Research Article

Current Trends in Ophthalmology

D-Serine Induced by Ocular Hypertension is Associated with Retinal Cell Death

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Abstract

Purpose: The purpose of this study is to investigate the role of free D-serine in the death of retinal cells caused by ocular hypertension.

Methods: Adult Wistar rats were used as an experimental model of ocular hypertension. Immunohistochemistry was used to identify the retinal sites and expression patterns of D-serine and serine racemase in the rat retina. The concentrations of free D-serine and L-serine in the retina were measured by two-dimensional high-performance liquid chromatography. Retinal cell death was investigated by Immunohistochemistry.

Results: D-serine was expressed on the retinal ganglion cell layer in the retinas of rats with ocular hypertension. A serine racemase was specifically expressed in the retinal ganglion cells. The ratio of free D-/L-serine in the retinas with ocular hypertension was higher than that in the retinas with normal tension. Annexing-V-positive cells were observed in the retinal ganglion cell layer in the retinas of the rats with ocular hypertension, and these cells were also co-localized with D-serine expression.

Conclusions: We suspect that the up-regulation of serine racemase expression induced by ocular hypertension leads to an increase in free D-serine converted from free L-serine in retinal ganglion cells and that retinal cell death is associated with D-serine expression.

Keywords: D-Serine; Retinal cell death; Rat; Glaucoma

Abbreviations: AMPA: α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid; NMDA: N-methyl-D-aspartate; PBS: Phosphate-buffered Saline; IOP: Intraocular Pressure; FITC: Fluorescein Isothiocyanate; 2D-HPLC: two-dimensional High-performance Liquid Chromatography; NBD: 7-nitro-2, 1, 3-benzoxadiazole; OH: Ocular Hypertension; NT: Normal Tension; mRNA: messenger Ribonucleic Acid; Ab: Antibody; IgG: Immunoglobulin G; RGL: Retinal Ganglion Cell Layer; IPL: Internal Plexiform Layer; INL: Internal Nuclear Layer; OPL: Outer Plexiform Layer; ONL: Outer Nuclear Layer; PHL: Photoreceptor Layer; SR: Serine Racemase antibody; ARVO: Association for Research in Vision and Ophthalmology

Introduction

Free D-serine and serine racemase, localized in the mammalian central nervous system, play an important

role in neuronal transmission [1]. The stimulation of AMPA (α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid) receptors by glutamate activates serine racemase

through phosphorylation in astrocytic glia [2-4]. Activated serine racemase converts L-serine to D-serine, and D-serine is released to neuronal synapses [5]. D-serine co-binds with glutamate to N-methyl-D-aspartate (NMDA) receptor in neuronal synapses. The stimulation of NMDA receptors leads to the translocation of calcium ions into neurons [6]. Thus, the serine, dependent on serine racemase, shuttles between astrocytic glia and neurons, and D-serine acts as a co-agonist for NMDA receptors in neuronal synapses [7].

In the vertebrate retina, D-serine has an important effect on the light-evoked NMDA receptor-mediated current in retinal ganglion cells [8]. D-serine is synthesized in Muller cells by serine racemase, which is dependent on AMPA receptors, and it activates NMDA receptors in retinal ganglion cells [9]. Light-evoked D-serine release is necessary for the optimal activation of NMDA receptors in the retina [10]. These findings indicate that the neuronal transmission by D-serine and serine racemase via NMDA receptors is also localized in the retina.

The dysfunction of D-serine regulation can cause neuronal abnormalities. Genetic analyses and biochemical analyses revealed that the D-serine and serine racemase levels were reduced in patients with schizophrenia [11,12]. Serine racemase knockout mice show reduced levels of cortical D-serine, resulting in hypo-functioning of NMDA receptors, and they exhibit behaviour similar to schizophrenia [13]. The mechanism that underlies the maintenance of the tolerance of D-serine is also important to maintain the physiological functioning of NMDA receptors in the central nervous system. The translocation of serine racemase to the membrane has an inhibitory effect on D-serine synthesis, resulting in a reduction of the over-activation of NMDA receptors [14]. The over-activation of NMDA receptors can cause cell death, which is suspected as the origin of several neuronal diseases. In glaucoma, an over-activation of NMDA receptors is thought to underlie glaucomatous retinal dysfunction and is considered to play a significant role in inducing the apoptosis of retinal cells [15].

