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Muscarinic activation attenuates abnormal processing of β-amyloid precursor protein induced by cobalt chloride-mimetic hypoxia in retinal ganglion cells

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ABSTRACT

β-Amyloid peptide (Aβ), the major pathological factor in Alzheimer's disease, has recently been reported to be implicated in the development of glaucoma. In this study, we explored the effect of muscarinic activation on abnormal processing of β-amyloid precursor protein (APP) induced by a risk factor hypoxia in retinal ganglion cells. Hypoxia mimetic compound cobalt chloride could increase the generation of Aβ via up-regulating the expression of APP as well as the expression of β-secretase and γ-secretase, whereas muscarinic receptor agonist pilocarpine could significantly attenuate this abnormal pathway, thereby resulting in a decreased amyloidogenic cleavage of APP. This finding may provide an insight into better understanding of pathophysiology for the retinal neurodegenerative disease and searching for its new modifying approach.

β-Amyloid peptide (Aβ), the major constituent of senile plaques in Alzheimer's disease (AD), has been reported to be implicated in the development of glaucoma, with evidence of abnormal β-amyloid precursor protein (APP) processing, increased expression of Aβ in retinal ganglion cells (RGCs) in experimental glaucoma [1] and decreased vitreous Aβ levels (consistent with retinal Aβ deposition) in patients with glaucoma [2]. Further evidence of a link between retinal neurodegenerative disease such as glaucoma and AD has emerged from studies showing that patients with AD have RGCs loss associated with typical glaucomatous changes, such as optic neuropathy and visual functional impairment [3–5].

Aβ is derived from APP by sequential proteolytic cleavages from β-site APP-cleaving enzyme 1 (BACE1) and presenilin-1 (PS1), the major proteases for β and γ-cleavage of APP separately. BACE1 is a membrane-bound aspartic protease, which is the rate-limiting enzyme in Aβ production from APP. Alternatively, APP can be cleaved by α-secretase within the Aβ domain to generate non-amyloidogenic soluble APPα (sAPPα). A disintegrin and metalloprotease domain 10 (ADAM10) is the major protease for α-cleavage of APP [6–8]. On the basis of the amyloid hypothesis, the neurodegenerative process comprises a series of events triggered by the abnormal processing of APP. It is well known that the retina is highly sensitive to reduction in oxygen tension and hypoxia is a major risk factor in the pathophysiology of glaucoma [9–11]. However, hypoxia-induced abnormal process of APP in RGCs remains to be identified.

The animal experiment has suggested that cortical cholinergic deafferentation may be the cause of age-related Aβ deposition [12]. In vitro studies have found that muscarinic receptor (M1 and M3) agonists could stimulate the non-amyloidogenic pathway of APP, resulting in increased release of sAPPα or reduced Aβ production [13–15]. The muscarinic receptor agonists were also the first class of agents used for the treatment of glaucoma by improving the trabecular outflow of aqueous fluid and exhibiting neuroprotective and trophic effects on RGCs [16,17]. However, the mechanisms of muscarinic receptor agonists on the regulation of APP metabolism in RGCs have not been defined. Therefore, it is of great interest to investigate the amyloidogenic pathway of APP under glaucoma related pathophysiological condition and pharmacological intervention of muscarinic receptor agonists.

Materials and methods

Treatment of cell cultures. The rat retinal ganglion cells (RGC-5 cells), purchased from American Type Culture Collection (Manassas, VA), which have been previously characterized as expressing ganglion cell markers and exhibiting ganglion cell-like behavior in culture [18]. Cells were cultured in DMEM (Invitrogen Life Technologies, Carlsbad, CA) containing 1 mM glucose, 100 U/ml penicillin/streptomycin (Invitrogen Life Technologies, Carlsbad, CA),

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2 mM glutamine and 10% of heat-inactivated fetal calf serum (Invitrogen Life Technologies, Carlsbad, CA) in a humidified incubator with 5% CO_2 at 37 °C. Cobalt chloride ($CoCl_2$) (Sigma–Aldrich, St. Louis, MO) was dissolved in distilled H_2O and sterilized through a 0.2 μm filter prior to use. After being pretreated with 1 μM pilocarpine (Sigma–Aldrich, St. Louis, MO) for 6 h, the cells were subjected to 200 μM $CoCl_2$ in the presence of 1 μM pilocarpine for 24 h. Treatment with 200 μM $CoCl_2$ was applied to the cultures to cause chemical hypoxia.

