厚生労働科学研究費補助金（感覚器障害研究事業）分担研究報告書

緑内障性網膜神経障害に対する新規治療薬の開発と新規眼圧測定原理の確立

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研究要旨

以下の２点を目的に下記の研究を行った結果、緑内障性網膜神経障害の関連タンパクと考えられるＧＡＢＡ受容体拮抗薬が有用であることが判明し、眼球剛性の測定器は従来の眼圧測定器と相関した値を示すことが明らかになった。

１．緑内障性網膜神経障害に対する新規治療法を開発すること

２．眼球剛性を考慮した真の眼圧測定を行うこと

Ａ．研究目的

1. 緑内障性網膜神経障害に関与するタンパクについてプロテオミクス解析により候補因子を確定しているが、本研究ではそれらの機能解析を行い新規神経保護薬を開発すること。
2. 正確な眼内圧測定が可能となることが期待される5000-1000 0フレーム/秒の測定が可能なスーパービジュアライズ装置を用いた新たな眼圧測定装置を開発すること。

Ｂ．研究方法

1.緑内障性網膜神経障害の関連タンパクの可能性のあるＧＡＢＡ受容体を主として解析した。In vitroでは、継代培養系の網膜神経細胞であるＲ２８細胞やＲＧＣ５細胞を用いて、ＧＡＢＡ受容体アンタゴニストであるビククリンやアゴニストであるムシモールを用いて、ＧＡＢＡ受容体による網膜神経細胞死への関与をＭＴＳアッセイ等を行って検討した。In vivoでは、マウス眼球を用いて、網膜神経節細胞死を誘導し、緑内障性網膜障害の誘導因子と考えられるグルタミン酸毒性や酸化ストレスに対するＧＡＢＡ受容体の関与について、組織免疫液染色法を用いて検討した。

2. スーパービジュアライズ装置を用いた眼圧測定装置（眼球剛性測定器）を活用し、研究協力者を対象にして、従来日常診療で多用されてきたゴールドマン眼圧測定器から得られた眼圧および角膜厚や角膜曲率との相関性について検討した。また、角膜の性状の影響が少ないとされるＤＣＴ（：Dynamic Contour Tonometer）眼圧測定器とゴールドマン眼圧測定器から得られた結果について検討した。

（倫理面への配慮）

文部科学省告示の「研究機関等における動物実験等の実施に関する基本指針」および，広島大学動物実験指針，広島大学動物実験実施規則に則った。

C．研究結果

1. In vitroでは、ＧＡＢＡ受容体アゴニストのムシモールが網膜神経節細胞死を誘導し、ビククリンが特異的にその細胞死を抑制することが判明した。In vivoでは、緑内障性網膜障害の誘導因子とされるグルタミン酸による網膜神経節細胞死をビククリンが抑制した。加えて、網膜神経節細胞死を誘導する酸化ストレスのシグナル・タンパクの活性化においても、ビククリンによる活性化阻害が確認された。
2. 眼球剛性は100.2±19.1 N/m (70.8 - 139.5 N/m)であり、最小と最大の間では1.97倍の差が認められ、眼球剛性は眼圧（p=0.02）と有意に相関したが、角膜厚・角膜曲率とは相関を認めなかった。また、ゴールドマン眼圧測定器とＤＣＴを用いた結果の差は、中心角膜厚による影響でないことが判明した。

D．考察

1.ビククリンは薬剤毒性が高いため、そのままでの臨床応用は危険度が高い。そのため、ビククリンの化合式を修飾した低毒性の化合物の合成、もしくは別のＧＡＢＡ受容体アンタゴニストの模索が試みられる。また、全く別の手法として、RNA interferanceを活用したＧＡＢＡ受容体のＲＮＡノックダウンも検討される。

2. 現用の様々な眼圧測定器を併用・活用して、眼球剛性に関与する因子をさらに検討していく必要性がある。

E．結論

1. 緑内障モデルマウスの解析よりＧＡＢＡ受容体が網膜神経節細胞死に関連している可能性が考えられたため、その詳細について検討した結果、ＧＡＢＡ受容体アンタゴニストであるビククリンが、網膜神経節細胞死を抑制することが判明した。
2. スーパービジュアライズ装置を用いた眼圧測定装置（眼球剛性測定器）を活用して、眼球剛性と眼圧が相関することが判明したが、中心角膜厚が眼球剛性の重要な因子でない可能性が示唆された。

G．研究発表

1.論文発表

1) Tamura H, Kawakami H, Kanamoto T, Kato T, Yokoyama T, Sasaki K, Izumi Y, Matsumoto M, Mishima HK..High frequency of open-angle glaucoma in Japanese patients with Alzheimer's disease. J Neurol Sci. 2006 Jul 15;246(1-2):79-83. 2006 Mar 6.

2) Tanimoto S, Kanamoto T, Mizukami M, Aoyama H, Kiuchi Y. Pigment epithelium-derived factor pro

motes neurite outgrowth of retinal cells. Hiroshima J Med Sci. 2006 Dec;55(4):109-16.

2.学会発表

1）Kanamoto T, et al. Proteomics analysis of the retina of EAAC1-knock out mice: Neuro-protective effect of PDGF against glutamate-induced excitotoxity in retinal ganglion cells. 2006. ARVO (Florida. USA)

2）杉本　英一郎　他：ゴールドマン眼圧計を用いた眼球剛性の測定、2006　第60回日本臨床眼科学会（京都）

H．知的財産権の出願・登録状況（予定を含む）

1.特許取得

なし。

2.実用新案登録

なし。

3.その他

別添５

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
なし							

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Yi-Ning Chen, Hideyuki Yamada, Wei Mao, Shigemi Matsuyama, Makoto Aihara, Makoto Araie.	ypoxia-Induced Retinal Ganglion Cell Death and the Neuroprotective Effects of Beta-adrenergic Antagonists	Brain Research		In press	2007
Tamura H, Kawakami H, Kanamoto T, Kato T, Yokoyama T, Sasaki K, Izumi Y, Matsumoto M, Mishima HK	High frequency of open-angle glaucoma in Japanese patients with Alzheimer's disease	J Neurol Sci	246	79-83	2006

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High frequency of open-angle glaucoma in Japanese patients with Alzheimer's disease

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Abstract

The clinical and genetic relationships between Alzheimer's disease (AD) and glaucoma remain obscure. The aim of this study was to determine the prevalence of open-angle glaucoma (OAG) in patients with AD and whether the apolipoprotein E (*APOE*) 4 allele is associated with AD, with or without OAG, in Japanese. The groups consisted of 172 patients with the diagnostic criteria of AD and 176 age-matched controls. Ophthalmic examinations were conducted, and genomic analysis was performed by PCR and digestion of products with an enzyme. OAG was found in 41 (23.8%) of the AD patients, which was a significantly ($p=0.0002$) higher prevalence than that in the controls (9.9%). Furthermore, there was no significant difference between intraocular pressures (IOPs) in AD patients with OAG and without OAG. The percentage of AD patients who carried an *APOE* ε4 allele (29.5%) was significantly ($p=0.0007$) higher than that of the controls (9.1%). However, the percentage of AD patients with OAG who carried an *APOE* ε4 allele (35.7%) was not significantly different than that of AD patients without OAG (27.7%, $p=0.42$). In summary, the prevalence of OAG is high in Japanese patients with AD, suggesting that common factors other than APOE may contribute to the two diseases.

Keywords: AD, Alzheimer's disease; OAG, open-angle glaucoma; APOE, apolipoprotein E; IOP, Intraocular pressure; Japanese; Prevalence; Polymorphism

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disease that is the most common cause of dementia in the elderly. AD is characterized neuropathologically by large extracellular beta-amyloid plaques and tau-containing intraneuroral neurofibrillary tangles. Early-onset AD may be explained by highly penetrate mutations in the genes encoding amyloid precursor protein, presenilin 1 and presenilin 2, and the majority of cases (90–95%) are late-onset AD (LOAD) in which several factors have been implicated. The ε4 allele of the gene encoding apolipoprotein E (*APOE*) is a major risk factor for LOAD in the general population [1–4].

Glaucoma is the second or third leading cause of visual loss worldwide. Open-angle glaucoma (OAG) is the most common type of glaucoma in many populations [5–8]. The pathogenesis of OAG is not yet known, but there are several known risk factors [9–11], one of which is elevated intraocular pressure (IOP). Lowering IOP is the current standard therapy for many kinds of glaucoma and effectively slows down disease progression [12]. However, even after pressure reduction, many patients have ongoing visual loss. In addition, 30% ~ 50% of OAG patients suffer from

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'normal-tension' glaucoma (NTG) in which there is optic nerve degeneration without elevation in IOP [8,12,13].

AD and glaucoma have common features. Both become more severe with advance of age, and they both occur more frequently in women than in men [5,14]. Neurons are affected in the brain of AD patients and the eye of glaucoma patients. In addition, it has been reported that patients with AD demonstrate widespread axonal degeneration of the optic nerves and loss of retinal cells, especially ganglion cells [7,15–17]. Moreover, the ε4 allele of *APOE* has been established as a genetic risk factor of AD. Some studies have shown that ε4 is a genetic risk factor of OAG, though the contribution of the ε4 allele remains controversial [18–21]. Therefore, AD and glaucoma might have some common risk factors, mechanisms, or pathways.

To clarify the relationship between AD and glaucoma, we investigated the frequency of OAG in AD patients and we evaluated APOE as a common risk factor for AD and OAG in Japanese.

2. Materials and methods

2.1. Prevalence of open-angle glaucoma in patients with AD

A total of 172 patients with sporadic AD (age: mean±S.D.=80.9±8.4 years; 35 men, 137 women) who were institutionalized residents in four Japanese hospitals or who visited those hospitals for treatment of AD were recruited into this study. The diagnosis of probable AD was based on clinical findings according to the National Institute of Neurological and Communicative Disorders Association (NINCDS–ADRDA) criteria. A total of 176 individuals (age: mean±S.D.=81.9±8.8 years; 42 men, 134 women) who were institutionalized residents without AD in another three Japanese hospitals or who visited these hospitals for treatment of diseases other than AD were randomly chosen to be control subjects. These patients were matched for age and sex (Table 1).

These patients received ophthalmic examinations including estimation of width of the angle of the anterior chamber, and indirect ophthalmoscopy was performed in dilated pupils in all eyes to evaluate optic nerve head cup-to-disc ratios. At the same time as the ophthalmic examinations, the IOP of each of the AD patients was measured three times and that of each of the control subjects was measured once or several times. An electric impression tonometer (Tono-pen®) using the principle of the Mackay-Marg tonometer, which can measure IOP with the patients in any position, was used for measurement of the IOP in AD patients because measurement in sitting position was difficult for many of the AD patients. The IOP of the control subjects was measured using a Goldmann applanation tonometer, which is a tonometer that operates based on Imbert–Fick's law and is suitable for outpatients. However, in almost all AD patients, no reliable data of visual field could be obtained. Probable OAG was diagnosed by width of the angle of the anterior chamber >grade 2 (method of Van Herick et al. [22]), a vertical cup-to-disc ratio of the optic nerve head >0.7 and/or difference between the vertical cup-to-disc ratio in the eyes >0.2 with characteristic glaucomatous disc change. In no instance was the diagnosis of OAG dependent on the level of IOP. Ophthalmic examination was performed and diagnosis was made by two glaucoma specialists who were not blinded to the clinical diagnosis (dementia/control).

