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Involvement of the carrier-mediated process in the retina-to-blood transport of spermine at the inner blood-retinal barrier

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ABSTRACT

The elimination of spermine, the end product of cellular polyamine, from the retina to the blood across the blood-retinal barrier (BRB) was investigated. The *in vivo* microdialysis study revealed that the elimination of [^{3}H]spermine from vitreous humor after vitreous bolus injection was in a biexponential manner. The rate constant for the elimination of [^{3}H]spermine during the terminal phase was estimated to be 1.67-fold greater than that of [^{14}C]D-mannitol, a bulk flow marker, and the difference in the terminal elimination rate constant between [^{3}H]spermine and [^{14}C]D-mannitol was reduced in the presence of 50 mM spermine, suggesting a retina-to-blood transport system for [^{3}H]spermine across the BRB. The retina-to-blood transport of [^{3}H]spermine was also supported by a study of the retinal uptake index (RUI). The *in vitro* transport study with TR-iBRB2 cells, a model cell line of the inner BRB, revealed time-, concentration- and temperature-dependent transport of [^{3}H]spermine, suggesting the involvement of carrier-mediated processes in spermine transport across the inner BRB. The *in vitro* study also suggested that the transport of spermine at the inner BRB is pH-, membrane potential- and Cl^--sensitive and Na^+-insensitive, and these functional properties of spermine transport suggest only a minor contribution of spermine transporters, such as CCC9 (SLC12A8), the expression of which was suggested at the inner BRB. In the inhibition study, [^{3}H]spermine transport was markedly inhibited by putrescine, spermidine, spermine and agmatine while substrates of a well-characterized organic cation transporter (OCTs/SLC22A) and a cationic amino acid transporter (CATs/SLC7A) had no effect, suggesting the involvement of unknown transporters in spermine elimination from the retina.

1. Introduction

Polyamines, such as putrescine, spermidine and spermine, are known as bioactive amines that are ubiquitously distributed in the animal tissues (de Vera et al., 1995; Moinard et al., 2005). Polyamines have been suggested to play an important role in cellular proliferation and differentiation (Tabor and Tabor, 1976; Pegg and McCann, 1982), and alterations of polyamine concentration, in cells, blood and urine, have been reported in patients with diseases including cancer, psoriasis, multiple sclerosis, Duchenne muscular dystrophy and chronic renal failure (Wallace and Caslake, 2001; Noga et al., 2012; Russell and Stern, 1981; Swendseid et al., 1980; Saito et al., 1983). In cancer cells, the cellular concentration of polyamine was reported to be elevated by the activation of ornithine decarboxylase that catalyzes the biosynthesis of putrescine (LaMuraglia et al., 1986; Cañizares et al., 1999; Hayasaka et al., 2011), and the proliferation of cancer cells was reported to be suppressed in the presence of an ornithine decarboxylase inhibitor, such as α-difluoromethylornithine (Quemener et al., 1992; Meyskens et al., 2008).

In the retina associated with vision sense, the physiological significance of polyamines has been reported, and the loss of cone cells, photoreceptor cells, is known to be caused by polyamine deficiency in the developing rabbit retina (Withrow et al., 2002). In a study with multiple sclerosis model mice, the cell loss in the retinal ganglion cell layer has been shown to be inhibited by the oral administration of spermidine, suggesting the antioxidative effect of polyamines (Guo et al., 2011). In addition, the involvement of spermine was suggested in the retinal degeneration observed in gyrate atrophy of the choroid and retina since the excess amount of spermine induced cell apoptosis in a study involving the bovine retina (Kaneko et al., 2007). These reports suggest that the regulation of polyamine concentration is important in the retina. In the

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cellular biosynthesis of polyamines, it is known that putrescine is produced from ornithine by ornithine decarboxylase (ODC), and putrescine is converted into spermidine by spermidine synthase (Sturman et al., 1976; Pegg and McCann, 1982; Seiler, 2004). ODC is one of the rate-limiting enzymes in biosynthesis of polyamine, and its cellular expression and activity were detectable in the retina (Yanagihara et al., 1996; Biedermann et al., 1998). In addition, the localization of ODC and spermine/spermidine in Müller cells was reported to suggest the role of spermine/spermidine in regulation of K^+ channels (Biedermann et al., 1998). Spermine synthase catalyzes the conversion of spermidine into spermine that is known to be the end product, suggesting the involvement of a spermine elimination system from the retina in the regulation of polyamine concentration (Sturman et al., 1976; Pegg and McCann, 1982; Seiler, 2004).

In the retina, the blood-retinal barrier (BRB) separates the retinal tissue and the circulating blood, and the BRB includes two barrier structures, the inner and outer BRB, that are formed by the retinal capillary epithelial cells and retinal pigment epithelial (RPE) cells, respectively. In order to maintain the retinal function, membrane transporters expressed by the BRB contribute to the nutrient supply and endobiotic removal across the BRB since the retinal capillary epithelial cells and RPE cells form the tight junction to restrict the paracellular solute transport at the inner and outer BRB, respectively (Stewart and Tuor, 1994; Cunha-Vaz, 2004; Hosoya et al., 2011, 2012). In particular, it is known that the inner BRB is involved in nourishing two-thirds of the retina (Tomi and Hosoya, 2010), and previous reports about retinal capillary endothelial cells revealed the expression of various membrane transporters, such as cationic amino acid transporter 1 (CAT1/SLC7A1), neutral and basic amino acid transporter (y^+LAT2/SLC7A6), monocarboxylate transporter 1 (MCT1/Slc16a1), equilibrative nucleoside transporter 2 (ENT2/SLC29A2), L (leucine-referring)-type amino acid transporter (LAT1/SLC7A5), taurine transporter (TAUT/SLC6A6) and glucose transporter (GLUT1/SLC2A1) (Tomi et al., 2009; Hosoya et al., 2001a; Nagase et al., 2006; Tomi et al., 2005, 2007a, 2007b; Usui et al., 2013; Takata et al., 1992; Kumagai et al., 1996).