Aβ ELISA assay. The supernatants of cultured RGC-5 cells were collected and the concentrations of secreted Aβ peptides (Aβ40 and Aβ42) in conditioned medium were quantified using commercial ELISA kits from Wako Chemicals GmbH (Neuss, Germany), following the manufacturer's protocols.

Western blot analysis. RGC-5 cells were washed twice in phosphate buffered saline and lysed in ice-cold SDS buffer (2% SDS, 30 mM Tris–HCl pH 6.8, 10% glycerol, 2 mM EDTA). Lysates were sonicated and protein concentrations were determined using the BCA Protein Assay Kit (Pierce Chemical, Rockford, IL, USA). Prior to loading, 2.5% 2-mercaptoethanol and 0.0125% bromphenol blue were added and lysates were boiled for 5 min. Proteins were separated by SDS–PAGE and transferred into PVDF membrane (Millipore Billerica, MA). The membranes were incubated overnight at 4 °C with the following primary antibodies: anti-rat-APP antibody (1:500, Chemicon, Temecula, CA), or anti-rat-actin antibody (1:1000, Santa Cruz Biotechnology, Santa Cruz CA, USA) as control. The membranes were incubated with the proper horseradish peroxidase conjugated secondary antibody (1:10,000, Pierce Chemical, Rockford, IL, USA) at room temperature for 1 h. The immunoblots were visualized by using an enhanced chemiluminescence detection kit (Pierce Chemical, Rockford, IL, USA). Relative levels of protein were quantitated by optical density analysis. To avoid interassay variations, values were normalized to the control value in each experiment.

Quantitative real-time polymerase chain reaction. RGC-5 cells were homogenized in Trizol Reagent (Invitrogen Life Technologies, Carlsbad, CA). Total RNA was extracted according to the manufacturer's instruction. SuperScript First-Strand Kit (Invitrogen Life Technologies, Carlsbad, CA) was used to synthesize first strand cDNA from samples with an equal amount of RNA. Real-time PCR was carried out with ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA). Nucleotide sequences of primers are shown in Table 1. APP, BACE1, PS1, ADAM10 mRNA levels were normalized to levels of β-actin. Specificity of the produced amplification product was confirmed by examination of dissociation reaction plots. A distinct single peak indicated that a single DNA sequence was amplified during PCR. Each sample was tested in triplicate and threshold cycle (CT) values were averaged from each reaction. The ΔΔCT method of relative quantification was used to determine the fold change in expression. This was done by first normalizing the resulting CT values of the target mRNAs to the CT values of the internal control β-actin in the same samples ($\Delta CT = CT_{Target} - CT_{\beta\text{-actin}}$). It was further normalized with the control ($\Delta\Delta CT = \Delta CT - CT_{Control}$). The fold change in expression was then obtained ($2^{-\Delta\Delta CT}$). Results obtained from at least three independent experiments were used to calculate the mean ± SEM.

Statistical analysis. The data are presented as the mean ± SEM. The statistical significance of differences between groups was assessed by using a Student–Newman–Keuls test, where $P < 0.05$ indicated a significant difference.

Table 1
Sequences of primers used in the present study.