Table 1
Age and sex of the prevalence study subjects

	AD (n=172)	Controls (n=176)	All subjects (n=348)	p-value
Age (year)[a]	80.9±8.4	81.9±8.8	81.4±8.7	0.11
Sex[b]				
Male	35	42	77	0.43
Female	137	134	271	

[a] Mann–Whitney's U test.
[b] χ^2 test for independence.

Table 2
Age and sex of the genomic study subjects

	AD (n=122)	Controls (n=77)	All subjects (n=199)	p-value
Age (year)[a]	81.6±8.6	79.7±8.9	80.9±8.8	0.23
Sex[b]				
Male	21	17	38	0.40
Female	101	60	161	

[a] Mann–Whitney's U test.
[b] χ^2 test for independence.

2.2. APOE genotyping

All of the AD patients and control subjects in the prevalence study who gave informed consent for the purpose and all procedures of this study, including collection of blood samples, were enrolled in the study. Blood samples were collected from 122 patients with sporadic AD (age: mean±S.D.=81.6±8.6 years; 21 men, 101 women) and 77 patients without AD as controls (age: mean±S.D.=79.7±8.9 years; 17 men, 60 women). They were examined in the same Japanese hospitals as those in which patients in the prevalence study were examined (Table 2). The study protocol was in accordance with the Ethical Principles for Human Genome Research of the Ministry of Education, Culture, Sports, Science and Technology, the Ministry of Health, Labor and Welfare and the Ministry of Economy, Trade and Industry of Japan.

Genomic DNA was extracted from peripheral blood leukocytes using commercially available kits (QIAmpDNA Blood Maxi kit, Qiagen, Germany). *APOE* isoforms were amplified by polymerase chain reaction (PCR) using oligonucleotide primers F4 (5′-AGAGAATTCGCGCCGG-CCTGGTACAC-3′) and F6 (5′-TAAGCTTGGCACGG-CTGTCCAAGGA-3′) as described by Hixon and Vernier

[23]. Each amplification reaction mixture contained 1 μl leukocyte DNA, 0.5 pmol/μl of each primer, 10% dimethyl sulfoxide, and 0.025 U/μl of *Taq* polymerase (Expand High Fidelity PCR System; Roche Diagnostics, Swiss) in a final volume of 30 μl. Each reaction mixture was heated at 95 °C for 3 min for denaturation and was subjected to 30 cycles of amplification by primer annealing (62 °C for 1 min), extension (72 °C for 2 min), and denaturation (95 °C for 1 min). After PCR amplification, 5 U of *Hha*I (New England Biolabs, USA) were added directly to each reaction mixture for digestion of the APOE sequences (3 h and 30 min at 37 °C). This process required purification of the PCR products. Each reaction mixture was loaded onto an 8% polyacrylamide non-denaturing gel and subjected to electrophoresis for 2 h under a constant current (45 mA). After electrophoresis, the gel was treated with ethidium bromide (0.1 mg/l) for 15 min, and DNA fragments were visualized by UV illumination. The size of the *Hha*I fragments was estimated by comparison with known size markers (20-bp DNA ladder marker; Takara Bio Inc, Japan).

2.3. Statistical methods

Age and IOP were compared between patients and controls and between AD cases with OAG and AD cases without OAG by means of Mann–Whitney's *U* test. Sex was compared by means of the Chi-square test for independence or Fisher's Exact Test as appropriate. Differences in genotype and allele frequency distributions among the AD cases and controls and among AD cases with OAG and AD cases without open-angle glaucoma were assessed with the χ^2 test for independence or Fisher's Exact Test as appropriate. These analyses were performed with standard statistical software (Stat View 5.0; SAS Institute, USA), and significance level was set at 0.05.

3. Results

Clinical characteristics of the AD patients and control subjects are shown in Table 3. Among the 172 patients with AD, 41 (23.8%) had OAG. In the control group, 16 subjects (9.9%) had OAG. The percentage of AD patients with OAG (23.8%) was significantly higher than the percentage of control subjects with OAG (9.9%; $p=0.0002$). There was no variation between the study group and control group as the difference between slit-lamp biomicroscopy and indirect ophthalmoscopy which could represent a source of error in diagnosis of OAG. A statistically significant difference was found between the mean age of the AD patients with OAG and that of AD patients without OAG ($p=0.025$). There was no significant difference between groups in male: female ratio. Although almost entire exam was not compromised by non-cooperation, IOP was not measured in 38 of the 172 patients with AD because of non-cooperation. We could not directly compare IOP of the AD patients with that of the control subjects because of the different methods used to measure IOP. However, IOPs in the two groups were not significantly different. IOP of the subjects with OAG was not significantly higher than that of the subjects without OAG in both the AD group ($p=0.09$) and the control group ($p=0.64$) (Table 3). Moreover, almost all subjects with OAG showed normal IOP (<21 mm Hg).

Table 3
Clinical characteristics of patients with Alzheimer's disease and controls

	AD ($n=172$)		Controls ($n=176$)	
	No Glaucoma	Glaucoma	No Glaucoma	Glaucoma
No. of patients[a]	131	41*	160	16
Age (year)[b]	80.0±11.0	84.0±7.6**	81.6±8.9	80.6±7.5
Sex[c]				
Male	26	9	38	4
Female	105	32	122	12
IOP (mm Hg)[b]	15.5±3.0	16.4±3.2	14.8±3.9	15.2±3.6

[a] χ^2 test for independence: AD vs. Controls.
[b] Mann–Whitney's *U* test: No Glaucoma vs. Glaucoma.
[c] χ^2 test for independence: No Glaucoma vs. Glaucoma.
* $p=0.0002$.
** $p=0.025$.

Table 4
Apolipoprotein E genotype in patients with Alzheimer's disease and controls

APOE genotype	AD			Controls
	Glaucoma (−)	Glaucoma (+)	Total	
	$n=94$ [n (%)]	$n=28$ [n (%)]	$n=122$ [n (%)]	$n=77$ [n (%)]
ε2/ε2	1 (1.1)	0 (0.0)	1 (0.8)	2 (2.6)
ε2/ε3	3 (3.2)	2 (7.1)	5 (4.1)	7 (9.1)
ε3/ε3	64 (68.0)	16 (57.1)	80 (65.6)*	61 (79.2)
ε3/ε4	25 (26.6)	8 (28.6)	33 (27.1)*	6 (7.8)
ε2/ε4	1 (1.1)	1 (3.6)	2 (1.6)	1 (1.3)
ε4/ε4	0 (0.0)	1(3.6)	1(0.8)	0 (0.0)
ε4 carrier	26 (27.7)	10 (35.7)	36 (29.5)*	7 (9.1)

* $p<0.05$, χ^2 test for independence.

The distribution of *APOE* genotyping is shown in Table 4. The ε3/ε3 genotype was found most frequently in all study groups. The relative proportion of the ε3/ε3 genotype was significantly lower in AD patients in both groups ($p=0.027$) than in the controls, and the relative proportion of the ε3/ε4 genotype was significantly higher in the AD patients ($p=0.0009$) than in the controls. Moreover, the percentage of AD patients with the ε4 allele (29.5%) was significantly greater than that of control subjects (9.1%, $p=0.0007$). However, the percentage of AD patients with OAG who had the ε3/ε3 genotype (57.1%) was not significantly different from that of AD patients without OAG who had the ε3/ε3 genotype (68.0%, $p=0.29$), and the percentage of AD patients with OAG who had the ε3/ε4 genotype (28.6%) was not significantly different than that of AD patients without OAG who had ε3/ε4 genotype (26.6%, $p=0.84$). The percentage of AD patients with OAG who had an *APOE* ε4 allele (35.7%) was not

Table 5
Apolipoprotein E allele frequency in patients with Alzheimer's disease and controls

APOE allele frequency	AD			Controls [*n* (%)]
	Glaucoma (−) [*n* (%)]	Glaucoma (+) [*n* (%)]	Total [*n* (%)]	
ε2	6 (3.2)	3 (5.4)	9 (3.7)	12 (7.8)
ε3	156 (83.0)	42 (75.0)	198 (81.1)	135 (87.7)
ε4	26 (13.8)	11 (19.6)	37 (15.2)*	7 (4.5)
Total	188 (100)	56 (100)	244 (100)	154 (100)

* $p<0.05$, χ^2 test for independence.

significantly different from that of AD patients without OAG who had an *APOE* ε4 allele (27.7%, $p=0.42$). The other genotyping distributions of these patients were not significantly different from each other.

The allele frequencies of the *APOE* gene are shown in Table 5. The ε3 allele frequency was not significantly lower in the AD patients (81.1%) than in the controls (87.7%, $p==0.086$). The ε4 allele frequency was higher in the AD patients (15.2%) than in the controls (4.5%, $p=0.001$). However, the ε4 allele frequency in AD patients with OAG (19.6%) was not significantly higher than that in AD patients without OAG (13.8%, $p=0.29$). The other allele frequency distributions in these patients were not significantly different from each other.

4. Discussion

The prevalence of OAG in the AD patients was 23.8%, which was significantly higher than that of the control subjects (9.9%). In the Tajimi study [24] (a study carried out to assess a population-based prevalence of glaucoma among residents aged 40 years or older in Tajimi City, located in central Japan, by simple random sampling without stratification, which was used to select 4000 subjects from the 54165 residents in Tajimi City), the prevalence of OAG increased with age from 2.0% for subjects aged 40–49 years to 7.6% for those aged 70 years or older. In our study, the mean age of patients was almost 80 years. The prevalence of probable OAG in our control group was almost the same as that in the Tajimi study. In Germany, the prevalences of probable OAG were reported to be 25.9% in AD patients and 5.2% in controls, with a high prevalence of glaucoma in patients with AD [25]. Our results are consistent with those of the study in Germany. Therefore, it is thought that AD and glaucoma have some common mechanisms or risk factors of disease onset.

Furthermore, there was no significant difference between IOPs in AD patients with OAG and without OAG, and almost all AD patients with OAG showed normal tension. These results may be contributed that approximately 90% of OAG patients in Japan suffer from NTG [5,24]. Moreover, relationships of damage to abnormalities of the optic nerve in blood viscosity, to vasospasm and immunoreactive tendencies also have been reported [9–11]. Considering these findings, factors other than IOP might contribute to the optic nerve degeneration in AD patients.

