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Hyperbaric pressure and increased susceptibility to glutamate toxicity in retinal ganglion cells in vitro

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Purpose: To investigate the effect of hyperbaric pressure on purified retinal ganglion cells (RGCs) and the additive effect of hyperbaric pressure on glutamate-induced RGC death.

Methods: An RGC primary culture from 8-day-old Wistar rats was prepared and cultured in a hyperbaric chamber. The RGC survival rate under various pressure conditions and with 5 or 25 µM of glutamate stimulation was determined and compared with that of RGCs under isobaric conditions. First, RGCs were cultured at atmospheric pressure (0 mmHg) and under hyperbaric pressure (+30 and +90 mmHg, with pressure fluctuations varying from 0 to +30 or +60 mmHg). Next, RGCs were cultured at +15, +30, and +90 mmHg with the addition of 5 or 25 µM of glutamate. The effects of N-Methyl-D-aspartic acid (NMDA) and 2-amino-3-(5-methyl-3-oxo-1,2- oxazol-4-yl)propanoic acid (AMPA)/kainate receptor antagonists, MK-801, and 6,7-dinitroquinoxaline-2,3-dione (DNQX), on cell survival were assessed. Additionally, types of cell death and the induction of Bcl-2-associated X protein (BAX) leading to apoptosis were studied under hyperbaric pressure conditions and/or with 5 µM of glutamate.

Results: RGC death was not induced under increasing or fluctuating pressure conditions. RGC death was induced by 25 µM of glutamate and increased as pressure increased. RGC death was not induced by 5 µM of glutamate but was induced by and increased with increasing pressure. MK-801 and DNQX significantly reduced glutamate-induced RGC death, and DNQX was more effective than MK-801. Under hyperbaric pressure conditions, the addition of 5 µM of glutamate resulted in the induction of apoptosis and BAX, which did not occur under hyperbaric pressure conditions or with the addition of glutamate alone.

Conclusion: In a rat RGC culture, hyperbaric pressure alone did not induce RGC death but increased RGC susceptibility to glutamate toxicity, which may be of relevance to ocular diseases with pressure-induced RGC death.

Retinal ganglion cell (RGC) death associated with structural changes in the optic nerve head is the cause of vision loss in glaucomatous optic neuropathy (GON). RGC death in GON is believed to occur as a result of an apoptotic mechanism triggered by multiple stimuli, such as the elevation of intraocular pressure (IOP), ischemia, oxidative stress, elevation of glutamate, excessive production of nitric oxide, or deprivation of neurotrophic factors [1,2]. Glutamate has a pivotal role in the neuronal system. At physiologic concentrations, glutamate transmits neuronal signals through several types of glutamate receptors comprised of ionotropic glutamate receptors, N-Methyl-D-aspartic acid (NMDA) and 2-amino-3-(5-methyl-3-oxo-1,2- oxazol-4-yl)propanoic acid (AMPA)/KA receptors, and metabotropic glutamate receptors (mGluRs) [3]. The release and uptake of glutamate are regulated by glutamate transporters in retinal cells; keeping the extracellular concentration of glutamate at physiologic levels and providing consecutive neurotransmission. However, numerous experiments support the hypothesis that excessive glutamate induces RGC death in vivo and in vitro [4,5]. Interestingly, ocular hypertension models mimicking glaucoma suggest that glutamate is at least partly involved in RGC death due to elevated intraocular pressure (IOP), because the glutamate receptor antagonists, MK801 or memantine, can ameliorate RGC death due to elevated IOP [6-10]. However, the exact mechanism for glutamate-induced RGC death under elevated IOP remains to be elucidated.

One of the leading hypotheses is that an excessive glutamate increase in the extracellular space is caused by the dysfunction of glutamate transporters due to ocular hypertension. This induces excessive increases in intracellular calcium ion concentration or oxidative stress and lead to apoptosis [11-15]. A recent report using glutamate transporter-knockout mice supports the idea that excessive glutamate concentrations in the retina may induce RGC death [16]. However, only a few reports show glutamate increases in rodent retina due to ocular hypertension [17,18], while other reports show no increase in vitreous glutamate concentration in glaucoma patients [19,20] or in animal models [19,21]. In addition, previous histological analyses of glutamate transporters in ocular hypertensive eyes reported conflicting results; some reported decreases in Gamma-ray Large Area Space

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Telescope (GLAST) and GLT1 immunoreactivities in RGCs and/or Muller cells [18,22], while others reported increases [15,23]. In human glaucoma eyes, GLAST (EAAT1) was decreased [11].

Another possible mechanism for glutamate-induced cell death under ocular hypertension is excessive glutamate sensitivity due to increased glutamate receptor expression [11,18,24-26], or other unknown biochemical mechanisms, leading to increased RGC susceptibility to glutamate toxicity.

Since glutamate transporters and receptors are expressed by and interact with each other—not only in neuronal cells, such as RGCs, but also in glial cells, such as Muller cells—it is challenging to investigate the effects of high pressure on the complicated system of glutamate homeostasis in the whole retina, in vivo. An alternative method is to study the effects of high pressure on the glutamate toxicity in isolated, cultured neural cells. In this study, we investigated whether various levels of pressure directly elicit RGC death or exacerbate glutamate-induced RGC death, using a rat primary RGC culture cultivated in a pressure-variable culture chamber.