Name	Sequence
β-Actin forward primer	5′-AATCCTGTGGCATCCATGAA-3′
β-Actin reverse primer	5′-GGACAGTGAGGCCAGGATAGA-3′
APP forward primer	5′-GGACTCTGTGCCAGCCAATAC-3′
APP reverse primer	5′-CCTGAATCATGTCCGAACTCC-3′
BACE1 forward primer	5′-TTGTCACGGCAGACATGGAA-3′
BACE1 reverse primer	5′-CATGAGGCAGAGTGGCAACA-3′
PS1 forward primer	5′-GCGATGATGGTGGCTTCAG-3′
PS1 reverse primer	5′-TCCTGGACAGCAGCTCTTGA-3′
ADAM10 forward primer	5′-GCACCTGTGCCAGCTCTGAT-3′
ADAM10 reverse primer	5′-TCCGACCATTGAACTGCTTGT-3′

Results

Muscarinic activation on Aβ generation in hypoxia-treated RGC-5 cells

As shown in Fig. 1, in RGC-5 cells, hypoxia markedly increased Aβ42 and Aβ40 levels to 252.8 ± 16.2% ($P < 0.01$) and 331.1 ± 24.6% ($P < 0.01$), respectively. With 1 μM pilocarpine pretreatment, the levels of Aβ42 and Aβ40 decreased to 121.1 ± 21.1% and 153.0 ± 14.8%, respectively. And incubation with 1 μM pilocarpine alone could also decrease the levels of Aβ.

Muscarinic activation on the expression of APP in hypoxia-treated RGC-5 cells

Western blot analysis indicated that the exposure to hypoxia resulted in a significant increase in APP expression compared with the control. Pretreatment with 1 μM pilocarpine completely prevented hypoxia-induced increase in APP expression (Figs. 2 and 3).

Muscarinic activation on the mRNA levels of BACE1, PS1 and ADAM10 in hypoxia-treated RGC-5 cells

BACE1 and PS1 but not ADAM10 mRNA levels could significantly increase in hypoxia-treated group. Pretreatment with 1 μM pilocarpine completely prevented hypoxia-induced increase in BACE1 and PS1 mRNA levels. And the incubation with 1 μM pilocarpine alone can decrease the BACE1 mRNA level in RGC-5 cells (Fig. 3).

Discussion

Hypoxia is one of the major risk factors for neurodegenerative diseases such as AD and glaucoma [19–21]. Although several stud-

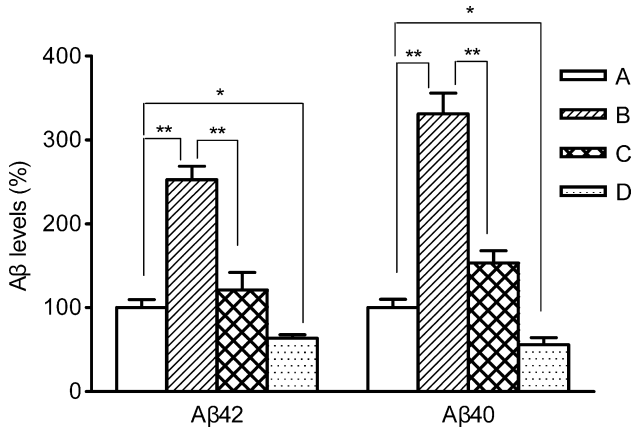

Fig. 1. Muscarinic activation attenuated the overexpression of secreted Aβ induced by hypoxia in RGC-5 cells. Conditioned media were collected and secreted Aβ (Aβ40 and Aβ42) was quantified using commercial ELISA kits. All data represent mean ± SEM of levels relative to that of control from three independent experiments. (A) Control. (B) Cells were treated with 200 μM $CoCl_2$ for 24 h. (C) Cells pretreated with 1 μM pilocarpine for 6 h were incubated with 200 μM $CoCl_2$ in the presence of 1 μM pilocarpine for 24 h. (D) Cells were treated with 1 μM pilocarpine alone for 24 h. $^{*}P < 0.05$, $^{**}P < 0.01$.