APOE ε4 allele carriers accounted for a significantly high ratio (29.5%) in *APOE* genotyping of AD patients (with and without OAG), and the *APOE* ε4 allele accounted for a significantly high ratio (15.2%) in *APOE* allele frequency of AD patients. These findings are similar to results of previous studies [3,4]. *APOE* genotyping of AD patients showed that the rate of *APOE* ε4 allele carriers was significantly greater among AD patients. However, there was no significant difference among the three AD groups (all AD cases, AD cases with OAG and AD cases without OAG), although a very large percentage of AD patients with OAG carried at least one *APOE* ε4 allele compared to the percentage of carriers in the control group. Therefore, the results of this study did not suggest that AD accompanied by OAG occurs with high frequency in *APOE* ε4 allele carriers. As another study, we also investigated *APOE* ε4 carriers in OAG patients without AD. The percentage of *APOE* ε4 carriers in these patients was high, but the difference was not significant. The ratio of *APOE* ε4 carriers was not different in AD patients with OAG and in non-AD controls with OAG in Japanese (Tamura et al., unpublished results). Therefore, *APOE* ε4 was not a sufficient risk factor of OAG in our Japanese study and the results suggested that *APOE* ε4 polymorphism may not be a common risk factor for both AD and OAG.

In this study, we revealed a clinical correlation between the two diseases, but we could not show the common risk factors of the diseases. There are several candidates in common risk factors. For example, genotypes of the *APOE* promoter were also reported to modify OAG [26,27] and they may be candidates. As well as genetic factors, chronic or repetitive intermittent intracranial pressure elevations may be common risk factors of AD and glaucoma [28].

In summary, we found that the prevalence of OAG was high in Japanese patients with AD. We expect that the two diseases have common causative factors other than APOE ε4. Therefore, careful attention should be given to the potential for OAG in AD patients not to give the impression to non-ophthalmic trained physicians or clinicians that OAG would be manifest through visual disturbance.

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References

[1] Thomas DB. Genetic factors in Alzheimer's disease. N Engl J Med 2005;352:862–4.

[2] Laws SM, Hone E, Gandy S, Martins RN. Expanding the association between the *APOE* gene and the risk of Alzheimer's disease: possible roles for *APOE* promoter polymorphisms and alteration in *APOE* transcription. J Neurochem 2003;84:1215–36.

[3] Saunders AM, Strittmatter WJ, Schmechel D, George-Hyslop PH, Pericak-Vance MA, Joo SH, et al. Association of apolipoprotein E allele epsilon 4 with late-onset familial and sporadic Alzheimer's disease. Neurology 1993;43:1467–72.

[4] Maruyama H, Izumi Y, Oda M, Torii T, Morino H, Sasaki K, et al. Lack of an association between cystain C gene polymorphisms in Japanese patients with Alzheimer's disease. Neurology 2001;57:337–9.

[5] Shiose Y, Kitazawa Y, Tsukahara S, Akamatsu T, Mizokami K, Futa R, et al. Epidemiology of glaucoma in Japan: a nationwide glaucoma survey. Jpn J Ophthalmol 1991;35:133–55.

[6] Leske MC, Connell AM, Schachat AP, Hyman L. The Barbados eye study. Prevalence of open angle glaucoma. Arch Ophthalmol 1994;112:821–9.

[7] Weinreb RN, Khaw PT. Primary open-angle glaucoma. Lancet 2004;363:1711–20.

[8] Klein BE, Klein R, Sponsel WE, Franke T, Cantor LB, Martone J, et al. Prevalence of glaucoma. Beaver Dam eye study. Ophthalmology 1992;99:1499–504.

[9] Trope GE, Salinas RG, Glynn M. Blood viscosity in primary open-angle glaucoma. Can J Ophthalmol 1987;22:202–4.

[10] Gasser P, Flammer J, Guthauser U, Mahler F. Do vasospasms provoke ocular disease? Angiology 1990;41:213–9.

[11] Cartwright MJ, Grajewski AL, Friedberg ML, Anderson DR, Richards DW. Immune-related disease and normal-tension glaucoma. A case–control study. Arch Ophthalmol 1992;110:500–2.

[12] Heijl A, Leske MC, Hyman L, Bengtsson B, Hyman L, Hussein M, et al. Early Manifest Glaucoma Trial Group. Reduction of intraocular pressure and glaucoma progression: results from the Early Manifest Glaucoma Trial Group. Arch Ophthalmol 2002;120:1268–79.

[13] Sommer A, Tielsch JM, Katz J, Quigley HA, Gottsch JD, Javitt J, et al. Relationship between intraocular pressure and primary open angle glaucoma among white and black Americans. The Baltimore eye survey. Arch Ophthalmol 1991;109:1090–5.

[14] Henderson VW. The epidemiology of estrogen replacement therapy and Alzheimer's disease. Neurology 1997;48:27–35.

[15] Sadun AA, Bassi CJ. Optic nerve damage in Alzheimer's disease. Ophthalmology 1990;97:9–17.

[16] Quigley HA, Dunkelberger GR, Green WR. Retinal ganglion cell atrophy correlated with automated perimetry in human eyes with glaucoma. Am J Ophthalmol 1989;107:453–64.

[17] Hinton DR, Sadun AA, Blanks JC, Miller CA. Optic-nerve degeneration in Alzheimer's disease. N Engl J Med 1986;315:485–7.

[18] Vickers JC, Craig JE, Stankovich J, McCormack GH, West AK, Dickinson JL, et al. The apolipoprotein ε4 gene is associated with elevated risk of normal tension glaucoma. Mol Vis 2002;8:389–93.

[19] Mabuchi H, Tang S, Ando D, Yamakita M, Wang J, Kashiwagi K, et al. The apolipoprotein E gene polymorphism is associated with open angle glaucoma in the Japanese population. Mol Vis 2005;11:609–12.

[20] Lake S, Liverani E, Desai M, Casson R, James B, Clark A, et al. Normal tension glaucoma is not associated with the common apolipoprotein E gene polymorphisms. Br J Ophthalmol 2004;88:491–3.

[21] Ressiniotis T, Griffiths PG, Birch M, Keers S, Chinnery PF. The role of apolipoprotein E gene polymorphisms in primary open-angle glaucoma. Arch Ophthalmol 2004;122:258–61.

[22] Van Herick W, Shaffer RN, Schwaltz A. Estimation of width of angle of anterior chamber: incidence and significance of the narrow angle. Am J Ophthalmol 1969;68:262–9.

[23] Hixon JE, Vernier DT. Restriction isotyping of human apolipoprotein E by gene amplification and cleavage with *Hha*I. J Lipid Res 1990;31:545–8.

[24] Iwase A, Suzuki Y, Araie M, Yamamoto T, Abe H, Shirato S, et al. The Tajimi Study Group, Japan Glaucoma Society. The prevalence of primary open-angle glaucoma in Japanese: the Tajimi study. Ophthalmology 2004;111:1641–8.

[25] Bayer AU, Ferrari F, Erb C. High occurrence rate of glaucoma among patients with Alzheimer's disease. Eur Neurol 2002;47:165–8.

[26] Copin B, Brezin AP, Valtot F, Dascotte JC, Bechetoille A, Garchon HJ. Apolipoprotein E-promoter single-nucleotide polymorphisms affect the phenotype of primary open-angle glaucoma and demonstrate interaction with the myocilin gene. Am J Hum Genet 2002;70:1575–81.

[27] Fan BJ, Wang DY, Fan DSP, Tam POS, Lam DSC, Tham CCY, et al. SNPs and interaction analyses of myocilin, optineurin, and apolipoprotein E in primary open angle glaucoma patients. Mol Vis 2005;11:625–31.

[28] Wostyn P. Can chronic increased intracranial pressure or exposure to repetitive intermittent intracranial pressure elevations raise your risk for Alzheimer's disease? Med Hypotheses 2004;62:925–30.

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Research Report

Hypoxia-induced retinal ganglion cell death and the neuroprotective effects of beta-adrenergic antagonists

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ABSTRACT

Hypoxia-induced retinal ganglion cell (RGC) death has been implicated in glaucomatous optic neuropathy. However, the precise mechanism of death signaling and how neuroprotective agents affect it are still unclear. The aim of this study is to characterize the mechanisms of hypoxia-induced apoptosis of cultured purified RGCs and to study the neuroprotective effects of beta-adrenergic antagonists. Rat RGCs were purified utilizing a modified two-step immuno-panning procedure. First, the extent of apoptosis in RGCs under hypoxia was quantified. Next, the effects of glutamate-channel antagonists (MK801 or DNQX), Bax inhibiting peptide (BIP), and beta-adrenergic antagonists (betaxolol, nipradilol, timolol or carteolol) on hypoxia-induced RGC death were investigated by the cell viability assay. Third, the effects of beta-adrenergic antagonists on hypoxia-induced increase of intracellular calcium concentrations ($[Ca^{2+}]_i$) and the additional effect of NO scavenger to nipradilol were evaluated. Apoptotic RGC percentages under hypoxia were significantly increased compared to the control. The viability of RGCs under hypoxia was not affected by MK801 or DNQX, whereas it was increased in a dose-dependent manner with exposure to BIP, and to betaxolol, nipradilol, timolol, but not to carteolol. These effective beta-adrenergic antagonists showed no significant change in hypoxia-induced $[Ca^{2+}]_i$ levels. The NO scavenger alleviated neuroprotective effect by nipradilol. In conclusion, purified RGC damage induced by hypoxia involves Bax-dependent apoptotic pathway, but mostly independent of glutamate receptor-mediated excitotoxicity. Betaxolol, timolol and nipradilol showed a protective effect against hypoxia-induced RGC death, which was thought to be irrelevant either to calcium channel or β-adrenoceptor blocking effects.

1. Introduction

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 has been thought to occur by an apoptotic mechanism triggered by multiple stimuli, such as an elevation of intraocular pressure (IOP), ischemia, oxidative stress, an elevation of glutamate,

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Abbreviations: RGCs, retinal ganglion cells; GON, glaucomatous optic neuropathy; IOP, intraocular pressure; BIP, Bax-inhibiting peptide

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excessive production of nitric oxide (NO), or deprivation of neurotrophic factors (Quigley, 1999; Wax and Tezel, 2002).

In vivo, ischemia is defined as an arrest of blood flow and consequent reduction of oxygen supply (Osborne et al., 1999b). Evidence has suggested that tissue hypoxia was present in glaucomatous retina and optic nerve head (Tezel and Wax, 2004), and an ischemic-like insult or the presence of vascular disorders that induce tissue hypoxia, such as vasospasm, systemic hypotension or impaired local perfusion, is partly involved in the development of glaucoma (Chung et al., 1999; Costa et al., 2003; Drance et al., 1988; Flammer, 1994; Osborne et al., 1999b; Tielsch et al., 1995). Consequently, there is a rationale to investigate the mechanism of hypoxia-induced RGC death and the neuroprotective effects on hypoxia-induced RGC death by several agents.

Hypoxia-induced release of glutamate from the isolated retina or cultured retinal cell has been reported (Neal et al., 1994; Ohia et al., 2001; Rego et al., 1996). Also, apoptosis may be induced by hypoxia in the retina through the activation of a caspase cascade (Tezel and Wax, 1999). However, it is still unclear how the two main pathways for apoptosis, the receptor-dependent (extrinsic) pathway and the mitochondrial-dependent (intrinsic) pathway, play their roles in hypoxia-induced RGC death (Kroemer and Martin, 2005; Li et al., 2000). Bax-inhibiting peptide (BIP) is a newly designed, membrane-permeable peptide composed of five amino acids of the Ku70 Bax-binding domain, which binds Bax and regulates Bax internalization into mitochondria (Sawada et al., 2003a,b; Yoshida et al., 2004). In a previous in vivo study, BIP was proven to prevent RGC apoptosis after optic nerve transection, suggesting that Bax plays a central role in RGC apoptosis (Qin et al., 2004). In the current study, we first examined whether hypoxia-induced RGC death was mainly by apoptosis, and evaluated the effects of BIP, MK 801, an NMDA-receptor antagonist, and DNQX, an AMPA-KA receptor antagonist, on hypoxia-induced RGC death.

