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The Glucose Transporter GLUT1 and the Tight Junction Protein Occludin in Nasal Olfactory Mucosa

Piret Hussar[1,2], Nomingerel Tserentsoodol[2,3], Haruko Koyama[2], Minako Yokoo-Sugawara[2,4], Toshiyuki Matsuzaki[2,3], Shigeru Takami[5] and Kuniaki Takata[2,3]

[1]Department of Histology, Institute of Anatomy, Tartu University, Tartu, Estonia, [2]Laboratory of Molecular and Cellular Morphology, Institute for Molecular and Cellular Regulation, Gunma University, Maebashi, Gunma 371-8512, [3]Department of Anatomy and Cell Biology and [4]Third Department of Medicine, Gunma University School of Medicine, Maebashi, Gunma 371-8511 and [5]Department of Anatomy, School of Health Sciences, Kyorin University, Hachioji, Tokyo 192-8508, Japan

Correspondence to be sent to: Kuniaki Takata, Department of Anatomy and Cell Biology, Gunma University School of Medicine, Maebashi, Gunma 371-8511, Japan. e-mail: takata@med.gunma-u.ac.jp

Abstract

The nervous cells in the brain and the peripheral nerves are isolated from the external environment by the blood–brain, blood–cerebrospinal fluid and blood–nerve barriers. The glucose transporter GLUT1 mediates the specific transfer of glucose across these barriers. The olfactory system is unique in that its sensory cells, olfactory receptor neurons, are embedded in the nasal olfactory epithelium and send their axons directly to the olfactory bulb of the brain. Only the apical parts of the olfactory receptor neurons are exposed to the lumen, and these serve as sensors for smell. Immunohistochemical examination showed that the tight junction protein occludin was present in the junctions of the olfactory epithelium. Endothelial cells in the blood vessels in the lamina propria of the olfactory mucosa were also positive for occludin. These observations suggest that the olfactory system is guarded from both the external environment and the blood. GLUT1 was abundant in these occludin-positive endothelial cells, suggesting that GLUT1 may serve in nourishing the cells of the olfactory system. Taken together, GLUT1 and occludin may serve as part of the machinery for the specific transfer of glucose in the olfactory system while preventing the non-specific entry of substances.

Introduction

Epithelia or endothelia constitute the structural basis of the blood–tissue barriers such as the blood–brain, blood–nerve, blood–retina, blood–aqueous and placental barriers (Takata *et al*., 1997). Tight junctions connecting the epithelial or endothelial cells or syncytial cell layers prevent the free exchange of substances between the blood and the compartments guarded by the barrier, and only a limited number of substances are allowed to pass through it. Specific transporters localized at plasma membranes of the cells of the barrier play pivotal roles in the specific and effective transfer of selected substances transcellularly across the barrier (Takata, 1996).

Glucose is one of the most important sources for ATP production as well as for the synthesis of a variety of cellular molecules. In the small intestine, dietary carbohydrates are hydrolyzed to monosaccharides, which are absorbed and transferred to the blood stream through absorptive epithelial cells (Takata *et al*., 1993; Takata, 1996). Most of the cells in the body utilize blood glucose, the level of which is strictly controlled. Glucose transporters, also called sugar transporters, are membrane proteins that serve in the transfer of sugars across the cellular membranes (Baldwin, 1993; Bell *et al*., 1993; Takata *et al*., 1993; Takata, 1996). Two types of glucose transporters have been identified: the SGLT family and the GLUT family. SGLT glucose transporters are sodium-dependent active transporters serving in the concentrative transport of sugars in the small intestine and the kidney. GLUT glucose transporters are passive facilitated-diffusion transporters that transport sugars according to their concentration gradient. We have shown previously that GLUT1, an isoform of the GLUT family, is abundant in the cells of blood–tissue barriers (Takata *et al*., 1990, 1997). GLUT1 is localized at both the apical and basolateral domains of the cells of the barriers, and appears to constitute a key molecule in the transcellular transfer of glucose from the blood to the specialized compartments guarded by the barriers. The importance of GLUT1 was made evident by a mutation of GLUT1 that was shown to be responsible

for seizures due to the decrease of the glucose level in the cerebrospinal fluid caused by defective glucose transport across the blood–brain barrier (Seidner *et al.*, 1998).