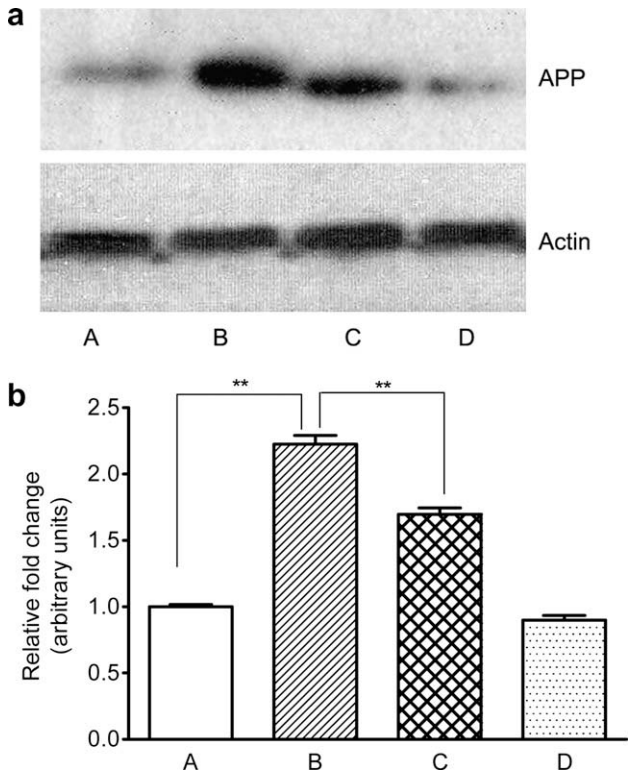

Fig. 2. Muscarinic activation attenuated the expression of APP induced by hypoxia in RGC-5 cells. (a) Immunoreactive bands of APP and Actin. (b) The densitometric quantitation ratio of APP normalized to Actin. (A) Control. (B) Cells were treated with 200 μM $CoCl_2$ for 24 h. (C) Cells pretreated with 1 μM pilocarpine for 6 h were incubated with 200 μM $CoCl_2$ in the presence of 1 μM pilocarpine for 24 h. (D) Cells were treated with 1 μM pilocarpine alone for 24 h. $^{**}P < 0.01$.

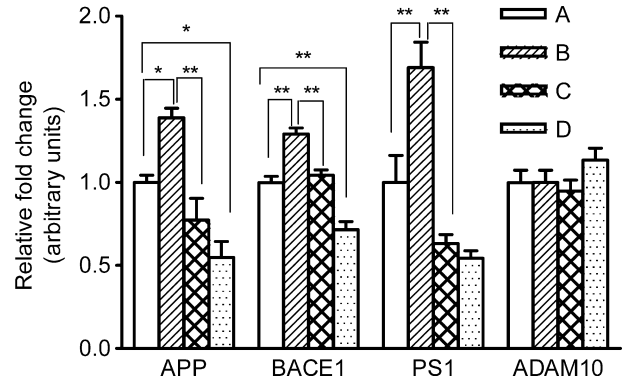

Fig. 3. Muscarinic activation attenuated the overexpression of APP, BACE1 and PS1 mRNA levels induced by hypoxia in RGC-5 cells. (A) Control. (B) Cells were treated with 200 μM $CoCl_2$ for 24 h. (C) Cells pretreated with 1 μM pilocarpine for 6 h were incubated with 200 μM $CoCl_2$ in the presence of 1 μM pilocarpine for 24 h. (D) Cells were treated with 1 μM pilocarpine alone for 24 h. $^{*}P < 0.05$, $^{**}P < 0.01$.

ies have addressed the effects of hypoxia on enzymes involved in APP processing/Aβ production [22,23], the results are still contradictory. Some studies reported that both activity and expression of BACE1 were significantly increased in rats under transient cerebral ischemia [24]. Hypoxia facilitated β-cleavage of APP and increased the neuritic plaque formation by enhancing BACE activity [21]. Whereas another study reported a drastic decrease in ADAM10 protein levels with unchanged BACE1 levels in human neuroblastoma SH-SY5Y cells subjected to chronic hypoxic treatments [25]. It is difficult to reconcile these results from different experimental procedures, such as cells/tissues examined, culture conditions, the methods and duration of hypoxic treatment. The present results indicated that hypoxia in RGCs could up-regulate the expression of APP, BACE1 and PS1, thus increase Aβ generation. It is well documented that accelerated Aβ generation and deposit in cholinergic neurons could lead to cell death and involve in the neurodegeneration of AD. Glaucoma is also a common neurodegenerative disease that affects RGCs. Thus we hypothesize that hypoxia-induced abnormal processing of APP in RGCs may be involved in the development of glaucoma.