METHODS

Animals: Wistar rats were purchased from Saitama Laboratory Animal Supply Inc. (Saitama, Japan). All experiments were conducted in accordance with the Animal Care and Use Committee and the ARVO Statement for Use of Animals in Ophthalmic and Vision Research.

Materials: Cell culture reagents were obtained from Gibco (Grand Island, NY). Calcein AM was obtained from Sigma (St. Louis, MO). A Vybrant® Apoptosis Assay Kit (V13241) and Hoechst 33342 were purchased from Molecular Probes (Eugene, OR). A cell-dissection kit of Papain was obtained from Worthington Biochemical (Lakewood, NJ). Recombinant neurotrophic factors (human brain-derived neurotrophic factor [BDNF] and rat ciliary neurotrophic factor [CNTF]) were obtained from Sigma. Monoclonal ascites IgG_{2a} antibody (OX-41) against rat macrophage and monoclonal IgG_1 antibody (OX-7) against rat and mouse Thy-1.1 were obtained from Chemicon (Temecula, CA). Staurosporine was purchased from Sigma. The AMPA-KA receptor antagonist, 6,7-dinitroquinoxaline-2,3-dione (DNQX), and the NMDA receptor antagonist, MK 801, were obtained from Tocris Bioscience (Ellisville, MO).

Purification and Culture of RGCs: RGCs were purified by a two-step immunopanning procedure as described previously [27-29]. Briefly, the dissociated retinal cells from 8-day-old Wistar rats were incubated in flasks (Nunc A/S, Roskilde, Denmark) coated with an anti-rat macrophage monoclonal antibody (1:50) to exclude macrophages, and were then incubated in tubes (Corning, Acton, MA) coated with an anti-rat Thy1.1 monoclonal antibody (1:300). RGCs that adhered to the tubes were collected by centrifugation at 600 rpm for 5 min and were seeded on 13 mm glass coverslips in a 24-well plate that had been coated with 50 µg/ml poly-L-lysine (Sigma-Aldrich, St. Louis, MO) and 1 µg/ml laminin (Invitrogen, Carlsbad, CA). Purified RGCs were plated at a density of approximately 1,000 cells per well. RGCs were cultured in a serum-free B27 complete medium containing a neurobasal medium (Invitrogen) with 1 mM L-glutamine (Sigma), B27 supplement (Invitrogen), 40 ng/ml human recombinant BDNF, 40 ng/ml rat recombinant CNTF, 10 µM forskolin (Sigma), 100 U/ml penicillin, and 100 µg/ml streptomycin. The plates were incubated in a tissue culture incubator with a humidified atmosphere containing 5% CO_2 and 95% air at 37 °C for 3 days.

Evaluation of RGC survival: In the present study, a surviving RGC was defined as a cell with a calcein-AM-stained cell body and a process extending at least three cell diameters from the cell body [27-29]. All surviving RGCs on each glass coverslip were counted at 200× magnification using an inverted fluorescence microscope (Nikon Eclipse TE300, Tokyo, Japan). In each experiment, the number of RGCs cultured in isobaric conditions (control group) was set at 100%. In the control culture, about 1,000 cells were counted in one assay. We have repeated at least six assays for each experiment. The percentage of surviving RGCs, after hyperbaric stress and /or glutamate stress, was calculated and normalized to the control group. Each percentage is expressed in the text and figures as the mean±standard deviation (SD). Compounds evaluated in the present study are described below.

Pressure variable chamber: The pressure variable chamber was purchased from Hirayama Company (Chiba, Japan) and was placed in the normal incubator (Figure 1 shows the effects of pressure and not the chamber setup). This type of pressure chamber contains a pressurized pipe allowing variations in the chamber's inner pressure where the culture wells are placed. To avoid pressure-dependent increases in O_2 concentration in the culture media, the inflated O_2 concentration was automatically controlled based on Henry's law as follows: the inflated O_2 concentration=20% × 760 mmHg/incubating pressure.

Effect of direct hyperbaric conditions on RGC death: To investigate the direct effects of hyperbaric stress on RGCs, the cells were cultivated under constant pressure. Three levels of pressure, 0 (atmospheric pressure), +30, and +90 mmHg were applied for 72 h and the survival rate was calculated.