Topical beta-adrenergic antagonists have been widely used in the treatment of glaucoma. Our previous studies and others suggested that topically instilled drugs, including some beta-adrenergic antagonists, penetrate to the posterior retina by local diffusion at concentrations of $\geq 10^{-8}$ M in normal rabbit eyes (Acheampong et al., 2002; Ichikawa et al., 2004; Ishii et al., 2003; Mizuno et al., 2001; Osborne et al., 1999a). Subsequent experimental studies confirmed betaxolol's neuroprotective potency in animal models of retinal ischemia or excitotoxicity (Gross et al., 2000; Hirooka et al., 2000; Osborne et al., 1997, 1999a; Wood et al., 2001), and revealed that topically applied nipradilol, metipranolol or timolol can also reduce the detrimental effects induced by ischemia/reperfusion or glutamate to rat RGCs in vivo or in vitro (Goto et al., 2002; Mizuno et al., 2001; Wood et al., 2003). Whether these beta-adrenergic antagonists can protect against hypoxia-induced RGC death at concentrations that can be achieved after topical instillation (Acheampong et al., 2002; Ichikawa et al., 2004; Ishii et al., 2003; Mizuno et al., 2001; Osborne et al., 1999a) may have more clinical relevance. Therefore, as a second part of the study, the effects of 4 commercially available beta-adrenergic antagonists, betaxolol, nipradilol, timolol and carteolol, on hypoxia-induced RGC death were investigated. Further, the effects of these drugs on intracellular calcium levels and the combination effect of nipradilol and c-PTIO, a NO-scavenger, were studied.

2. Results

2.1. *Immunocytochemical identification of cultured* RGCs *(Fig. 1)*

The purity of isolated RGCs was assessed by immunocytochemical identification for Thy-1.1 (Fig. 1). The immunopositive rate for Thy-1.1 is 92.5% (2302/2489 cells). Cultured RGCs showed a high immunopositive rate for Thy-1.1.

2.2. *Detection of apoptotic and necrotic RGCs after hypoxic stimuli (Figs. 2 and 3)*

Photomicrographs of purified RGCs in the same field after 2-h hypoxic stress are shown in Fig. 2. The cells were stained by annexin V, propidium iodide and Hoechst 33342 under blue (A), green (B), and ultraviolet light (C) excitation, respectively. Plate D was the phase-contrast image of the same field. Apoptotic cell was visualized in plates A, C and D (white arrows). Necrotic cells were visualized in plates A, B, C and D (white arrowheads). Alive cells were visualized in plates C and D (yellow arrows).

After a 2-h incubation, the apoptotic RGC percentages were 33.5±6.3% in the hypoxic group and 37.6±6.8% in the staurosporine-treated group (Fig. 3). Both values were significantly higher than 16.8±6.1, that of the control group ($n=8$, $p<0.05$ by Dunnet test) but there was no significant difference between the hypoxic and the staurosporine-treated group. The necrotic RGC percentages in the staurosporine-treated and hypoxic groups were 36.1±11.6 and 23.0±8.2%, respectively. Only the percentage in the staurosporine-treated group was significantly higher than 19.9±12.1%, that of the control group ($p<0.05$ by Dunnet test). The necrotic RGC percentages showed no inter-group differences between the hypoxic and the control groups.

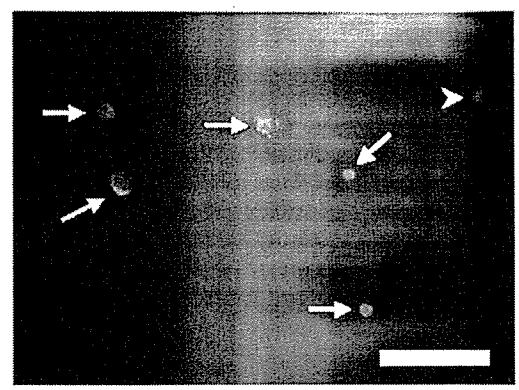

Fig. 1 – Immunocytochemical images of cultured RGCs. This is a photomicrograph of Thy-1.1 labeling. Thy-1.1 positive RGCs were stained green by Alexa Fluor® 488 and their nuclei were stained red by propidium iodide (arrows). Thy-1.1 negative cell was stained by propidium iodide only (arrowhead). Cultured RGCs showed a high immunopositive rate for Thy-1.1. Scale bar, 50 μm.

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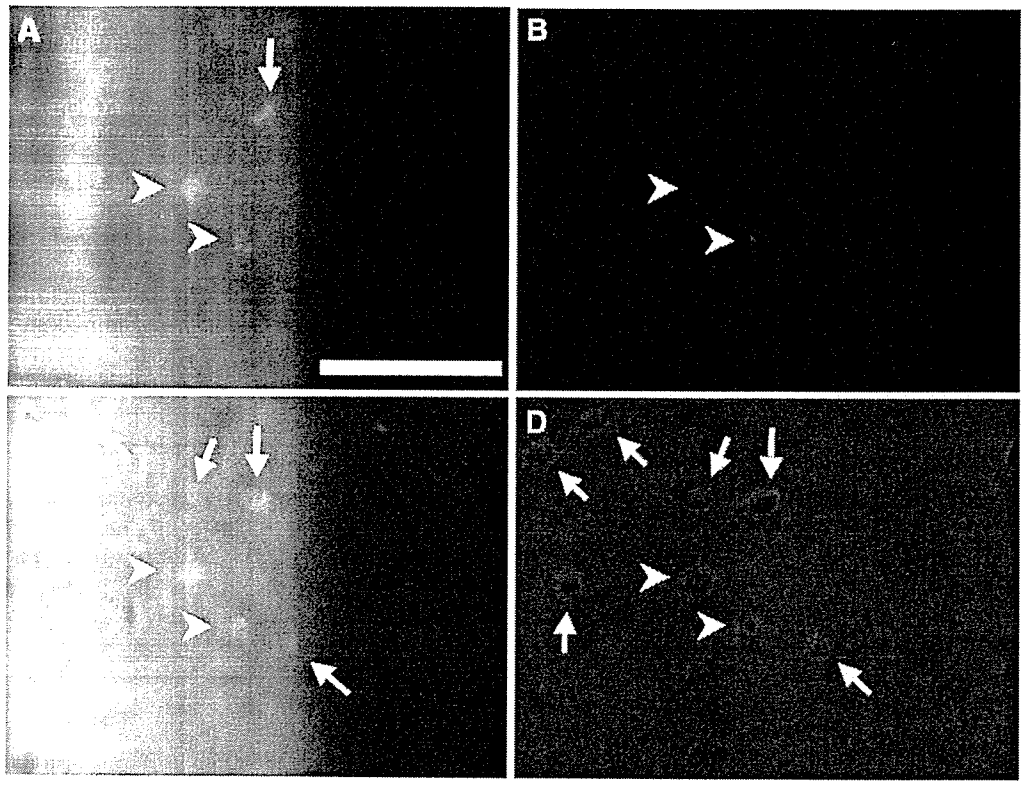

Fig. 2 – Fluorescein-labeled and phase-contrast photomicrographs of purified RGCs after hypoxic stimuli. After 2-h hypoxic stimuli, cultured RGCs were stained by annexin V, propidium iodide and Hoechst 33342 under blue (A), green (B), and ultraviolet light (C) excitation, respectively. Annexin V labeled RGCs (green) can be observed in plate A and propidium iodide labeled RGCs (red) can be observed in plate B. Hoechst 33342 stained RGCs (blue green) can be visualized in plate C. In plates A and B, both annexin V+/propidium iodide– apoptotic RGCs (white arrow) and annexin V+/propidium iodide+ necrotic RGCs (white arrowheads) were detectable. In plate C, Annexin V–/propidium iodide–/Hoechst 33342+ RGCs were observed and regarded as living cells (yellow arrows). Plate D is the phase-contrast image of the same field and shows the morphology of isolated RGCs. Scale bar, 50 μm.

2.3. *Effects of glutamate receptor antagonists (Fig. 4)*

After 12 h of hypoxia stress, cell viability was reduced to 74.8% in the non-treated control group. The viability of the DNQX- and MK-801-treated groups was 73.0% and 72.9% after a 12-h incubation, respectively. There was no significant difference between the non-treated control, DNQX, and MK-801-treated groups (Fig. 4).

2.4. *Effect of VPTLK, a Bax-inhibiting peptide (Fig. 5)*

Fig. 5 shows the protective effects of VPTLK (Bax-inhibiting peptide) on hypoxia-induced RGC damage. The viability of RGC cultures after 12 h of hypoxia was 71.2% without VPTLK treatment while viability increased in a dose-dependent manner with exposure to VPTLK (10^{-5} M: 77.1%, 5×10^{-5} M: 83.7%, 2×10^{-4} M: 89.0%, $n=8$). The addition of VPTLK showed a significant neuroprotective effect at concentrations of 5×10^{-5} M and 2×10^{-4} M ($p<0.05$ vs. control by Dunnet test). On the other hand, the negative control peptide of Bax-inhibiting peptide, KLPVM, showed no protective effect on hypoxia-induced cell damage of the purified RGCs.

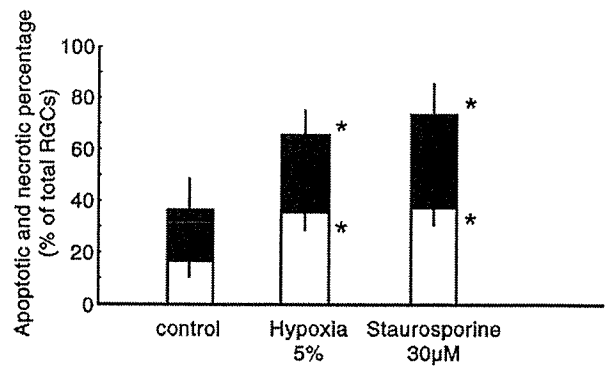

Fig. 3 – Detection of apoptotic and necrotic RGCs after hypoxia stimuli or staurosporine induction. Purified RGCs were plated on coverslips and cultured for 2 h. The white part of each bar represents apoptotic while the black part represents necrotic. Each value represents mean±S.D. ($n=8$). Asterisk indicates $p<0.05$ vs. control (Dunnet test).

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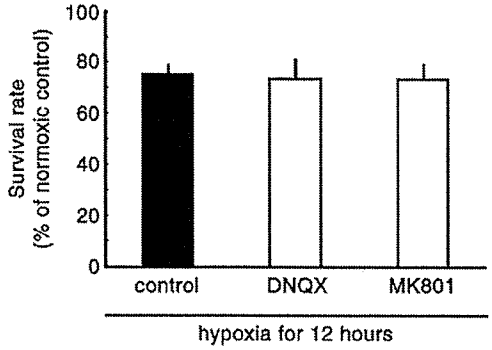

Fig. 4 – Effects of DNQX and MK-801 on hypoxia-induced cell damage of purified RGCs. Each value represents mean±S.D. (n=5). No significant difference was observed between the hypoxia-induced and DNQX- and MK-801 treated groups.

