemically determined the localization of arginase 1 and 2 in the olfactory bulb (OB) of the roe dear (*Capreolus pygargus*). Reverse transcription PCR revealed that the mRNAs encoding both arginase 1 and 2 were expressed in the OB. Arginase 1 was localized to olfactory nerve axons, calcitonin gene-related peptide-positive mitral/tufted cells (excitatory neurons), and glutamate acid decarboxylase 65/67-immunopositive periglomerular cells of the main olfactory bulb. The arginase 2 immunoreactivities in the OB tissues were similar to those of arginase 1. Furthermore, both arginases were detected in the accessory olfactory bulb. These findings suggest that both arginase 1 and 2 are potentially associated with excitatory and inhibitory neurotransmitter activities in animal OBs, including those of the roe deer.

1. Introduction

Arginases catalyze the hydrolysis of arginine to ornithine and urea and exist in two isoforms: arginases 1 and 2 (Yu et al., 2001). Arginase 1, which is a hepatic arginase, and arginase 2, which is an extrahepatic arginase, are differentially expressed in various tissues of the rat and mouse based on RT-PCR, *in situ* hybridization, and immunohistochemistry (Choi et al., 2012; Nakamura et al., 1999; Yu et al., 2001).

Recent studies have reported that the main olfactory system in ungulates is deeply involved in maternal (Keller and Levy, 2012; Kendrick et al., 1992), reproductive-sexual (Baum and Cherry, 2015), social (Keller and Levy, 2012; Sanchez-Andrade and Kendrick, 2009; Villagran and Ungerfeld, 2013), and fear-related (Osada et al., 2014) behaviors. The Korean roe deer, *Capreolus pygargus*, is an ungulate and is the most abundant wild animal on Jeju Island, South Korea. Previously, we reported the morphological characteristics of the vomeronasal system (Park et al., 2014), chemosensory olfactory mucosae (Park et al., 2015), and γ-amino butyric acid (GABA) transmission in the main olfactory bulb (MOB) (Kim et al., 2015). However, little is known about arginase in the main olfactory system of wild ungulates, including roe deer. In this study, we examined the distribution of arginases 1 and 2 in the olfactory bulbs (OB) of *C. pygargus* by immunohistochemistry.

2. Materials and methods

2.1. Tissue preparation

Five male Korean roe deer (three, 2–3 years old; two, 2 months old) were obtained from the Jeju Wildlife Rescue Center. The age was calculated based on the number of horn branches. The OB was removed and fixed in 10% buffered formalin for 48 h. The OB of the Sprague–Dawley rat (3-month-old, male) (Orient Bio, Kyunggi, Korea) was compared with that of the roe deer. The Institutional Animal Care and Use Committee of Jeju National University approved all study protocols. The protocols for the care and handling of animals conformed to current international laws and policies (NIH Guide for the Care and Use of Laboratory Animals, NIH Publication No. 85-23, 1985, revised 1996).

The olfactory bulbs containing the MOB and accessory olfactory bulb (AOB) of roe deer and rat were dehydrated in a graded ethanol series (70%, 80%, 90%, 95%, and 100%), cleared in xylene, embedded in paraffin, and sectioned at in 5-μm thicknesses. Sagittal serial sections

Abbreviations: AOB, accessory olfactory bulb; CGRP, calcitonin gene-related peptide; ePL, external plexiform layer; g, glomerulus; GABA, γ-amino butyric acid; GAD, glutamic acid decarboxylase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GL, glomerular layer; GrL, granule cell layer; iPL, inner plexiform layer; ML, mitral cell layer; MOB, main olfactory bulb; M/T cell, mitral and tufted cell; M/TcL, mitral and tufted cell layer; OB, olfactory bulb; ONL, olfactory nerve cell layer

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Table 1
Characteristics of antibodies used.

Antigen	Immunogen	Manufacturer, species, antibody type	Dilution
Arginase 1	Human arginase 1 aa. 53–207	BD Transduction Laboratories™ (610708, Lot. 4275868), mouse, monoclonal	1:200 (1:1000)[a]
Arginase 2	Human arginase 2 aa. 291–354	Santa Cruz (SC20151, Lot. #K2613), rabbit, polyclonal	1:100 (1:1000)[a]
CGRP	Synthetic CGRP (rat) conjugated to KLH	Sigma-Aldrich (C8198, Lot. 113M4760), rabbit, polyclonal	1:10,000
GAD	Synthetic peptide from rat [C]DFLIEEIERLGQDL	Millipore (AB1511, Lot. 2324512), rabbit, polyclonal	1:2000
β-actin	Synthetic β–cytoplasmic actin N-terminal peptide conjugated to KLH	Sigma-Aldrich (a5441, Lot. 028K4826), mouse, monoclonal	1:10,000
Secondary antibodies for immunohistochemistry			
Biotinylated goat anti-rabbit IgG		Vector Laboratories (PK-6101, VECTASTAIN Elite ABC Rabbit IgG Kit)	1:100
Biotinylated horse anti-mouse IgG		Vector Laboratories (PK-6102, VECTASTAIN Elite ABC Mouse IgG Kit)	1:100
Secondary antibodies for Western blot analysis			
Peroxidase anti-rabbit IgG (H + L)		Vector Laboratories (PI-1000, Lot. Y1108)	1:1000
Peroxidase anti-mouse IgG (H + L)		Vector Laboratories (PI-2000, Lot. ZC1212)	1:1000

Abbreviations: aa, amino acid; CGRP, calcitonin gene-related peptide; GAD, glutamic acid decarboxylase, IgG; immunoglobulin.
[a] Antibody dilution used for Western blotting.