We hypothesized that understanding the failure in regulating D-serine in the retina induced by ocular hypertension may help clarify the mechanism of retinal degeneration in glaucoma. Our present study revealed that ocular hypertension increased the expression of D-serine and serine racemase in the retina of rats and that retinal cell death was associated with D-serine expression.

Methods

Animal study design

All experiments were performed in accordance with the Association for Research in Vision and Ophthalmology's statement on the use of animals in ophthalmic research. These experiments were approved by the Animal Use Committee of Hiroshima University.

In total, twenty-four adult Wistar male rats, obtained from CLEA (Tokyo, Japan), were studied. The rats were housed in clear plastic cages that contained pine bedding and were kept at 21°C on a 12-h light and 12-h dark cycle.

The preparation of a model rat with ocular hypertension was performed as described [16]. Briefly, the rat was anesthetized by a mixture of ketamine-xylazine (10 and 4 mg/kg, respectively) injected intracamerally with 10 µL of 35% India ink (Becton Dickinson, Cockeysville, MD, USA) in 0.01 M Phosphate-buffered Saline (PBS). At 1 week after the injection, a round of 200 laser burns were delivered on a dark circular band at limbos, which was visualized as the pigmented trabecular meshwork by the aggregation of carbon particles. The argon laser settings were 200 mm diameter, 150–200 mW, for 0.2-s durations (Ultima argon laser 2000 SE; Coherent, Tokyo). Munemasa Y, et al. reported that the IOP is increased immediately within a week after the application of Lasers, and the ocular hypertension is sustained at least 5 weeks after laser treatment [16]. At 2 weeks after the laser treatment, the rats' IOP was measured in an awake state with a portable tonometer (Tonolab, Icare Finland, Helsinki, Finland).

The IOP range in the left eyes was 13 ~ 22 mmHg. The treated eyes of four rats did not exhibit increased IOP, which were 17 mmHg ~ 22 mmHg, and they were excluded. Three eyes had enlarged corneas and almost ruptured because IOP was suspected to be extraordinarily high, and the cornea was seriously damaged. Finally, seventeen rats were successfully defined as model rats with ocular hypertension, and their IOP range was 29 ~ 37 mmHg. Six rats were used for immunohistochemistry, and eleven were used for liquid chromatography (HPLC).

Immunohistochemistry

Rat eyes were enucleated at 2 week after the application of Lasers, washed with PBS three times, and fixed in 4% par formaldehyde. The fixed eyes were sequentially placed in 12%, 15%, and 18% sucrose. The eyes were then sectioned to 20 µm on a cryostat, and the sections were incubated with a rabbit polyclonal anti-D-serine antibody

(Millipore, Temecula, CA), a rabbit polyclonal anti-serine racemase antibody (Santacruz Biotechnology, Santa Cruz, CA), and a monoclonal anti-NeuN antibody (Millipore, Temecula, CA). Staining was visualized via incubation with fluoresce in isothiocyanate (FITC)-conjugated anti-IgG (Alexa Fluor: Invitrogen Corp., Carlsbad, CA.) and Texas Red-conjugated anti-IgG (Alexa Fluor). The sections were labelled with fluoresce in-tagged Annexin-V (Invitrogen, Carlsbad, CA) as specified by the manufacturer. The slides were mounted on microscope slides with Fluoromount-G (Southern Biotech, Birmingham, AL) and examined with a confocal laser microscope (BZ-8100: Keyence, Osaka, Japan).

Measurement of D-serine and L-serine in the rat retina

The retinas were carefully isolated from enucleated eyes. D-serine and L-serine levels in the rat retina were measured with a two-dimensional high-performance liquid chromatography (2D-HPLC) system [17-19] with some modifications. Briefly, a × 50 volume of water was added to the retina, which was then homogenized with a micro-homogenizing system (Micro Smash™ MS-100R, Tomy, Tokyo). After deproteinization with methanol, the amino acids were derivatized with 4-fluoro-7-nitro-2, 1, 3-benzoxadiazole (NBD-F). The reaction mixture was then subjected to the 2D-HPLC system (NANOSPACE SI-2 series, Shiseido, Tokyo) that included a micro bore-monomolithic ODS column (0.53 mm ID × 1000 mm, prepared in a fused silica capillary, provided by Shiseido) and a narrow-bore enantioselective column (KSAACSP-001S, 1.5 mm ID × 250 mm, self-packed, material provided by Shiseido). With a micro bore-ODS column, the fraction of NBD-Serine was isolated as the D + L mixture, and the enantiomers were separated and determined with a narrow-bore enantioselective column. Fluorescence detection of the NBD-amino acids was carried out at 530 nm with excitation at 470 nm.