2.5. *Effects of betaxolol, nipradilol, timolol and carteolol (Fig. 6)*

After 12 h of hypoxia stress, cell viability increased in a dose-dependent manner with exposure to betaxolol (0M: 72.9%, 10^{-8} M: 77.4%, 10^{-7} M: 83.3%, 10^{-6} M: 86.7%, n=8), nipradilol (0 M: 72.7%, 10^{-8} M: 80.7%, 10^{-7} M: 82.6%, 10^{-6} M: 86.3%, n=8), and timolol (0 M: 73.3%, 10^{-8} M: 78.4%, 10^{-7} M: 82.2%, 10^{-6} M: 83.3%, n=8). Betaxolol, nipradilol, and timolol showed a significant neuroprotective effect at the concentrations of 10^{-7} M, 10^{-8} M, and 10^{-7} M, respectively (p<0.05 vs. control by Dunnet test). Carteolol showed no significant effect on RGC viability at all concentrations examined (0M: 67.3%, 10^{-8} M: 69.0%, 10^{-7} M: 70.1%, 10^{-6} M: 67.2%, n=8) (Fig. 6).

2.6. *Effects of concomitant incubation with c-PTIO and nipradilol (Fig. 7)*

To clarify the mechanism of the neuroprotective effect of nipradilol on hypoxia-induced RGC damage, the influence of c-PTIO, a NO scavenger, was studied. c-PTIO (10^{-5} M) partially inhibited the neuroprotective effect of 10^{-6} M nipradilol on hypoxia-induced RGC damage. After 12 h of hypoxia stress, cell viability of the non-treated control group was reduced to 75.1%. However, the viability of nipradilol-treated and nipradilol+c-PTIO group was 89.4% and 82.5%, respectively (vs. control, p<0.05 by Dunnet test, n=12). The viability of the nipradilol+c-PTIO group was significantly lower than that of the nipradilol group (p<0.05 by Tukey test). On the other hand, the viability of the 10^{-5} M c-PTIO group as a negative control was 74.2% (vs. control, NS, n=12). These results suggested that the effect of nipradilol against hypoxia-induced RGC damage is partially suppressed by c-PTIO, a NO scavenger (Fig. 7).

2.7. *$[Ca^{2+}]i$ change in RGCs after 6-h hypoxia and the effect of beta-adrenergic antagonists (Fig. 8)*

$[Ca^{2+}]_i$ was significantly increased in the hypoxia group while nimodipine (10^{-6} M) inhibited the increase of $[Ca^{2+}]_i$. At a concentration of 10^{-6} M, all other β-receptor antagonists tested showed no influence on $[Ca^{2+}]_i$ levels after 6 h in hypoxic conditions (Fig. 8).

3. Discussion

Our results show that even if under normoxic conditions, 16.8% of the cultured RGCs are apoptotic and 19.9% are necrotic. Spontaneous cell deaths of purified RGCs can be observed in our study and both apoptosis and necrosis contribute to these spontaneous cell deaths. The trend of progressive decrease in numbers of purified RGCs should be taken into consideration if we want to use this model to investigate the long-term effects of any neuroprotective agents. The present study revealed that the percentages of apoptotic cells in RGCs under hypoxia was higher than of that in RGCs under normoxia, and similar to that of the staurosporine-treated cultured RGCs. This observation provides evidence that hypoxia-induced RGC death involves the apoptotic pathway.

Ischemia–hypoxia is implicated in the development of glaucoma (Chung et al., 1999; Costa et al., 2003; Drance et al.,

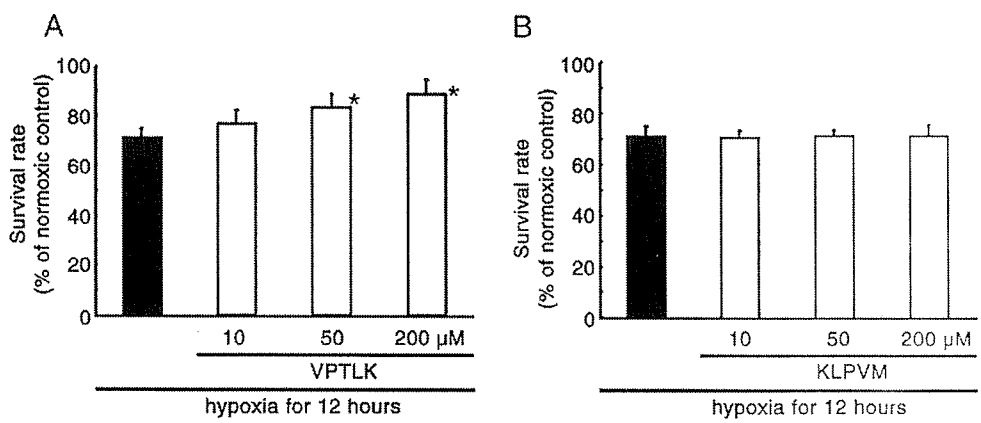

Fig. 5 – Effects of VPTLK, the Bax-inhibiting peptide and KLPVM, the negative control peptide, against hypoxia-induced cell damage of purified RGCs. RGC viability increased in a dose-dependent manner with exposure to VPTLK. Each value represents mean±S.D (n=8). Asterisk indicates p<0.05 vs. hypoxia control (black bar) by Dunnet test.

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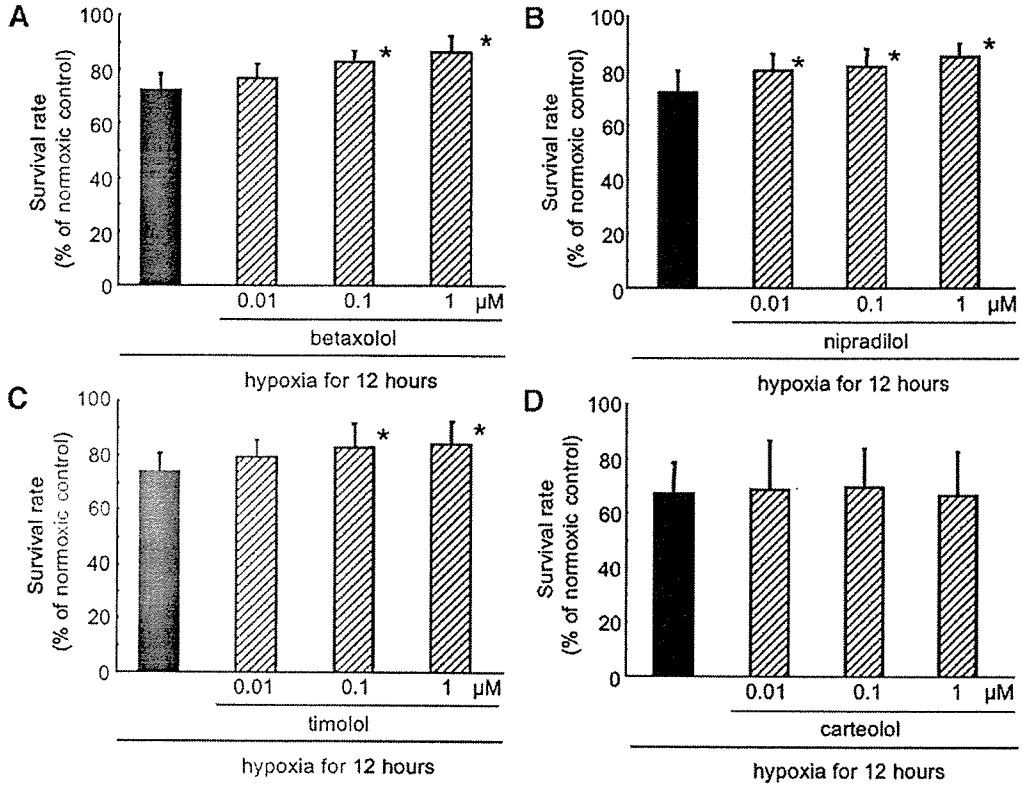

Fig. 6 – Effects of betaxolol, nipradilol, timolol and carteolol against hypoxia-induced cell damage of purified RGCs. RGC viability increased in a dose-dependent manner with exposure to betaxolol, nipradilol and timolol but not to carteolol. Each value represents mean ± S.D. (n=8). Asterisk indicates $p<0.05$ vs. hypoxia control (black bar) by Dunnet test.

1988; Flammer, 1994; Osborne et al., 1999b; Tielsch et al., 1995; Tezel and Wax, 2004), and hypoxia-induced release of glutamate from isolated retina or cultured retinal cells has been reported (Neal et al., 1994; Ohia et al., 2001; Rego et al., 1996). However, the primary role of glutamate and NMDA receptors in glaucoma is still open to debate. Some researchers

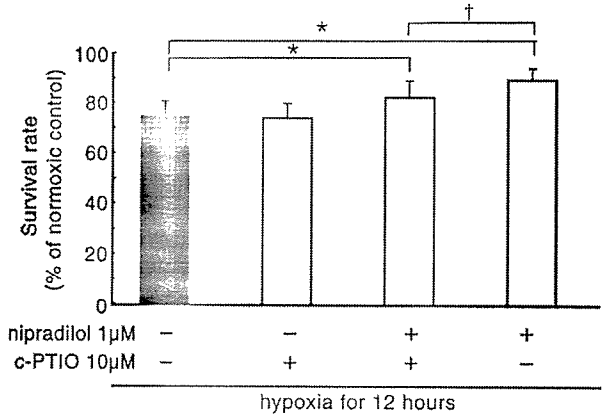

Fig. 7 – Effects of concomitant incubation with c-PTIO and nipradilol on hypoxia-induced cell damage of purified RGCs. Four groups were prepared: nipradilol (10^{-6} M), nipradilol (10^{-6} M)+c-PTIO (10^{-5} M), c-PTIO (10^{-5} M) and non-treated control. C-PTIO alleviated the neuroprotective effects of nipradilol. Each value represents mean ± S.D. (n=12). *$p<0.05$ vs. hypoxia control (black bar) (Dunnet test). †$p<0.05$ (Tukey test).

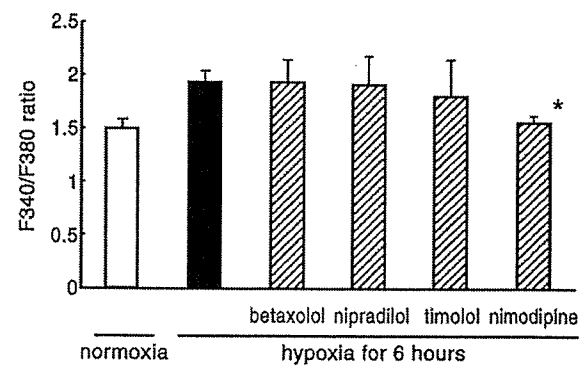

Fig. 8 – Changes in $[Ca^{2+}]_i$ after 6 h of hypoxic stress and the effects of 10^{-}super 6 M betaxolol, nipradilol, timolol and nimodipine. After hypoxic incubation for 6 h, $[Ca^{2+}]_i$ increase was noted in purified RGC cultures. Nimodipine (a calcium channel blocker) inhibited this $[Ca^{2+}]_i$ increase while β-receptor antagonists showed no influence on the increase in $[Ca^{2+}]_i$ after 6 h in hypoxic conditions. Each value represents mean ± S.D. (n=5). *$p<0.05$ vs. hypoxia control (black bar) (Dunnet test).