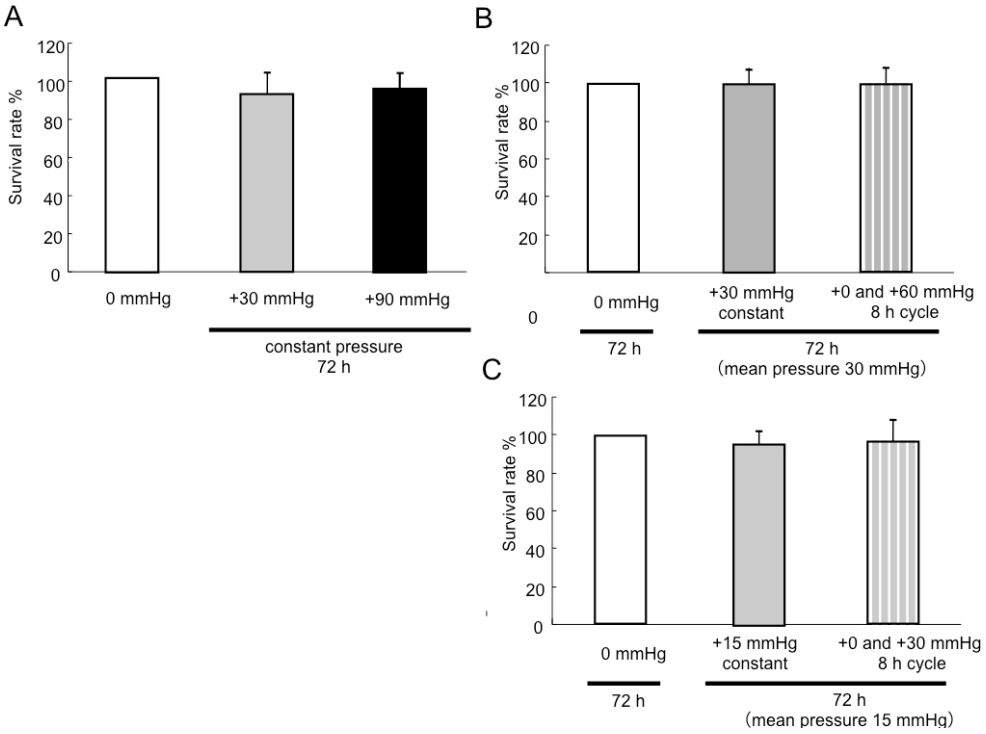

Figure 1. The effect of constant or variable pressure on RGC survival. **A**: RGCs were cultured under constant +30 and +90 mmHg hyperbaric pressure and were compared to the atmospheric pressure in 72 h. A constant hyperbaric pressure had no effect on RGC survival. **B**: RGCs were cultured under +0 and +60 mmHg of variable pressure with an 8 h cycle and under +30 mmHg pressure as a control. Repeated variable pressure also had no effect on RGC survival. The data indicates a mean±SD, which is not significantly different from the control (white bar), using Dunnett's test, n=6. **C**: RGCs were cultured under +0 and +30 mmHg of variable pressure with an 8 h cycle and under +15 mmHg of pressure as a control. Repeated variable pressure also showed no effect on RGC survival. The data indicates a mean±SD, which is not significantly different from the control (white bar), using Dunnett's test, n=6.

Next, to investigate the direct effects of pressure variation on RGCs, the cells were alternately cultivated under 0 mmHg and +60 or +30 mmHg with an 8-h cycle for 72 h, and the survival rate was compared to that of RGCs cultivated under constant +30 or +15 mmHg hyperbaric pressure, which is the average pressure variation value.

Effects of hyperbaric conditions on glutamate-induced RGC death: To investigate the additive effects of hyperbaric pressure on glutamate-induced cell death, 5 or 25 µM of glutamate was simultaneously added to the four constant pressure groups (0, +15, +30, and +90 mmHg) and the survival rate was measured after 72 h. Next, the AMPA-KA receptor-selective antagonist, DNQX, or the NMDA receptor antagonist, MK 801, was added to the culture medium at a final concentration of 10 µM. This concentration was found to completely inhibit the respective receptor subtypes [30-32].

Detection of apoptotic and necrotic RGCs: We used a Vybrant® Apoptosis Assay Kit to quantify the extent of apoptosis in RGSs under stress. RGCs were incubated under stress and then Alexa Fluor 488-conjugated annexin V binding combined with propidium iodide labeling was performed. Apoptotic RGCs were stained as annexin V+/propidium iodide-, and necrotic RGCs were stained as annexin V+/propidium iodide+. Undamaged RGCs remained negative for both staining [33,34]. At the end of the double staining, 8 µM of Hoechst 33342 was added to the culture medium. Cells were counted in at least 10 random fields in each well at 200× magnification using a fluorescence microscope (Nikon Eclipse TE300). The percentages of apoptotic RGCs and necrotic RGCs were quantified by determining the ratio of (annexin V+/propidium iodide-) cells and (annexin V+/propidium iodide+) cells to Hoechst 33342-positive RGCs, respectively.

RNA extraction and quantitative RT–PCR of BAX: To verify the activation of the mitochondrial apoptotic pathway by hyperbaric stress (0, +15, +30, and +90 mmHg) or glutamate toxicity (0, 5, and 25 µM), the expression of BAX was evaluated by quantitative RT–PCR (LightCycler system; Roche Diagnostics, Mannheim, Germany) with specific fluorescein hybridization probes. Total RNA was extracted from cultured RGC cells using ISOGEN (Nippon gene, Toyama, Japan) and according to the product's specifications. cDNA was made using a reverse transcriptase (Super Script II; Gibco Grand Island, NY).

The detection probes were two independent, single-labeled oligonucleotides that hybridized adjacently on the amplicon, internal to the flanking PCR primers. The upstream primer was labeled with a fluorescent dye at the 3′ terminus

and the downstream primer was labeled with a red dye (LightCycler Red 640; Roche) at the 5′ end. The sequences of these probes were complementary to the antisense strands of glucose-3 phosphate dehydrogenase (*G3PDH*) and *BAX*. The primers for *G3PDH* and *BAX* were 5′ACC ACA GTC CAT GCC ATC AC and 5′-TCC ACC ACC CTG TTG CTG TA, and 5′-AGA CAG GGG CCT TTT TGT TAC and 5′-GAG GAC TCC AGC CAC AAA GAT, respectively.