Results

Induction of D-serine expression by ocular hypertension

To determine whether D-serine is expressed in the retina, we used immunostaining with an anti-D-serine antibody in rat retinas with ocular hypertension or normal tension. The results demonstrated that D-serine was expressed on the retinal ganglion cell layers in the retinas of rats with ocular hypertension and those with normal tension and that D-serine expression was apparently increased in ocular hypertension compared with that in

normal tension (Figure 1).

Figure 1: The expression of D-serine in the retina of rats with ocular hypertension or normal tension. The retina of a rat with ocular hypertension (OH, panel a) and the retina of a rat with normal tension (NT, panel b) are labeled with an anti-D serine antibody (D-serine Ab) and sections stained with only the secondary antibody, an FITC-conjugated anti-rabbit IgG, as the negative control (panels c, d). IOP: intraocular pressure, RGL: retinal ganglion cell layer, IPL: internal Plexiform layer, INL: internal nuclear layer, OPL: outer Plexiform layer, ONL: outer nuclear layer, PHL: photoreceptor layer.

Expression of serine racemase in the retina of rats with ocular hypertension

To investigate the mechanism of D-serine up-regulation induced by ocular hypertension, rat retinas were also immunostained with an anti-serine racemase antibody. The results showed that serine racemase was expressed on the retinal ganglion cell layer in the retina with ocular hypertension, but it was only lightly expressed in the retina with normal tension (Figure 2A).

To ascertain the localization of serine racemase in retinal ganglion cells, we performed double staining of serine racemase and NeuN, a marker of retinal ganglion cells. Serine racemase was co-localized with NeuN on the retinal ganglion cell layer, which showed that serine racemase is specifically expressed in retinal ganglion cells (Figure 2B).

We also directly measured the concentrations of free D-serine and L-serine in the retina. We prepared a pool of retinas to reduce the individual differences among rats. Four rats were used for the group with ocular hypertension, and four rats with normal ocular tension were also prepared. The concentration of serine was measured with the two-dimensional HPLC system. The result showed that the D-serine / L-serine ratio in the

retina with ocular hypertension was higher than in the retina with normal tension, and indicated that D-serine expression was induced by ocular hypertension in the retina (Table 1).

Table 1: The concentration of free D-serine and free L-serine in the retina of rat with ocular hypertension or normal tension, measured by two-dimensional HPLC system.

	Ocular hypertension (OH)	Normal tension (NT)
Number of eyes	4	4
Total weight (mg)	10.5	9.8
Average of IOPs (mmHg)	32.33	15.91
D-serine (nmol/g)	33.72	27.18
L-serine (nmol/g)	2656	3158
D- 1L-serine ratio (%)	1.270	0.861

These results indicate that the up-regulated serine racemase expression induced by ocular hypertension increases the conversion of free D-serine from free L-serine in retinal ganglion cells.

Retinal cell death with D-serine expression in the retina with ocular hypertension

To observe the association of D-serine expression in retinal cell death induced by ocular hypertension, we stained rat retinas exposed to ocular hypertension with fluoresce in-tagged Annexing-V labelling (FITC-Annexing-V). Annexing-V, labelled with a fluorophore, specifically binds to phosphatidylserine in apoptotic cells, which is translocated from the inner to the outer leaflet of the plasma membrane. Annexing-V-positive cells were observed in the retinal ganglion cell layer in the retinas of rats with ocular hypertension, and they were co-localized with D-serine expression (Figure 3).

These results demonstrate that retinal cell death was associated with D-serine expression.

Discussion

Our analyses revealed that ocular hypertension increased the expression of D-serine and serine racemase in the retina. We also found that this change leads to retinal cell death.