Tight junctions play important roles in the barrier function of the epithelial and endothelial sheets (Anderson *et al.*, 1995). Recent studies have revealed that tight junctions have a specialized membrane structure in which a number of specific proteins assemble, such as zonula occludens-1 (ZO-1), ZO-2, ZO-3, 7H6 antigen, cingulin, symplekin, occludin and claudins (Denkar and Nigam, 1998; Tsukita and Furuse 1999; Cereijido *et al.*, 2000; Tsukita *et al.*, 2001). We showed that occludin and GLUT1 were specifically expressed in the cells of the blood–ocular (Tserentsoodol *et al.*, 1998) and blood–nerve (Tserentsoodol *et al.*, 1999) barriers. These two molecules may constitute the machinery for the selective transfer of glucose across the barriers while preventing the non-specific flow of blood constituents.

The olfactory system is a unique extension of the central nervous system (Doucette, 1990). The sensory cells of the olfactory system, olfactory receptor neurons (ORNs), are embedded in the olfactory epithelium of the nasal mucosa, and protrude their dendrites to the lumen (Graziadei, 1973, 1977; Farbman, 1992). Freeze-fracture replica examination has shown that ORNs and supporting cells are sealed by rows of well-developed tight junction strands (Kerjaschki and Hörandner, 1976). The ORNs project their axons directly to the olfactory bulb. In order to clarify whether these specialized parts of the nervous system are surrounded by tight junctions and whether glucose transporters are present in the barrier layer, we immunolocalized occludin and GLUT1 in the rat olfactory mucosa. To identify the olfactory mucosa in tissue sections, antisera to tubulin and protein gene product 9.5 (PGP) were used. PGP is a useful marker for various types of neurons (Thompson *et al.*, 1983; Doran *et al.*, 1993) including mammalian ORNs (Takami *et al.*, 1993, 1995; Taniguchi *et al.*, 1993).

Materials and methods

Antibodies

Rabbit anti-GLUT1 and guinea pig anti-GLUT1 antibodies were raised using synthetic partial peptides of human GLUT1 and characterized as described previously (Takata *et al.*, 1990; Shin *et al.*, 1996). Rabbit anti-chicken tubulin was from S.J. Singer (University of California at San Diego) (Rogalski and Singer, 1984). Mouse anti-PGP was from Ultra Clone (Rossiters Farm House, Wellow, Isle of Wight, UK) (Bonfanti *et al.*, 1992) and mouse anti-occludin was from Zymed (San Francisco, CA) (Tserentsoodol *et al.*, 1999). Fluorescein isothiocyanate-labeled donkey anti-guinea pig immunoglobulin G (IgG), dichlorotriazinyl amino fluorescein-labeled and rhodamine red X-labeled donkey anti-rabbit IgG, and Cy3-labeled donkey anti-mouse IgG were products of Jackson Immunoresearch (West Grove, PA).

Immunofluorescence staining

Male Wistar rats, 4 weeks old (supplied from the Animal Breading Facility, Gunma University), were anesthetized with an i.p. injection of sodium pentobarbital. Mucous membranes of the olfactory region were taken under a dissecting microscope. Specimens were fixed with 1–3% paraformaldehyde in phosphate-buffered saline (PBS) at 4°C for 3–24 h. In some cases, rats were perfused with the same fixative from the left ventricle under anesthesia, and specimens removed were further fixed in the same way as in immersion fixation. Specimens were washed with PBS, infused with 20% sucrose in 0.1 M sodium phosphate buffer, pH 7.4, containing 0.02% sodium azide, frozen in liquid nitrogen, and stored at –80°C until use. Cryostat sections, 4–8 μm thick, were cut and mounted on glass slides coated with poly-L-lysine. For occludin staining, unfixed fresh tissue specimens were directly embedded in OCT compound and rapidly frozen in liquid nitrogen. Cryostat sections were cut, mounted on glass slides, and fixed in acetone and ethanol (Tserentsoodol *et al.*, 1999). Immunofluorescence staining was carried out basically as described previously (Tserentsoodol *et al.*, 1999). In short, sections were first covered with 5% normal goat serum, then sequentially incubated with the primary antibody and the fluorescence-labeled secondary antibody. For double-immunofluorescence labeling, specimens were sequentially incubated with a mixture of the primary antibodies raised in different animal species, then with a mixture of fluorescence-labeled species-specific secondary antibodies. Immunolabeled samples were mounted in 22% polyvinylalcohol in 56 mM Tris–HCl buffer, pH 9.0, 11% glycerol and 5% 1,4-diazabicyclo[2,2,2]octane (Shin *et al.*, 1996), and examined with an AX-70 epifluorescence microscope (Olympus, Tokyo, Japan). Images were captured with a PXL1400 cooled-CCD camera (Photometrics, Tucson, AZ) using an IPLab Spectrum software (Signal Analytics, Vienna, VA). Confocal observation was carried out with an Olympus BX-50 epifluorescence microscope equipped with an MRC-1024 laser confocal system (Bio-Rad, Hercules, CA). Images were captured and processed with Laser Sharp software (Bio-Rad).