The optimum PCR reaction conditions were determined by melting curve analysis using green fluorescent dye (SYBR Green I; Roche) before performing sample quantification.

The 20 µl PCR reaction mix contained 4 mM of $MgCl_2$, 0.5 µM of each primer, a 0.4 mM fluorescent probe, a 0.4 mM red probe, a 1 µl cDNA sample, and a 10% volume master hybridization mixture (LightCycler DNA; Roche) in a LightCycler capillary. Amplification began with a 10 min denaturation step at 95 °C, followed by 45 cycles of 5 s at 95 °C, 15 s at 54 °C, and 13 s at 72 °C. For G3PDH, amplification began with a 2 min denaturation step at 95 °C, followed by 45 cycles of 5 s at 95 °C, 15 s at 54 °C, and 13 s at 72 °C. The fluorescein density of the red, reflecting the amount of PCR product, was read at the end of each annealing step.

The quantification procedure was as follows: Standard curves were made for the target gene (*BAX*) and the housekeeping gene (*G3PDH*) using the dilution series of the target or *G3PDH* plasmids. The log concentrations of target (A) and *G3PDH* (B) transcripts were calculated from a standard curve. The expression levels were standardized as A/B and the relative amount was calculated in comparison with the control. This experiment was repeated in 5 samples and the data was presented as the means±SD. The induction of *BAX* in RGCs cultured under hyperbaric pressure with or without glutamate, was indicated by the relative amount compared to that of RGCs cultured under atmospheric pressure without glutamate.

Statistical analysis: The results are expressed as the mean±SD and a *t*-test was used with Bonferroni's correction. Data were analyzed using one-way analysis of variance (ANOVA) followed by Dunnett's test for the comparison of pressure-dependent damage, and Tukey's test for making a comparison among glutamate channel inhibitors. Steel's test was used to compare the ratio of cell death types under stress with that of the control culture. A p value <0.05 was evaluated as being of statistical significance.

RESULTS

Effect of constant hyperbaric pressure on RGC death: After 72 h of incubation at +30 and +90 mmHg, the RGC survival rate was 92.9±10.4% and 96.0±7.7% compared to 0 mmHg, respectively (Figure 1A). All hyperbaric groups showed no significant difference from the control group ($p=0.876$; Dunnett's test).

Effect of variable hyperbaric pressure on RGC death: After 72 h of incubation, the survival rates of RGCs in the high variable pressure group (0 to 60 mmHg, 8 h cycle) and in the constant pressure group (+30 mmHg), were 98.2±6.4% and 97.1±6.4%, respectively (Figure 1B). Additionally, the survival rates of RGCs in the low variable pressure group (0 to 30 mmHg, 8-h cycle) and in the constant pressure group (+15 mmHg), were 96.5±5.8% and 98.6±8.6%, respectively (Figure 1C). There was no difference between the variable pressure and the constant pressure group ($p>0.10$; *t*-test).

Effect of hyperbaric pressure on glutamate-induced RGC death under high and low glutamate concentrations: Without glutamate, the survival rates of the +15, +30, and +90 mmHg groups were similar to that of the 0 mmHg group (Figure 2A). With 25 µM of glutamate, the survival rates of the 0, +15, +30, and +90 mmHg groups were 79.6±14.9%, 64.2±11.1%, 63.6±9.6%, and 62.9±12.8%, respectively (Figure 2B). The survival rate of RGCs in the presence of 25 µM of glutamate was significantly reduced when compared to that of RGCs cultured without glutamate under the same pressure conditions ($p<0.05$; *t*-test with Bonferroni's correction). In addition, the survival rates at the three levels of hyperbaric pressure with 25µM of glutamate were significantly lower than that at atmospheric pressure with 25 µM of glutamate ($p<0.05$; Dunnett's test), although there was no difference among the three hyperbaric pressure groups.

Next, we applied a lower glutamate concentration (5 µM). The survival rates of the 0, +15, +30, and +90 mmHg groups with 5 µM of glutamate were 100±11.1v, 97.6±11.1%, 74.5±11.1%, and 62.2±11.1%, respectively (Figure 2B). Under atmospheric and +15 mmHg pressures, the survival rates of RGCs with 5 µM of glutamate were similar to those of RGCs cultured without glutamate. In contrast, the survival rates of the +30 and +90 mmHg groups with 5 µM of glutamate were significantly reduced when compared to RGCs cultured without glutamate. The survival rates of the +30 and +90 mmHg groups with 5 µM of glutamate were significantly lower than those at atmospheric pressure with 5 µM of glutamate ($p<0.05$; Dunnett's test), while those of the +15 mmHg group with 5 µM of glutamate were not significantly different from the control ($p>0.10$). RGCs cultured under hyperbaric pressure conditions over +30 mmHg were vulnerable at lower