During neuronal transmission in the central nervous system, glial cells, not neuronal cells, play a role in the production of D-serine, and a similar phenomenon occurs in the retina. The Muller cell line (which is generated from

the mouse retina) expresses serine racemase and the concentration of D-serine is higher in these cells [20]. In the salamander retina, light-evoked NMDA receptor activation was mediated by D-serine synthesis [8].

Figure 2: The expression of serine racemase in the retina of rats with ocular hypertension and normal tension. A: The retina of a rat with ocular hypertension (panel a) and that of a rat with normal tension (b) were labeled with an anti-serine racemase antibody (SR-Ab) and sections stained with only the secondary antibody, an FITC-conjugated anti-rabbit IgG, as the negative control (c, d). B: The retina of a rat with ocular hypertension was co-immunostained with an anti-serine racemase antibody and an anti-NeuN antibody, followed by secondary antibodies, a Texas Red-conjugated anti-rabbit IgG antibody and an FITC-conjugated anti-goat IgG antibody. Serine racemase immunoreactive cells are shown by red stain (e) and NeuN-positive cells by green (f). Yellow-stained cells show the co-expression of serine racemase and NeuN (g).

Figure 3: Co-immunostaining with D-serine and a cell death marker in the retina with ocular hypertension. The upper panels show a rat retina with ocular hypertension (OH), and the lower panels show a retina with normal tension (NT). D-serine, labeled with anti-D serine antibody, was visualized with a Texas Red-conjugated anti-rabbit IgG, and is represented by the red stain (a, d). Retinas were labeled with FITC-tagged anti-Annexin V antibody by the green stain (b, e). Overlay images are also shown (c, f), and the arrow indicates a cell co-expressing D-serine and Annexin-V.

However, regarding ocular hypertension, our results showed different patterns of D-serine and serine racemase expression in the retina. Retinal cells in the retinal ganglion cell layer expressed serine racemase and produced D-serine, which acts on itself. It is possible that retinal ganglion cells, not glial cells, self-produce D-serine, because the mRNA of serine racemase was expressed in both glial cells and neuronal cells [21]. We suspect that ocular hypertension induces the translation, not transcription, of serine racemase mRNA in retinal ganglion cells and the conversion to D-serine when the protein expression of serine racemase is reduced in normal ocular tension.

In amyotrophic lateral sclerosis, an adult-onset

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neurodegenerative disease, excessive D-serine induces NMDA receptor-dependent neurotoxicity and causes neuronal death [22]. D-serine acts on NMDA receptors [23] and increases calcium incorporation via NMDA receptors in retinal ganglion cells [24] under normal physiological conditions, but an excessive concentration of D-serine in the retinal ganglion cell layer may induce an over-activation of NMDA receptors in retinal ganglion cells, leading to retinal ganglion cell death. Our results suggest that an up-regulation of D-serine in ocular hypertension may be one of the mechanisms underlying glaucomatous retinal degeneration.

D-amino acid oxidase is an important enzyme in the regulation of the concentration of D-serine in the retina [25]. D-amino acid oxidase degrades D-serine and mediates the normal activity of NMDA receptors in retinal ganglion cells [26]. Regarding the mechanism underlying the association of ocular hypertension with D-serine up-regulation, it is possible that ocular hypertension reduces D-amino oxidase activity in the retinal ganglion cell layer.

Our present study has several limitations. Because we treated glaucoma model rats for acute ocular hypertension, the evaluation for any correlation between time and levels of D-serine increase with increasing duration of the induced ocular hypertension was quite difficult, and our findings cannot be applied directly to the physiology of chronic glaucoma patients. In addition, our study lacks retinal function data such as that obtained by electroretinograms, and it remains necessary to investigate the effect of D-serine regulation on retinal function in future studies.

Conclusions

In summary, we found that ocular hypertension increases D-serine and retinal cell death in the retinal ganglion cell layer, which may contribute to the mechanism of retinal degeneration in glaucoma.

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Compliance with ethical standard

Conflict of Interest

All authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or nonfinancial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

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Competing Interests

The authors declare that they have no competing interests.

Ethical approval

All animal procedures conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and they were approved by the Animal Use Committee of Hiroshima University.

Informed consent

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

TK was primarily responsible for experimental concept, design, and drafting of the manuscript. YK collected the animal samples. HS performed data acquisition and analysis. YM, YI, KT, and YK were involved in drafting of the manuscript. All authors read and approved the final manuscript.

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