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reported no difference in vitreous glutamate concentrations between patients with glaucoma and control subjects (Carter-Dawson et al., 2002; Honkanen et al., 2003). Conflicting results have also been obtained with rat models of ischemia (Lagreze et al., 1998) optic nerve crush (Yoles and Schwartz, 1998) and experimental glaucoma (Levkovitch-Verbin et al., 2002). Our results showed no significant effect of DNQX and MK-801 on hypoxia-induced RGC death, which implied that the mechanism of hypoxia-induced RGC death in the current in vitro model is mostly independent of excitotoxicity through glutamate receptors. However, in vivo, tissue glutamate levels may increase by release from other neuronal or glial cells (Kageyama et al., 2000), or dysfunction of glutamate uptake by glial cells (Vorwerk et al., 2000). The contribution of glutamate to hypoxic RGC death in vivo awaits future investigation.

Our study showed that BIP had protective effects against hypoxia-induced RGC death. Since Bax is known to activate the mitochondrial-dependent (intrinsic) cell death pathway (Kroemer and Martin, 2005), the current study indicated that hypoxia-induced RGC death should involve mitochondrial-dependent apoptosis. This result is consistent with a previous report that mitochondrial dysfunction is involved in RGC death induced by hypoxia (Tezel and Yang, 2004). Since BIP can counteract hypoxia-induced RGC death and readily penetrate cell membranes, it may also have the potential to be used as a therapeutic agent in neurodegenerative diseases, including glaucoma.

In recent years, the neuroprotective effect of beta-adrenergic antagonists has attracted researchers' interests. The antagonists currently examined were composed of selective (betaxolol) and non-selective (nipradilol, timolol, carteolol) beta-adrenergic receptor antagonists. Although it is possible for betaxolol or timolol to exert their neuroprotective actions by suppressing intracellular calcium increase in RGCs, the IC_{50} value was as high as 235.7 μM for betaxolol (Zhang et al., 2003). These concentrations are 10^3 to 10^4 times higher than 0.1 μM, the concentration at which betaxolol and timolol were currently found to show protective effects against hypoxia-induced RGC death. The $[Ca^{2+}]_i$ change in RGCs under hypoxia stress currently observed also indicated that the effects of betaxolol, nipradilol or timolol were independent of $[Ca^{2+}]_i$ change. Previous researchers attributed betaxolol's neuroprotective effects to its blocking potency on sodium channels (Hirooka et al., 2000) or the ability to stimulate production of BDNF (Wood et al., 2001). However, the IC_{50} value that betaxolol blocks sodium channels was reported to be 9.8 μM (Osborne et al., 2004), which is about 10^2 times higher than 0.1 μM, the concentration at which betaxolol was currently found to protect against hypoxia-induced RGC death. Therefore, the neuroprotective effects of betaxolol in this study can not be attributed to its calcium channel blocking or to its sodium channel blocking activity. Whether the neuroprotective effects currently observed are related to production of BDNF needs further investigation.

Nipradilol is a nonselective beta and selective α_1-adrenergic antagonist and previous reports showed that it has neuroprotective effects on glutamate induced RGC death in vitro (Kashiwagi et al., 2002). Nipradilol can generate NO from a nitroxy residue (Ohira et al., 1985; Okamura et al., 1996; Shirasawa et al., 1985; Uchida et al., 1983). The current study showed that nipradilol improved the survival rate of cultured RGCs in vitro, and that c-PTIO partially inhibited it, indicating that NO generated from nipradilol may partly accounts for neuroprotective effects of this compound. The present data showed that the neuroprotective effects of nipradilol could not be attributed to its blocking of calcium channels. Other α_1-adrenergic antagonists (such as bunazosin hydrochloride, prazosin hydrochloride, naftopidil dihydrochloride) were also reported to be protective to rat primary retinal cultures against glutamate-induced cell death at concentrations of 10^{-6} M or higher (Goto et al., 2004). However, it is difficult to attribute the neuroprotective effects of nipradilol to the α_1 adrenoceptor antagonistic ability because nipradilol's α_1-adrenoceptor antagonistic activity is not evident at a concentration between 10^{-9} and 10^{-7} M (Okamura et al., 2002). Thereafter, neuroprotective mechanisms of nipradilol need further investigation.

Timolol shows some affinity for potassium channels, sodium channels, calcium channels and GABA A receptors (Chidlow et al., 2000; Goto et al., 2002; Osborne et al., 2004). Although previous reports suggested that the inhibitory action of timolol on sodium or calcium channels could explain its neuroprotective effect (Chidlow et al., 2000), the IC_{50} value of timolol to block sodium channels is 79.7 μM (Osborne et al., 2004), which is 10^3 times higher than 0.1 μM, the concentration at which timolol was currently found to protect against hypoxia-induced RGC death. Its neuroprotective effects are unlikely to be relevant to its sodium channel blocking ability. As the results show, it is also unlikely that calcium channel blocking effects are responsible for the neuroprotective effect of timolol in the current model.

There is no evidence showing that β-adrenoceptors are expressed in RGCs. Although our findings suggested that some of the β-adrenergic antagonists partly inhibited hypoxia-induced RGC death, their neuroprotective effects are probably irrelevant to β-adrenoceptor receptors, since carteolol, a nonselective β-adrenergic antagonist, showed no effect.

The current study showed that under hypoxic stress, betaxolol, nipradilol, and timolol could improve the survival rates of purified RGCs in culture at a concentration of 10^{-8} M for nipradilol and 10^{-7} M for betaxolol and timolol. Several previous studies showed that the concentrations of betaxolol, nipradilol or timolol in normal rabbit retina could reach 10^{-7} M when these agents were topically applied (Goto et al., 2002; Mizuno et al., 2001; Osborne et al., 1999a). There may be the possibility that, in addition to their IOP-lowering effects, these 3 drugs can directly and favorably affect RGCs in vivo, although clinical trials aiming to demonstrate the favorable effects of betaxolol on the visual field of glaucoma eyes were yielded inconclusive results (Drance, 1998; Kaiser et al., 1994; Messmer et al., 1991).

In summary, purified RGC death induced by hypoxia involves the apoptotic pathway and is mostly independent of excitotoxicity through glutamate receptors. Since BIP was protective against hypoxia-induced RGC death, Bax-mediated apoptosis is thought to play an important role in hypoxia-induced damage. Betaxolol, nipradilol and timolol showed protective effects against hypoxia-induced RGC death in purified RGCs at a concentration range that can be achieved in the posterior retina of normal rabbits after their ipsilateral

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instillation. These effects could not be attributed to either to the calcium channel blocking activity or to β-adrenoceptor antagonistic activity.

4. Experimental procedures

4.1. *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.

4.2. *Materials*

Cell culture reagents were obtained from Gibco (Grand Island, NY). Calcein AM was obtained from Sigma (St. Louis, MO). 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. Betaxolol, nipradilol, timolol and carteolol were kindly provided by Alcon Japan (Tokyo, Japan), Kowa (Nagoya, Japan), Merck (Rahway, NJ) and Otsuka Pharmaceutical Co (Tokyo, Japan), respectively. 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). Bax-inhibiting peptide (BIP), VPTLK (H-Val-Pro-Thr-Leu-Lys-OH), and its negative control peptide, KLPVM (H-Lys-Leu-Pro-Val-Met-OH), were provided by the Department of Medicine, Case Western Reserve University (Cleveland, OH). C-PTIO (2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium salt), was obtained from Dojindo Molecular Technologies (Gaithersburg, MD).

4.3. *Purification and culture of RGCs*

RGCs were purified by a two-step immunopanning procedure as described previously (Otori et al., 1998; Uchida et al., 2003; Yamada et al., 2006). Briefly, the dissociated retinal cells from 6- to 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 then incubated in tubes (Corning, Acton, MA) coated with an anti-rat Thy-1.1 monoclonal antibody (1:300). RGCs adherent to the tubes were collected by centrifugation at 600 rpm for 5 min and 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 1000 cells/well. RGCs were cultured in serum-free B27 complete medium containing neurobasal medium (Invitrogen) with 1 mM L-glutamine (Sigma), B27 supplement (Invitrogen), 40 ng/mL human recombinant brain-derived neurotrophic factor (BDNF), 40 ng/mL rat recombinant ciliary neurotrophic factor (CNTF), 10 μM forskolin (Sigma), 100 U/mL penicillin, and 100 μg/mL streptomycin. Plates were incubated in a tissue culture incubator with humidified atmosphere containing 5% CO_2 and 95% air at 37 °C for 3 days. Hypoxia was achieved by placing the plates in a dedicated culture incubator with a controlled flow of N_2 and CO_2 at a setting of 5% CO_2 and 5% O_2 (Yamada et al., 2006).

4.4. *Immunocytochemical identification of cultured RGCs*

We used monoclonal IgG_1 antibody (OX-7) against rat and mouse Thy-1.1 as the primary antibody. Alexa Fluor® 488 goat anti-mouse IgG (H+L) purchased from Molecular Probes was used to detect the Thy-1.1 immunolabeled cultured cells. After RGCs were cultured for 3 days, they were washed with phosphate-buffered saline (PBS), fixed with 10% formaldehyde solution (Wako Pure Chemical Industries, Ltd., Osaka, Japan) for 10 min, washed 3 times with PBS, and blocked with 1% bovine serum albumin (Sigma) in PBS for 30 min. Then, RGCs were incubated for 60 min at room temperature with primary antibody diluted 200 times by PBS. After being washed three times with PBS, cells were incubated for 30 min at room temperature with Alexa Fluor® 488 goat anti-mouse IgG, and again washed three times with PBS. Finally, propidium iodide with a final concentration of 1.5 μM was added to stain the nuclei of the cells. Thy-1.1 positive RGCs were stained green by Alexa Fluor® 488 and their nuclei are stained red by propidium iodide. Thy-1.1 negative cells are stained red by propidium iodide. Cells were observed and counted in at least 5 random fields of 10 wells at 200× magnification using a fluorescence microscope (Nikon Eclipse TE300, Nikon Engineering Co., Tokyo, Japan). The immunopositive rate for Thy-1.1 was calculated.

4.5. *Detection of apoptotic and necrotic RGCs after hypoxic stimuli*

We used the Vybrant® Apoptosis Assay Kit to quantify the extent of apoptosis in RGCs that suffered from hypoxic stress (Schutte et al., 1998; Tezel and Yang, 2004). RGCs were incubated under hypoxic conditions (5% O_2, 5% CO_2, 37 °C) for 2 h 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 stains (Schutte et al., 1998; Tezel and Yang, 2004). At the end of the above double staining, Hoechst 33342 was added to the culture medium at 8 μM. Cells were counted in at least 10 random fields of 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. In addition, 2 control groups of RGCs of an identical passage were prepared simultaneously and cultured in normoxic conditions (95% air and 5% CO_2). Staurosporin (final concentration of 30 μM) was

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added to the medium of one group but not to the other. After a 2-h incubation in normoxic conditions, the percentages of apoptotic RGCs and necrotic RGCs were also quantified using the method described above.