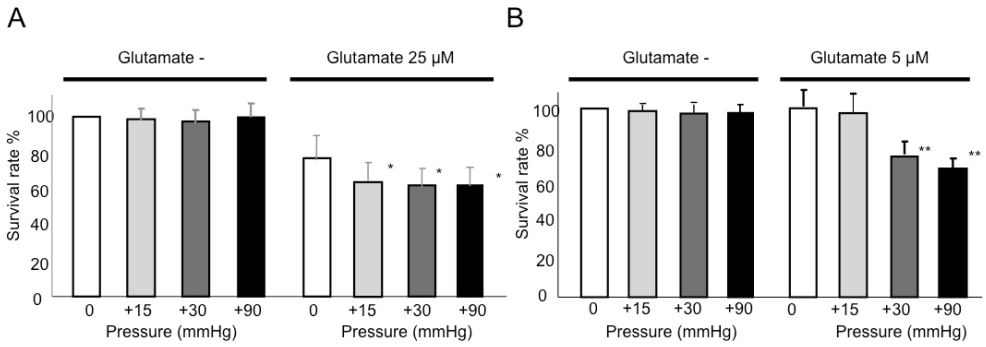

Figure 2. The effect of constant hyperbaric pressure on RGC survival with 25 and 5 μM of glutamate. **A**: RGCs were cultured under atmospheric and hyperbaric pressure, with or without 25 μM of glutamate. Glutamate of 25 μM induced-RGC death under atmospheric pressure conditions, which was deteriorated by hyperbaric pressure. **B**: RGCs were cultured under atmospheric and hyperbaric pressure, with or without 5 μM of glutamate. Glutamate of 5 μM did not induce RGC death under atmospheric pressure conditions. However, RGC death was induced by hyperbaric pressure. Data indicates mean±SD, *; p<0.05 by Dunnett's test vs control (atmospheric pressure with glutamate), n=8.

concentrations of glutamate, which were insufficient to elicit glutamate-induced cell death at normal or +15 mmHg of pressure.

Effects of MK-801 and DNQX on glutamate-induced RGC death under hyperbaric pressure conditions: To investigate the mechanism of hyperbaric pressure-induced increased susceptibility to glutamate toxicity, MK-801 and DNQX—an NMDA receptor and an AMPA/kainate receptor antagonist—were applied.

With 5 μM of glutamate, the survival rates of the 0 mmHg, 0 mmHg with MK801, and 0 mmHg with DNQX groups were similar to that of the control culture without glutamate (100.4±3.9% and 99.1±2.9% versus 100.3±4.0%). With 25 μM of glutamate, the survival rates of the 0 mmHg, 0 mmHg with MK801, and 0 mmHg with DNQX groups were 69.1±8.9%, 88.2±7.9%, and 98.7±3.9%, respectively ($p<0.05$ and $p<0.01$, respectively; Dunnett's test; Figure 3A).

At +15 mmHg pressure with 5 μM of glutamate, the survival rates of the groups under +15 mmHg, +15 mmHg with MK801, and +15 mmHg with DNQX pressure conditions were similar to those of the control culture without glutamate (98.7±6.1%, 98.7±4.5%, and 97.3±7.2%, respectively). With 25μM of glutamate, the survival rates of the +15 mmHg, +15 mmHg with MK801, and +15 mmHg with DNQX groups