Regarding polyamine transport, polyamine transporters have been identified in various organisms, including prokaryotes and eukaryotes (Igarashi and Kashiwagi, 2010). In mammals, several transport systems for polyamines have been reported in tissues, such as lymphocytes, intestine, kidney and brain (Kakinuma et al., 1988; Iseki et al., 1991; Kobayashi et al., 1999; Masuko et al., 2003), and organic cation transporter 1 (OCT1/SLC22A1), L-carnitine transporter 2 (CT2/SLC22A16) and the splice variant of cation-chloride cotransporter 9 (CCC9/SLC12A8) have been reported to recognize spermine as a substrate (Busch et al., 1996; Aouida et al., 2010; Daigle et al., 2009). However, little is known about the mechanism for the elimination of spermine from the retina to the circulating blood, and any clarification of spermine elimination will be beneficial in maintenance of the retina and the clinical treatment of retinal diseases, such as diabetic retinopathy and gyrate atrophy of choroid and retina (Matsushita et al., 2010; Nicoletti et al., 2003; Kaneko et al., 2007).

In this study, *in vivo* and *in vitro* studies were performed to investigate the elimination of spermine from the retina to the circulating blood. In the *in vivo* study, the blood-to-retina and retina-to-blood transports of spermine were analyzed by means of the retinal uptake index (RUI) and microdialysis, respectively. In the *in vitro* study, spermine transport was analyzed in a conditionally immortalized cell line of retinal capillary endothelial cells (TR-iBRB2 cells), an *in vitro* model cell line of the inner BRB (Hosoya et al., 2001a, 2001b; Hosoya and Tomi, 2005).

2. Materials and methods

2.1. Animals

Male Wistar rats (160–300 g) were purchased from Japan SLC (Hamamatsu, Japan). The use of experimental animals followed the guidelines instituted by the Animal Care Committee in the University of Toyama and by the Association for Research in vision and Ophthalmology (ARVO) Statement.

2.2. Reagents

Chemicals were of reagent grade and available commercially, and [1-^{14}C]D-mannitol ([^{14}C]D-mannitol, 55 mCi/mmol) and [1-^{14}C]*n*-butanol ([^{14}C]*n*-butanol, 2 mCi/mmol) were obtained from American Radiolabeled Chemicals (St. Louis, MO). [Terminal methylene-^{3}H]spermine ([^{3}H]spermine, 50 Ci/mmol) was obtained from Moravek Biochemicals (Brea, CA).

2.3. Microdialysis study

A microdialysis study was performed as described elsewhere (Katayama et al., 2006; Hosoya et al., 2009; Yoneyama et al., 2010; Akanuma et al., 2013). In brief, after anesthetizing rats (250–300 g) with sodium pentobarbital (60 mg/kg), their heads were placed on a stereotaxic frame (Narishige, Tokyo, Japan). To prevent eye blinking, 2% xylocaine was instilled to locally anesthetize their eyelids, and a 25G needle was inserted through the pars plana at a depth of 3.0 mm. After removal of the needle, 1 mL Ringer-HEPES solution (141 mM NaCl, 4 mM KCl, 2.8 mM $CaCl_2$, 10 mM HEPES, pH 7.4) containing [^{3}H]spermine (2.0 μCi) and [^{14}C]D-mannitol (0.2 μCi) were administered by means of a microsyringe (Hamilton, Reno, NE) at a depth of 3.0 mm from the eye surface. Immediately after, a microdialysis probe (TEP-50; Eicom, Kyoto, Japan) was implanted into the vitreous chamber, and the probe was fixed on the eye surface by means of surgical glue (Daiichi-Sankyo, Tokyo, Japan). To continuously supply Ringer-HEPES solution to the probe (2 μL/min, 37 °C), an infusion pump (Harvard, Holliston, MA) and polyethylene tubing (Natsume, Tokyo, Japan) were used. The radioactivity in the dialyzate collected at designated times was determined with a liquid scintillation counter (LSC-5200, Aloka, Tokyo, Japan).

Data analysis was performed as described previously (Yoneyama et al., 2010; Akanuma et al., 2013), and the data obtained were used in the calculation of C_P, the vitreous concentrations normalized by the injected dose (% of dose/mL), by means of Eq. (1), where $\mathrm{Dose}_{\mathrm{tracer}}$ and C_T are the total radioactivity in the solution after injection (dpm) and the concentration in the dialyzate (dpm/mL), respectively.

$$C_P = C_T/\mathrm{Dose}_{\mathrm{tracer}} \times 100 \quad (1)$$

$C_P(t)$ defined as the C_P at time t, was subjected to the nonlinear least-square regression analysis program, MULTI (Yamaoka et al., 1981), and was fitted to Eq. (2), where α and β are the apparent first-order rate constants for the initial and terminal phase, respectively, and A and B are intercepts on the *y*-axis for each exponential segment.

$$C_P(t) = A \times e^{-\alpha t} + B \times e^{-\beta t} \quad (2)$$

The probe recovery was estimated from Eq. (3), and C_V (dpm/mL) is the concentration in the test solution. In the present study, the recovery values were constant over 180 min, and the value for [^{3}H]spermine and [^{14}C]D-mannitol were 5.22% and 8.69%, respectively.