Table 2
Primer sequences used in the present study.

Primer	Sequence	Amplicon size (bp)
Primers for cloning		
Arginase 1-Forward	5′-GGTGGATGCTCACACTGACA-3′	568
Arginase 1-Reverse	5′-CAACCCCAAAGCAAGCCATA-3′	
Arginase 2-Forward	5′-CGTCCGGAAGAAGTCTGTC-3′	488
Arginase 2-Reverse	5′-CTGGGAGCTGTGGTACCTTG-3′	
Primers for RT-PCR		
Argianse 1-Forward	5′-TCAGCAGAAGTCCACACCAG-3′	272
Arginase 1-Reverse	5′-ATCTTGGCGATCCACCATC-3′	
Arginase 2-Forward	5′-CGTGGTTGTTGTGTGTTCG-3′	177
Argianse 2-Reverse	5′-GAACAGAGAAAACGGGGAGA-3′	

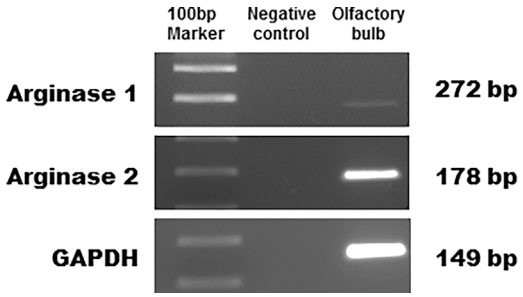

Fig. 1. The levels of the mRNAs encoding arginase 1 and 2 in the olfactory bulb of Korean roe deer. GAPDH served as the internal standard. The negative controls lacked cDNA. Marker: 100-bp ladder.

of the MOB were mounted on glass slides coated with silane (Sigma-Aldrich, St. Louis, MO, USA).

2.2. Characterization of antibodies

Table 1 summarizes the characteristics of the antibodies used in this study. The specificities of the anti-arginase 1 and 2 antibodies used to evaluate the OBs of roe deer and rats were validated by Western blotting, as described in our earlier report (Choi et al., 2012). Briefly, the OBs of roe deer and rats were homogenized in TNN lysis buffer containing protease and phosphatase inhibitors (1 mM Na_3VO_4, 1 mM PMSF, 10 μg/mL aprotinin, and 10 μg/mL leupeptin). Proteins (40 μg) were subjected to 10% (w/v) SDS-PAGE and transferred to a nitrocellulose membrane (Schleicher and Schuell, Keene, NH, USA) via a 100-V gradient applied for 2 h at 4 °C. The membranes were blocked by incubation with 5% (v/v) skim milk in Tris-buffered saline for 1 h and then incubated with the primary antibodies (anti-arginase 1, 1:1000 dilution; anti-arginase 2, 1:1000 dilution; anti-β-actin, 1:10,000) and

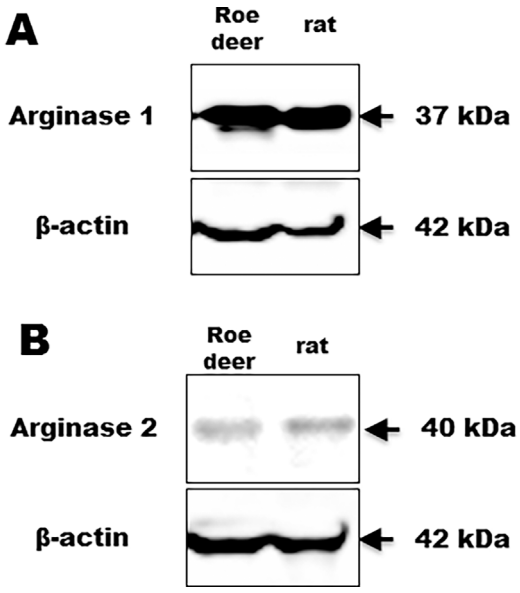

Fig. 2. Western blotting to detect arginase 1 and 2 in the olfactory bulbs of Korean roe deer (A and B, left lanes) and the rat (A and B, right lanes). β–actin served as the internal standard. Anti-arginase 1 and 2 antibodies cross-reacted when used to detect the roe deer proteins.

an anti-peptide antibody overnight at 4 °C. After washing, the membranes were incubated with appropriate secondary antibodies for 1 h. Bound antibodies were detected using a chemiluminescent substrate (a component of the BS ECL Plus Kit, catalog # W6002; Biosesang™, Gyeonggi, Korea), according to the manufacturer's instructions.

2.3. Immunohistochemistry

First, 5-μm-thick sections of paraffin-embedded tissues were deparaffinized and heated in a microwave oven (700 W) in citrate buffer (0.01 M, pH 6.0) for 3 min. After cooling for 20 min at room temperature, the sections were exposed to aqueous 0.3% hydrogen peroxide in methyl alcohol for 20 min to block endogenous peroxidase activity. After three washes with phosphate-buffered saline (PBS, pH 7.4), the sections were incubated with the matching blocking serum (10% normal goat or horse serum, Vector ABC Elite kit; Vector Laboratories, Burlingame, CA, USA), diluted in PBS for 45 min, and then incubated overnight at 4 °C with the primary antibody: mouse monoclonal anti-arginase 1; rabbit polyclonal anti-arginase 2; rabbit polyclonal anti-glutamate acid decarboxylase 65/67 (GAD 65/67); or rabbit polyclonal anti-calcitonin gene-related peptide (CGRP). As a