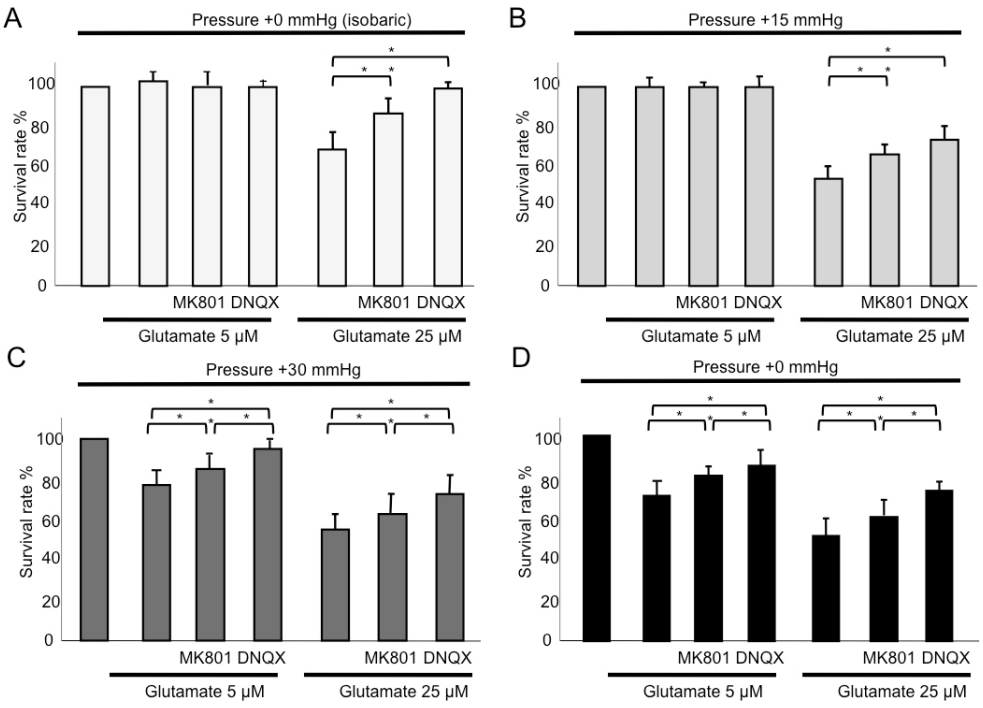

Figure 3. The effects of glutamate receptor antagonists, MK801 and DNQX, on glutamate toxicity at 0 (isobaric; **A**), +15 (**B**), +30 (**C**), and +90 mmHg (**D**) pressure. Both MK801 and DNQX partially inhibited glutamate-induced cell death, and its protective effect was higher in DNQX than in MK801. Data indicates mean±SD, *; p<0.05, **; p<0.01 from Tukey's test, n=8.

were 55.8±6.5%, 66.4±5.6%, and 75.2±7.7%, respectively ($p<0.05$ and $p<0.01$, respectively; Dunnett's test; Figure 3B).

At +30 mmHg pressure with 5 µM of glutamate, the survival rates of the +30 mmHg, +30 mmHg with MK801, and +30 mmHg with DNQX groups were 73.4±6.5%, 84.7±6.6%, and 91.2±4.1%, respectively ($p<0.05$ and $p<0.01$, respectively; Dunnett's test). With 25 µM of glutamate, the survival rates of the +30 mmHg, +30 mmHg with MK801, and +30 mmHg with DNQX groups were 54.6±8.8%, 64.9±8.6%, and 75.4±7.7%, respectively ($p<0.05$ and $p<0.01$, respectively; Dunnett's test; Figure 3C).

At +90 mmHg hyperbaric pressure, with 5 µM of glutamate, the survival rates of the +90 mmHg, +90 mmHg with MK801, and +90 mmHg with DNQX groups were 72.7±8.6%, 82.7±5.7%, and 87.4±8.6%, respectively. With 25 µM of glutamate, the survival rates of the +90 mmHg, +90 mmHg with MK801, and +90 mmHg with DNQX groups were 53.6±7.6%, 62.0±8.1%, and 73.8±4.5%, respectively ($p<0.05$ and $p<0.01$, respectively; Dunnett's test; Figure 3D).

Both MK801 and DNQX significantly increased the survival rates of RGCs treated with 5 or 25 µM of glutamate, when compared to groups without an antagonist at atmospheric (0 mmHg) or hyperbaric (+15, +30, +90 mmHg) pressures. Although both MK801 and DNQX were effective in reducing RGC death, DNQX was more effective than MK801 under all pressure conditions in which glutamate toxicity was observed (all pressure groups with 25 µM of glutamate and the +30 and +90 mmHg groups with 5 µM of glutamate; $p<0.05$; Tukey's test).

Types of cell death induced by hyperbaric pressure and a low dose of glutamate: Necrotic cell death without stress, under hyperbaric pressure only, with 5 µM of glutamate only, and under hyperbaric pressure with the addition of glutamate, were 14.6±3.7%, 10.0±4.0%, 10.1±3.5%, and 18.2±8.6%. There was no significant increase by three kinds of stress ($p>0.10$; Steel test). On the other hand, apoptotic cell death without stress, under hyperbaric pressure, with 5 µM of glutamate, and under hyperbaric pressure with the addition of glutamate, were 7.8±3.0%, 7,6±1.9%, 7.8±2.8%, and 16.1±4.2%. Apoptotic cell death significantly increased under hyperbaric pressure with the addition of glutamate ($p=0.01$; Steel test).

Induction of BAX in RGCs cultured at hyperbaric pressure in the presence of glutamate: Without glutamate, *BAX* RT–PCR amplicons from RGCs cultured at +15, +30, and +90 mmHg were 102.5±7.9%, 104.7±15.2%, and 109.9±33.4%, respectively, when compared to atmospheric pressure conditions without glutamate (N.S. ANOVA). With 5 µM of glutamate, *BAX* RT–PCR transcripts from RGCs cultured at 0, +15, +30, and +90 mmHg were 105.3±6.5%, 195.8±33.6%, 339.9±81.0%, and 531.8±175.5%, respectively, of the atmospheric pressure without glutamate ($p<0.05$; *t*-test). Glutamate significantly induced *BAX* expression ($p<0.01$; unpaired *t*-test) in RGCs under increased hyperbaric pressure conditions. Among the groups with 5 µM of glutamate, *BAX* was induced in a pressure-dependent manner in RGCs cultured at +15, +30, and +90 mmHg ($p<0.05$; Tukey's test).

DISCUSSION

No RGC death under hyperbaric conditions: One of the most important results from this study is that increased pressure alone had no effect on RGC survival in our culture conditions. We tried three levels of constant pressure, +15, +30, and +90 mmHg (Figure 2, Figure 4, Figure 5). A pressure of +15 mmHg may mimic chronic in vivo conditions such as primary open angle glaucoma, while +90 mmHg may mimic conditions such as acute angle closure glaucoma. Additionally, we tried to mimic the large fluctuation of IOP by changing the pressure periodically (Figure 3). There are limitations to investigating the effects of high pressure in this in vitro culture system for longer periods, since we could not maintain the viability of primarily isolated RGCs over five days. Our finding that high pressure itself had no direct effect on RGC death is unsurprising given the absence of structural stress induced by the high pressure occurring in the optic disc with lamina cribrosa, and it is also unlikely that RGCs can discern a 5% difference in the ambient pressure.