Results

Anti-tubulin antibody strongly stained the apices of the olfactory epithelial cells where numerous cilia are present (Figures 1 and 2). Nerve fiber bundles emanating from the epithelium and thicker fibers formed by the further bundling thereof were also strongly positive for tubulin (Figure 1). Anti-PGP antibody stained ORNs in the olfactory epithelium and the nerve fibers running underneath (Figure 3). These results show that PGP serves as a marker for the olfactory epithelium and nerve fibers emanating thereof, and that tubulin acts as a marker for the nerve fibers.

The tight junction membrane protein occludin was concentrated in the tight junctions connecting adjacent

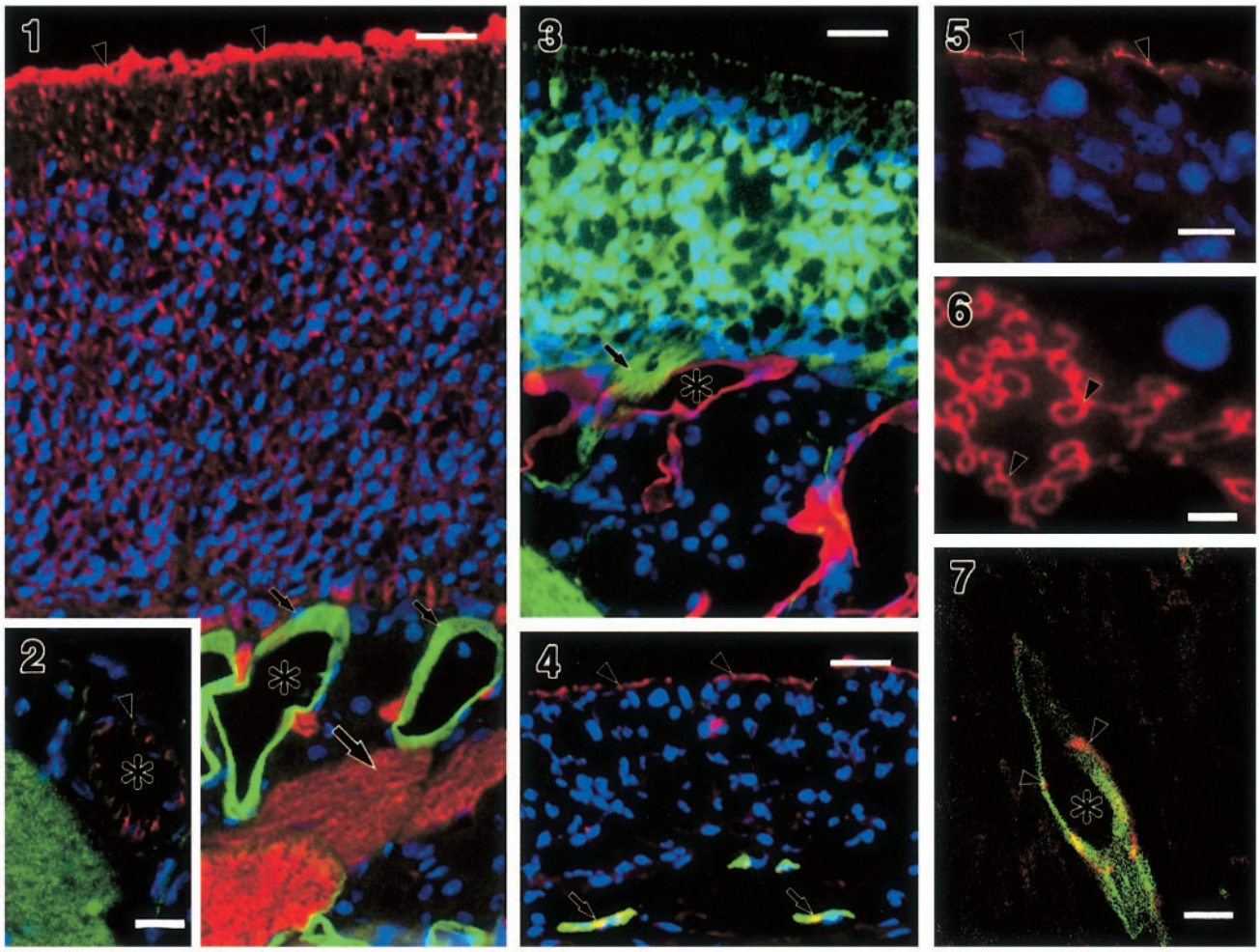

Figure 1 Immunofluorescence localization of tubulin (red) and GLUT1 (green) in the olfactory mucosa. Nuclei were counterstained with DAPI (blue). Tubulin is highly concentrated in the apical border of the epithelium (arrowheads) and nerve fiber bundles (large arrow). GLUT1 is abundant in the endothelial cells (small arrows) of the blood vessels (*). Bar: 20 μm.

Figure 2 Immunofluorescence localization of tubulin (green) and occludin (red) in the lamina propira. Nuclei were counterstained with DAPI (blue). Occludin is positive in the endothelium (arrowhead) of a blood vessel (*), whereas a bundle of nerve fibers (arrow) is negative. Bar: 10 μm.

Figure 3 Immunofluorescence localization of PGP (green) and GLUT1 (red) in the olfactory mucosa. Nuclei were counterstained with DAPI (blue). PGP is positive in the epithelial cells and nerve fiber bundles (arrow). GLUT1 is concentrated in the endothelial cells of the blood vessels (*). Bar: 20 μm.

Figure 4 Immunofluorescence localization of occludin (red) and GLUT1 (green) in the olfactory mucosa. Nuclei were counterstained with DAPI (blue). Occludin is localized in tight junctions in the epithelium (arrowheads), as well as in the endothelial cells of the blood vessels (arrows) in the lamia propria. Note that blood vessels positive for occludin are also positive for GLUT1. Bar: 20 μm.