4.6. *Assay of RGC survival*

The viable cells were stained with 1 μM calcein AM as previously described (Otori et al., 1998; Tezel and Wax, 2000; Uchida et al., 2003; Yamada et al., 2006). All surviving RGCs on each glass coverslip were counted at 200× magnification under an inverted fluorescence microscope. 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 two cell diameters from the cell body. Fluorescent images of stained RGCs were captured using a fluorescence microscope (Nikon Eclipse TE300). In each experiment, a control group of RGCs of an identical passage was prepared simultaneously and cultured in normoxic conditions (95% air and 5% CO_2). The number of RGCs after 3 days + 12 h culture in normoxic conditions was set at 100%. The percentage of surviving RGCs after hypoxic stress was calculated and normalized to the normoxic group. Each percentage is expressed in the text and figures as the mean ±standard deviation (S.D.). Compounds evaluated in the present study are described below. A non-treated group under hypoxia served as a control.

4.6.1. *Effects of glutamate receptor antagonists*

AMPA-KA receptor-selective antagonist, DNQX, or *N*-methyl-D-aspartate (NMDA) receptor antagonist, MK 801, was added to the culture medium at the final concentration of 10^{-5} M, and cell viabilities after 12 h of hypoxic stress were evaluated. This concentration of antagonists was found to completely inhibit their respective receptor subtypes (Kubrusly et al., 1998; Munsch and Deitmer, 1997; Zeevalk and Nicklas, 1990).

4.6.2. *Effects of VPTLK, a Bax-inhibiting peptide, on hypoxia-induced RGC damage*

For every adherent cell, 1–3 h of pre-incubation is necessary to observe the effects of BIP (Sawada et al., 2003a,b; Yoshida et al., 2004). Thus, we incubated the RGCs with VPTLK (0 M, 10^{-5} M, 5×10^{-5} M, 2×10^{-4} M) or KLPVM (0 M, 10^{-5} M, 5×10^{-5} M, 2×10^{-4} M), the negative control peptide, at 37 °C in a humidified atmosphere containing 5% CO_2 (normoxic conditions) for 2 h before the RGCs were put in hypoxic stress. After 12 h of hypoxia stress, the viability of each group was evaluated.

4.6.3. *Effects of beta-adrenergic antagonists on hypoxia-induced RGC damage*

At final drug concentrations of 0 M, 10^{-8} M, 10^{-7} M, and 10^{-6} M respectively, betaxolol, nipradilol, timolol or carteolol was added to the culture medium, and RGCs were incubated under hypoxic conditions (5% O_2, 5% CO_2, 37 °C) for 12 h. The survival rate of each group was then calculated.

4.6.4. *Effects of concomitant incubation with c-PTIO and nipradilol on hypoxia-induced RGC death*

Since one of the possible neuroprotective mechanisms of nipradilol is NO releasing action (Kashiwagi et al., 2002; Mizuno et al., 2001) while the other beta-adrenergic antagonists do not have such potency, a NO scavenger, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium salt (c-PTIO), was added to the culture medium of purified RGCs. In accordance with previous reports (Kashiwakura et al., 1998; Kawasaki et al., 1999; Parsons et al., 1999) and preliminary results, it was determined that a concentration of 10^{-5} M c-PTIO was sufficient to scavenge NO generated by nipradilol without influencing the RGCs. Four groups were prepared: 1) nipradilol (10^{-6} M); 2) nipradilol (10^{-6} M) + c-PTIO (10^{-5} M); 3) c-PTIO (10^{-5} M); and 4) control. Because c-PTIO is easily dissolved in the culture medium, the cultures without c-PTIO and nipradilol were used for control samples. After 12 h of hypoxia stress, the viability of each group was evaluated.

4.7. *$[Ca^{2+}]i$ imaging*

Hypoxia-induced changes in intracellular calcium concentration ($[Ca^{2+}]_i$) were evaluated by calcium-imaging techniques. With a final drug concentration of 10^{-6} M, betaxolol, nipradilol, timolol or nimodipine, a Ca^{2+} channel antagonist, were added to the culture medium and RGCs were then incubated under hypoxic conditions (5% O_2, 5% CO_2, 37 °C). Since $[Ca^{2+}]_i$ of neuronal cells will increase in only a few hours under ischemic conditions (Won et al., 2002), we exposed the purified RGCs to hypoxic stress for 6 h. The normoxic group was prepared using an identical passage of RGCs and incubated in a regular incubator with 95% air and 5% CO_2 also for 6 h. After being cultured under hypoxic or normoxic conditions for 5 h, purified RGC coverslips were moved to Hepes-Tyrode Buffer medium (composition in mM: 140.0 NaCl, 2.7 KCl, 1.0 $CaCl_2$, 12.0 $NaHCO_3$, 5.6 D-glucose, 0.37 NaH_2PO_4, and 25.0 HEPES, pH 7.4 at 25 °C) containing 0.1% BSA and 5 μM fura-2/AM, a fluorescent Ca^{2+} indicator. These RGCs were incubated under hypoxic (5% O_2, 5% CO_2, 37 °C) or normoxic (5% CO_2, 95% air, 37 °C) conditions for 40 min. Cells were subsequently washed three times with the Hepes-Tyrode Buffer to remove the dye, and then left under hypoxic (5% O_2, 5% CO_2, 37 °C) or normoxic (5% CO_2, 95% air, 37 °C) conditions for a further 20 min before Ca^{2+} imaging was performed. Fluorescence microscopy was performed at 25 °C. Emission fluorescence of fura-2 was imaged using a fluorescent objective lens (Nikon Plan Fluor 10×). The fura-2 absorption shift that occurred upon binding was determined by scanning the excitation spectra between 340 and 380 nm while monitoring emission at 510 nm. The resultant fluorescent images were analyzed from individual cells with a fluorescence analyzer (Argus 50, Hamamatsu Photonics, Shizuoka, Japan) using an ultrahigh sensitivity television camera (CCD). The fluorescence ratio (F340/F380) was obtained by dividing the 340-nm by the 380-nm images on a pixel-by-pixel basis, after background subtraction. Bright and round fluorescent spots with an area of 40–300 mm^2 were considered RGCs. The mean ratio over the area of individual RGCs that were separated from the edge of other RGCs was measured. Approximately 60 RGCs were analyzed in each experimental group.

4.8. *Statistical analysis*

Results are expressed as the mean ± S.D. Data were analyzed using one-way analysis of variance followed by the Dunnet test, or the Tukey test, with $p<0.05$ indicating significance.

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REFERENCES

Acheampong, A.A., Shackleton, M., John, B., Burke, J., Wheeler, L., Tang-Liu, D., 2002. Distribution of brimonidine into anterior and posterior tissues of monkey, rabbit, and rat eyes. Drug Metab. Dispos. 30, 421–429.

Carter-Dawson, L., Crawford, M.L., Harwerth, R.S., Smith III, E.L., Feldman, R., Shen, F.F., Mitchell, C.K., Whitetree, A., 2002. Vitreal glutamate concentration in monkeys with experimental glaucoma. Invest. Ophthalmol. Visual Sci. 43, 2633–2637.

Chidlow, G., Melena, J., Osborne, N.N., 2000. Betaxolol, a beta(1)-adrenoceptor antagonist, reduces Na(+) influx into cortical synaptosomes by direct interaction with Na(+) channels: comparison with other beta-adrenoceptor antagonists. Br. J. Pharmacol. 130, 759–766.

Chung, H.S., Harris, A., Evans, D.W., Kagemann, L., Garzozi, H.J., Martin, B., 1999. Vascular aspects in the pathophysiology of glaucomatous optic neuropathy. Surv. Ophthalmol. 43, S43–S50.

Costa, V.P., Harris, A., Stefansson, E., Flammer, J., Krieglstein, G.K., Orzalesi, N., Heijl, A., Renard, J.P., Serra, L.M., 2003. The effects of antiglaucoma and systemic medications on ocular blood flow. Prog. Retin. Eye Res. 22, 769–805.

Drance, S.M., 1998. A comparison of the effects of betaxolol, timolol, and pilocarpine on visual function in patients with open-angle glaucoma. J. Glaucoma 7, 247–252.

Drance, S.M., Douglas, G.R., Wijsman, K., Schulzer, M., Britton, R.J., 1983. Response of blood flow to warm and cold in normal and low-tension glaucoma patients. Am. J. Ophthalmol. 15, 37–39.

Flammer, J., 1994. The vascular concept of glaucoma. Surv. Ophthalmol. 38, S3–S6.

Goto, W., Otori, Y., Morikawa, H., Otori, Y., Hara, H., Kawazu, K., Mizuno, K., Tano, Y., 2002. Protective effects of timolol against retinal damage induced by glutamate and ischemia in the rat retina. Brain Res. 958, 10–19.

Goto, W., Morikawa, M., Tanaka, E., Hara, H., Araie, M., 2004. Bunazosin hydrochloride reduces glutamate-induced neurotoxicity in rat primary retinal cultures. Brain Res. 1003, 119–127.

Gross, R.L., Peachey, S.H., Gao, F., Yang, X.L., Dai, S.C., Wu, S.M., 2[illegible] timolol on light responses and membrane conductance in retinal ganglion cells. Invest. Ophthalmol. Visual Sci. 41, 727–728.

Hirooka, K., Tokuda, M., Baldridge, W.H., Barnes, S., 2000. Suppressive effects of betaxolol on ionic currents in retinal ganglion cells may explain its neuroprotective effects. Exp. Eye Res. 70, 611–621.

Honkanen, R.A., Tsuda, S., Zimmerman, M.B., Khanna, C.L., Weaver, Y.K., Hedlewicz, J., Wazid, R., Gehrs, K.M., Weingeist, T.A., Polk, H.C., Folk, J.C., Russell, S.R., Kwon, Y.H., 2003. Vitreous amino acid concentrations in patients with glaucoma undergoing vitrectomy. Arch. Ophthalmol. 121, 183–188.

Ichikawa, M., Ishida, Y., Asai, Y., Hara, H., Ishii, K., Araie, M., 2004. Effects of topically instilled bunazosin, an alpha1-adrenoceptor antagonist, on constrictions induced by phenylephrine and ET-1 in rabbit retinal arteries. Invest. Ophthalmol. Visual Sci. 45, 4041–4048.

Ishii, K., Matsuo, H., Fukaya, Y., Tanaka, S., Sakaki, H., Waki, M., Araie, M., 2003. Iganidipine, a new water-soluble Ca2+ antagonist: ocular and periocular penetration after instillation. Invest. Ophthalmol. Visual Sci. 44, 1169–1177.

Kageyama, T., Ishikawa, A., Tamai, M., 2000. Glutamate elevation in rabbit vitreous during transient ischemia–reperfusion. Jpn. J. Ophthalmol. 44, 110–114.