So far, there is only one report on RGC death being induced by a slight increase in the pressure over a 48 h time period, which was smaller than that used in the current study [35]. In that report, rat primary cultures of RGCs were stressed under one directional pressure using a centrifuge with a special attachment for rotating the culture wells. Even if the culture conditions in the centrifuge are strictly regulated, the centrifugal force that is perpendicular to the bottom of the culture well differs from the conditions created in our system and may not be suited to studies of the effect of ambient pressure on cultured RGCs.

The higher susceptibility of RGCs to glutamate toxicity under hyperbaric conditions: The second interesting finding in our study was that RGCs cultured at hyperbaric pressure became more susceptible to glutamate toxicity. Additionally, we found that glutamate-induced RGC death was partially inhibited by the ionotropic glutamate receptor antagonists, MK801 and DNQX. The mechanism of this higher susceptibility may be due to a change in the expression of glutamate receptors, the affinity of the ionotropic glutamate receptor to

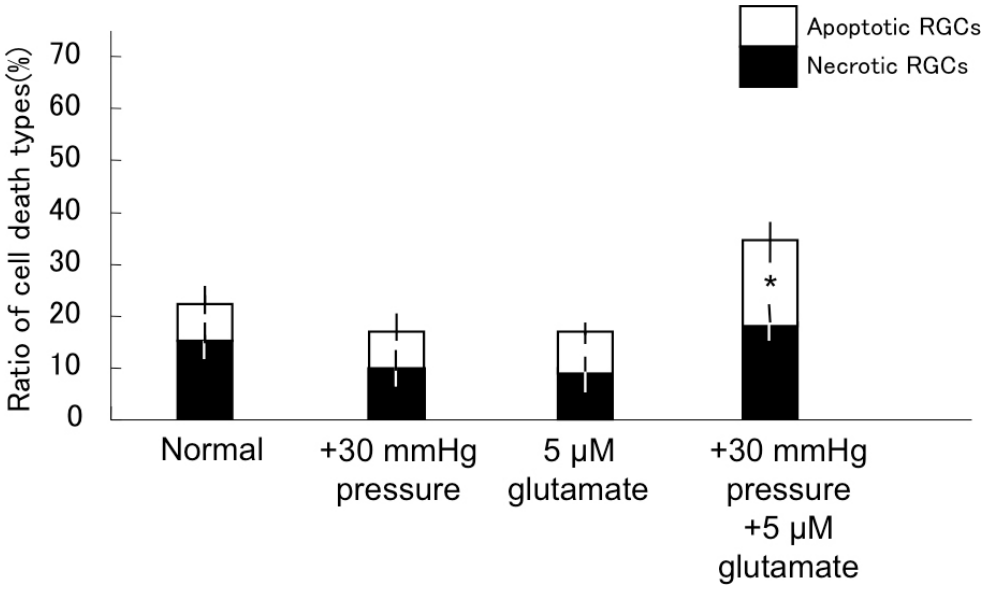

Figure 4. The detection of cell death induced by hyperbaric stress and 5 μM of glutamate. Hyperbaric stress and 5 μM of glutamate did not induce apoptosis and necrosis, but both hyperbaric stress and 5 μM of glutamate significantly increased apoptotic cell death. Necrosis was constant and independent of each stress. Data indicates mean±SD, *; p<0.05 from Steel's test. n=7–8.

glutamate, or the expression of RGC glutamate transporters, GLT1 or EAAC1. A previous report indicated that NMDA receptor 1 was upregulated in a rat ocular hypertension model [25]. However, the expression of the glutamate transporter GLT1, in ocular hypertension models is still controversial. In a DBA2J mouse with chronic ocular hypertension due to pigment dispersion and angle closure, GLT1 was down-regulated [18], whereas in human glaucomatous eyes and a rat glaucoma model, GLT1 was upregulated [15]. Since EAAC1 knockout mice showed glaucoma-like optic nerve degeneration [16,36], expression of EAAC1 may be affected by hyperbaric pressure. Further studies are required to investigate the effect of hyperbaric pressure on glutamate receptors and glutamate transporters in vivo and in vitro.