Figure 5 Olfactory mucosal surface stained for occludin (red) and GLUT1 (green). Occludin (red) is localized in the tight junctions (arrowheads) in the epithelium, which is negative for GLUT1. Nuclei were counterstained with DAPI (blue). Bar: 10 μm.

Figure 6 Tangential section of the olfactory mucosal surface stained for occludin (red) by immunofluorescence microscopy. Note the characteristic small circles of occludin staining representing tight junctions encircling the apical portions of olfactory receptor neurons. A nucleus that was counterstained with DAPI (blue) is also seen. Bar: 10 μm.

Figure 7 Blood vessels in the olfactory mucosa stained for occludin (red) and GLUT1 (green). Endothelial cells in the blood vessels (*) in the lamina propria are positive for both GLUT1 and occludin (arrowheads). This is a confocal microscopic image. Bar: 10 μm.

epithelial cells (Figures 4 and 5). Observation of tangential sections of the epithelia revealed that occludin encircles the apical portions of the ORNs (Figure 6). Occludin was also found in the cells of the ducts of Bowman's glands (data not shown), and was seen to be concentrated in the endothelial cells of blood vessels running in the connective tissue of the subepithelial lamina propria (Figures 2, 4 and 7). Nerve fiber bundles were negative for occludin (Figure 2).

The glucose transporter GLUT1 is abundant in the blood vessels in the olfactory mucosa (Figures 1, 3 and 4). Double labeling for GLUT1 and occludin revealed that GLUT1 is abundant in the endothelial cells connected by tight junctions of occludin (Figures 4 and 7). Blood vessels in the non-olfactory regions were negative for GLUT1 (data not shown). Axons of ORNs leave the epithelium as thin nerve fiber bundles, which then bundle together into thicker ones, forming olfactory nerves, which enter the olfactory bulb of the brain by passing through pores of the ethmoid bone. Double-immunofluorescence labeling for GLUT1 and tubulin, or for GLUT1 and PGP, revealed that thin nerve fiber bundles inside the epithelium and nerve fiber bundles in the underlying lamina propria were negative for GLUT1 (Figures 1 and 3). Some of thick nerve fiber bundles were weakly positive for GLUT1 (data not shown).