Kaiser, H.J., Flammer, J., Stumpfig, D., Hendrickson, P., 1994. Long term visual field follow-up of glaucoma patients treated with beta-blockers. Surv. Ophthalmol. 38, S156–S159 (discussion S160).

Kashiwagi, K., Iizuka, Y., Tsukahara, S., 2002. Neuroprotective effects of nipradilol on purified cultured retinal ganglion cells. J. Glaucoma 11, 231–238.

Kashiwakura, I., Kuwabara, M., Murakami, M., Hayase, Y., Takagi, Y., 1998. Effect of carboxy-PTIO, a nitric oxide scavenger, on the proliferation of murine hematopoietic progenitor cells in vitro. Res. Commun. Mol. Pathol. Pharmacol. 99, 329–337.

Kawasaki, Y., Fujikado, T., Hosohata, J., Tano, Y., Tanaka, Y., 1999. The effect of nitric oxide on the contractile tone of Muller cells. Ophthalmic Res. 31, 387–391.

Kroemer, G., Martin, S.J., 2005. Caspase-independent cell death. Nat. Med. 11, 725–730.

Kubrusly, R.C., de Mello, M.C., de Mello, F.G., 1998. Aspartate as a selective NMDA receptor agonist in cultured cells from the avian retina. Neurochem. Int. 32, 47–52.

Lagreze, W.A., Knorle, R., Bach, M., Feuerstein, T.J., 1998. Memantine is neuroprotective in a rat model of pressure-induced retinal ischemia. Invest. Ophthalmol. Visual Sci. 39, 1063–1066.

Levkovitch-Verbin, H., Martin, K.R., Quigley, H.A., Baumrind, L.A., Pease, M.E., Valenta, D., 2002. Measurement of amino acid levels in the vitreous humor of rats after chronic intraocular pressure elevation or optic nerve transection. J. Glaucoma 11, 396–405.

Li, Y., Schlamp, C.L., Poulsen, K.P., Nickells, R.W., 2000. Bax-dependent and independent pathways of retinal ganglion cell death induced by different damaging stimuli. Exp. Eye Res. 71, 209–213.

Messmer, C., Flammer, J., Stumpfig, D., 1991. Influence of betaxolol and timolol on the visual fields of patients with glaucoma. Am. J. Ophthalmol. 112, 678–681.

Mizuno, K., Koide, T., Yoshimura, M., Araie, M., 2001. Neuroprotective effect and intraocular penetration of nipradilol, a beta-blocker with nitric oxide donative action. Invest. Ophthalmol. Visual Sci. 42, 688–694.

Munsch, T., Deitmer, J.W., 1997. Intracellular Ca2+, Na+ and H+ transients evoked by kainate in the leech giant glial cells in situ. Neurosci. Res. 27, 45–56.

Neal, M.J., Cunningham, J.R., Hutson, P.H., Hogg, J., 1994. Effects of ischaemia on neurotransmitter release from the isolated retina. J. Neurochem. 62, 1025–1033.

Ohia, S.E., Awe, O.S., Opere, C.A., LeDay, A.M., Harris, L.C., Sharif, N.A., 2001. Hypoxia-induced [(3)H]D-aspartate release from isolated bovine retina: modulation by calcium-channel blockers and glutamatergic agonists and antagonists. Curr. Eye Res. 23, 386–392.

Ohira, A., Wada, Y., Fujii, M., Nakamura, M., Kasuya, Y., Hamada, Y., Shigenobu, K., 1985. Effects of nipradilol (K-351) on alpha-adrenoceptor mediated responses in various isolated tissues. Arch. Int. Pharmacodyn. Ther. 278, 61–71.

Okamura, T., Kitamura, Y., Uchiyama, M., Toda, M., Ayajiki, K., Toda, N., 1996. Canine retinal arterial and arteriolar dilatation induced by nipradilol, a possible glaucoma therapeutic. Pharmacology 53, 302–310.

Okamura, T., Fujioka, H., Ayajiki, K., 2002. Effects of nipradilol on

alpha-adrenoceptor function in ocular arteries. Pharmacology 65, 110–118.

Osborne, N.N., Cazevieille, C., Carvalho, A.L., Larsen, A.K., DeSantis, L., 1997. In vivo and in vitro experiments show that betaxolol is a retinal neuroprotective agent. Brain Res. 751, 113–123.

Osborne, N.N., DeSantis, L., Bae, J.H., Ugarte, M., Wood, J.P., Nash, M.S., Chidlow, G., 1999a. Topically applied betaxolol attenuates NMDA-induced toxicity to ganglion cells and the effects of ischaemia to the retina. Exp. Eye Res. 69, 331–342.

Osborne, N.N., Ugarte, M., Chao, M., Chidlow, G., Bae, J.H., Wood, J.P., Nash, M.S., 1999b. Neuroprotection in relation to retinal ischemia and relevance to glaucoma. Surv. Ophthalmol. 43, S102–S128.

Osborne, N.N., Wood, J.P., Chidlow, G., Casson, R., DeSantis, L., Schmidt, K.G., 2004. Effectiveness of levobetaxolol and timolol at blunting retinal ischaemia is related to their calcium and sodium blocking activities: relevance to glaucoma. Brain Res. Bull. 62, 525–528.

Otori, Y., Wei, J.Y., Barnstable, C.J., 1998. Neurotoxic effects of low doses of glutamate on purified rat retinal ganglion cells. Invest. Ophthalmol. Visual Sci. 39, 972–981.

Parsons, A.M., Sorman, J.L., El-Fakahany, E.E., 1999. Regulation of M1 muscarinic receptor-mediated signaling in intact cells by exogenous, but not endogenously produced, nitric oxide. Neurochem. Res. 24, 85–94.

Qin, Q., Patil, K., Sharma, S.C., 2004. The role of Bax-inhibiting peptide in retinal ganglion cell apoptosis after optic nerve transection. Neurosci. Lett. 372, 17–21.

Quigley, H.A., 1999. Neuronal death in glaucoma. Prog. Retin. Eye Res. 18, 39–57.

Rego, A.C., Santos, M.S., Oliveira, C.R., 1996. Oxidative stress, hypoxia, and ischemia-like conditions increase the release of endogenous amino acids by distinct mechanisms in cultured retinal cells. J. Neurochem. 66, 2506–2516.

Sawada, M., Sun, W., Hayes, P., Leskov, K., Boothman, D.A., Matsuyama, S., 2003a. Ku70 suppresses the apoptotic translocation of Bax to mitochondria. Nat. Cell Biol. 5, 320–329.

Sawada, M., Hayes, P., Matsuyama, S., 2003b. Inhibition of Bax-mediated apoptosis by the peptide designed from Bax-biding domain in Ku70. Nat. Cell Biol. 5, 352–357.

Schutte, B., Nuydens, R., Geerts, H., Ramaekers, F., 1998. Annexin V binding assay as a tool to measure apoptosis in differentiated neuronal cells. J. Neurosci. Methods 86, 63–69.

Shirasawa, Y., Fujii, M., Nakamura, M., 1985. Venodilating action of nipradilol (K-351) in the pithed rat pretreated with dihydroergotamine. Jpn. J. Pharmacol. 39, 77–82.

Tezel, G., Wax, M.B., 1999. Inhibition of caspase activity in retinal cell apoptosis induced by various stimuli in vitro. Invest. Ophthalmol. Visual Sci. 40, 2660–2667.

Tezel, G., Wax, M.B., 2000. Increased production of tumor necrosis factor-alpha by glial cells exposed to simulated ischemia or elevated hydrostatic pressure induces apoptosis in cocultured retinal ganglion cells. J. Neurosci. 20, 8693–8700.

Tezel, G., Wax, M.B., 2004. Hypoxia-inducible factor 1alpha in the glaucomatous retina and optic nerve head. Arch. Ophthalmol. 122, 1348–1356.

Tezel, G., Yang, X., 2004. Caspase-independent component of retinal ganglion cell death, in vitro. Invest. Ophthalmol. Visual Sci. 45, 4049–4059.

Tielsch, J.M., Katz, J., Sommer, A., Quigley, H.A., Javitt, J.C., 1995. Hypertension, perfusion pressure, and primary open-angle glaucoma. A population-based assessment. Arch. Ophthalmol. 113, 216–221.

Uchida, Y., Nakamura, M., Shimizu, S., Shirasawa, Y., Fujii, M., 1983. Vasoactive and beta-adrenoceptor blocking properties of 3,4-dihydro-8-(2-hydroxy-3-isopropylamino) propoxy-3-nitroxy-2H-1-benzopyran (K-351), a new antihypertensive agent. Arch. Int. Pharmacodyn. Ther. 262, 132–149.

Uchida, S., Suzuki, Y., Araie, M., Kashiwagi, K., Otori, Y., Sakuragawa, N., 2003. Factors secreted by human amniotic epithelial cells promote the survival of rat retinal ganglion cells. Neurosci. Lett. 341, 1–4.

Vorwerk, CK., Naskar, R., Schuettauf, F., Quinto, K., Zurakowski, D., Gochenauer, G., Robinson, M.B., Mackler, S.A., Dreyer, E.B., 2000. Depression of retinal glutamate transporter function leads to elevated intravitreal glutamate levels and ganglion cell death. Invest. Ophthalmol. Visual Sci. 41, 3615–3621.

Wax, M.B., Tezel, G., 2002. Neurobiology of glaucomatous optic neuropathy: diverse cellular events in neurodegeneration and neuroprotection. Mol. Neurobiol. 26, 45–55.

Won, S.J., Kim, D.Y., Gwag, B.J., 2002. Cellular and molecular pathways of ischemic neuronal death. J. Biochem. Mol. Biol. 35, 67–86.

Wood, J.P., DeSantis, L., Chao, H.M., Osborne, N.N., 2001. Topically applied betaxolol attenuates ischaemia-induced effects to the rat retina and stimulates BDNF mRNA. Exp. Eye Res. 72, 79–86.

Wood, J.P., Schmidt, K.G., Melena, J., Chidlow, G., Allmeier, H., Osborne, N.N., 2003. The beta-adrenoceptor antagonists metipranolol and timolol are retinal neuroprotectants: comparison with betaxolol. Exp. Eye Res. 76, 505–516.

Yamada, H., Chen, Y.N., Aihara, M., Araie, M., 2006. Neuroprotective effect of calcium channel blocker against retinal ganglion cell damage under hypoxia. Brain Res. 1071, 75–80.

Yoles, E., Schwartz, M., 1998. Elevation of intraocular glutamate levels in rats with partial lesion of the optic nerve. Arch. Ophthalmol. 116, 906–910.

Yoshida, T., Tomioka, I., Nagahara, T., 2004. New Bax-inhibiting peptides (BIPs) derived from mouse and rat Ku70. Biochem. Biophys. Res. Commun. 321, 961–966.

Zeevalk, G.D., Nicklas, W.J., 1990. Action of the anti-ischemic agent ifenprodil on *N*-methyl-D-aspartate and kainate-mediated excitotoxicity. Brain Res. 522, 135–139.

Zhang, J., Wu, S.M., Gross, R.L., 2003. Effects of beta-adrenergic blockers on glutamate-induced calcium signals in adult mouse retinal ganglion cells. Brain Res. 959, 111–119.

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