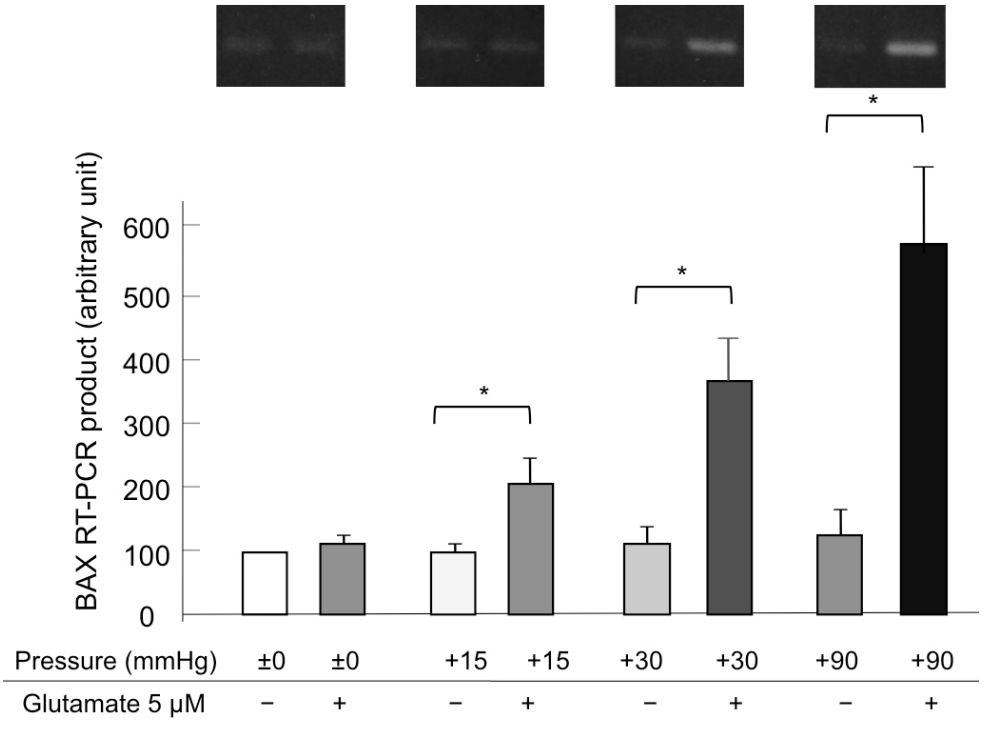

Figure 5. The induction of *BAX* by hyperbaric stress and 5 μM of glutamate. *BAX* was not induced by 5 μM of glutamate alone, but was significantly induced under hyperbaric pressure with glutamate. Data indicates mean±SD, *; p<0.01 by *t*-test. n=5. The representative PCR-band is indicated.

Our results from using specific antagonists for NMDA or kainite receptors suggest that both receptors contribute to glutamate-induced cell death under hyperbaric pressure. So far, the contribution of glutamate receptors to RGC toxicity is still the subject of debate. Some studies have reported that both NMDA and kainate receptors contribute to RGC death, as we reported [37], while others showed that only the kainate and not the NMDA receptor is responsible for RGC toxicity [27]. For example, some studies claimed that the NMDA receptor is critical for excitotoxicity in RGC [4]. Yet another group demonstrated that RGCs are resistant to glutamate or NMDA toxicity [38]. Otherwise, the culture medium for neurons, such as the neurobasal/B27 medium, may affect the susceptibility to glutamate toxicity [39]. The reason for these discrepancies has not been clarified, but the subtle differences among culture conditions or experimental procedures may be critical to the results.

Although our in vitro approach also suggests that the dysfunction of glutamate homeostasis may occur under ocular hypertension conditions in RGCs, it was difficult to study the mechanism in more detail given the limited numbers of isolated RGCs to allow a quantitative analysis of the expression of glutamate transporters or glutamate receptors to be made. Thus, further studies are needed to clarify the molecular mechanisms of RGC susceptibility to glutamate at hyperbaric pressure.

Pressure dependent apoptotic cell death under non-toxic, low glutamate concentrations: The third finding in this study is that apoptosis and *BAX* were induced in isolated RGC under 5 μM of glutamate stimulation. It is well known that glutamate stimulates the mitochondria-dependent apoptotic pathway involving the induction of *BAX* [40,41]. Our findings suggest that hyperbaric pressure increases the susceptibility of RGCs to glutamate toxicity through *BAX* expression. *BAX* induction may be caused by an increase in intracellular calcium ion concentration following the enhanced sensitivity to glutamate or by the increase in pressure itself. Since many molecules are related to the apoptotic pathway, further studies are needed to clarify the mechanism by which pressure modifies glutamate susceptibility.

Implication of the current results: The increased susceptibility of RGCs to glutamate toxicity under hyperbaric pressure conditions described by the current study may have in vivo implications. Under isobaric conditions in our culture system, RGCs survived stimulation with 5 μM of glutamate. However, once RGCs were exposed to abnormal hyperbaric conditions, they became susceptible to lower levels of glutamate that were otherwise non-toxic to RGCs. Thus, RGCs may be more susceptible to small increases in the concentration of glutamate. In living eyes, an increase in glutamate concentration in the extracellular space may be caused by the impaired uptake of glutamate by Müller cells through GLAST or other glutamate transporters in neuronal cells, which may be attributed to disturbances in blood flow, oxidative stress, or inflammatory processes [42-44]. If intraocular pressure is abnormally high, then otherwise non-toxic, small increases in extracellular glutamate might further exacerbate RGC damage. Based on the current results, the distribution and expression of glutamate receptors and the susceptibility of RGCs to glutamate toxicity may warrant further study using experimental ocular hypertensive animals.

In conclusion, we demonstrated that hyperbaric pressure increases the susceptibility of RGCs to glutamate toxicity using an in vitro rat primary RGC culture. In view of the complicated regulation system of glutamate in the retina, which consists of many neuronal and glial cells, further studies are required to clarify the involvement of glutamate-induced cell death under increased ocular pressure conditions.

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