Discussion

The ORNs are unique bipolar neurons whose cell bodies are located in the olfactory epithelium with dendrites protruding to the nasal cavity and axons directly extending all the way to the olfactory bulb in the central nervous system (Doucette, 1990). The central nervous system and peripheral nerves are guarded against free access from the outside by the blood–brain, blood–cerebrospinal fluid and blood–nerve barriers (Takata *et al*., 1993, 1997). Eyes are specialized extensions of the central nervous system and are guarded by the blood–ocular barrier, composed of retinal pigment epithelium, the epithelium of the ciliary body and the endothelial cells of the intraocular blood vessels (Raviola, 1977; Takata *et al*., 1997). Glucose is a ubiquitous nutrient used in the cells of the body. GLUT1 is abundant in the cells of the barrier, serving as the specific transfer machinery of glucose across these barriers (Harik *et al*., 1990; Takata *et al*., 1990, 1997). It was an open question whether GLUT1 and tight junctions are present in the olfactory system as well. We have shown in this work that GLUT1 is abundant in the endothelial cells of blood vessels in the lamina propria of the olfactory mucosa. The tight junction membrane protein occludin was present in these endothelial cells. ZO-1 was reported to be present in the blood vessels in the olfactory mucosa (Miragall *et al*., 1994). These observations suggest that the endothelia in the blood vessels in the lamina propria serve as the barrier layer. In blood–tissue barriers, such as in the brain, the retina, the iris and the peripheral nerve fiber bundles, abundance of GLUT1 in endothelial cells has been shown to be restricted to the cells of the barrier (Takata *et al*., 1997; Tserentsoodol *et al*., 1999). The presence of both occludin and GLUT1 in the endothelial cells of blood vessels in the lamina propria of the olfactory mucosa suggests that these molecules constitute a part of the machinery for the prevention of the non-specific flow of substances while allowing specific transfer of glucose in this region.

Tight junctions with well-developed strands of intramembranous particles in the apicolateral portion of the olfactory epithelium have been reported in the mouse (Kerjaschki and Hörandner, 1976) and the rat (Menco, 1988). ZO-1 was localized between the apical dendritic portions of the olfactory neurons and the supporting cells (Miragall *et al*., 1994). We show here that occludin is also present in these regions. These observations indicate that, like other epithelia, olfactory epithelium serves as a barrier layer against the luminal constituents, thereby maintaining the environment of the epithelium and lamina propria underneath.

ORNs are bipolar neurons that extend their axons up to the olfactory bulb. The axons leave the epithelium as thin fiber bundles, which are further bundled to form thicker olfactory nerves prior to penetration through the ethmoid bone (Farbman, 1992). Nerve fiber bundles were basically negative for occludin or GLUT1. Only weak labeling for GLUT1 was seen in the ensheathing cells in thick nerve fiber bundles. Although the presence of another tight junction protein ZO-1 was reported in the glial fibrillar acidic protein-positive ensheathing cells (Miragall *et al*., 1994), absence of occludin indicates that these cells do not constitute the barrier property. These observations suggest that nerve fibers in the olfactory mucosa are exposed to the environment of the lamina propria. Endothelial cells positive for both GLUT1 and occludin cells may serve as the primary site of the barrier, and glucose passes through this barrier via abundant GLUT1. This makes a marked contrast to the blood–nerve barrier in typical peripheral nerves such as the sciatic nerve (Tserentsoodol *et al*., 1999), where perineurial cells positive for occludin and GLUT1 serve as the barrier, and the surrounding blood vessels are negative for occludin and GLUT1.

In summary, we suggest that the environment of the olfactory system may be maintained as follows. The olfactory epithelium containing ORNs and supporting cells is protected from the harsh environment of the nasal cavity by well-developed tight junctions, as commonly seen in other types of epithelia. The axons of the olfactory nerves running in the lamina propria underneath are isolated from the blood constituents by the impermeable blood vessels lined with occludin-positive endothelial cells. Abundant GLUT1 in these endothelial cells may serve for the selective supply of glucose to the olfactory system, including the olfactory epithelium, as well as to the bundles of axons.

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