It was reported that cholinergic activity could regulate the cellular APP processing in several types of cells. In particular, α-secretase processing of APP can be accelerated by the stimulation of muscarinic receptors, thus the formation of Aβ is concomitantly decreased [12–14]. However, in our study, it is firstly demonstrated that muscarinic activation could attenuate hypoxia-induced abnormal APP processing in RGCs. This finding may provide an insight into better understanding of pathophysiology and modifying approach of glaucoma.

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References

[1] S.J. McKinnon, D.M. Lehman, L.A. Kerrigan-Baumrind, C.A. Merges, M.E. Pease, D.F. Kerrigan, N.L. Ransom, N.G. Tahzib, H.A. Reitsamer, H. Levkovitch-Verbin, H.A. Quigley, D.J. Zack, Caspase activation and amyloid precursor protein cleavage in rat ocular hypertension, Invest. Ophthalmol. Vis. Sci. 43 (2002) 1077–1087.
[2] S. Yoneda, H. Hara, A. Hirata, M. Fukushima, Y. Inomata, H. Tanihara, Vitreous fluid levels of beta-amyloid ((1–42)) and tau in patients with retinal diseases, Jpn. J. Ophthalmol. 49 (2005) 106–108.
[3] V. Parisi, R. Restuccia, F. Fattapposta, C. Mina, M.G. Bucci, F. Pierelli, Morphological and functional retinal impairment in Alzheimer's disease patients, Clin. Neurophysiol. 112 (2001) 1860–1867.
[4] P.K. Iseri, O. Altinas, T. Tokay, N. Yuksel, Relationship between cognitive impairment and retinal morphological and visual functional abnormalities in Alzheimer disease, J. Neuroophthalmol. 26 (2006) 18–24.
[5] L. Guo, T.E. Salt, V. Luong, N. Wood, W. Cheung, A. Maass, G. Ferrari, F. Russo-Marie, A.M. Sillito, M.E. Cheetham, S.E. Moss, F.W. Fitzke, M.F. Cordeiro, Targeting amyloid-beta in glaucoma treatment, Proc. Natl. Acad. Sci. USA 104 (2007) 13444–13449.
[6] H. Zheng, E.H. Koo, The amyloid precursor protein: beyond amyloid, Mol. Neurodegener. 3 (2006) 1–5.
[7] P.C. Wong, Translational control of BACE1 may go awry in Alzheimer's disease, Neuron 60 (2008) 941–943.
[8] T.L. Kukar, T.B. Ladd, M.A. Bann, P.C. Fraering, R. Narlawar, G.M. Maharvi, B. Healy, R. Chapman, A.T. Welzel, R.W. Price, B. Moore, V. Rangachari, B. Cusack, J. Eriksen, K. Jansen-West, C. Verbeeck, D. Yager, C. Eckman, W. Ye, S. Sagi, B.A. Cottrel, J. Torpey, T.L. Rosenberry, A. Fauq, M.S. Wolfe, B. Schmidt, D.M. Walsh, E.H. Koo, T.E. Golde, Substrate-targeting gamma-secretase modulators, Nature 453 (2008) 925–929.
[9] L.V. Johnson, W.P. Leitner, A.J. Rivest, M.K. Staples, M.J. Radeke, D.H. Anderson, The Alzheimer's A beta-peptide is deposited at sites of complement activation in pathologic deposits associated with aging and age-related macular degeneration, Proc. Natl. Acad. Sci. USA 99 (2002) 11830–11835.
[10] A. Agar, S. Li, N. Agarwal, M.T. Coroneo, M.A. Hill, Retinal ganglion cell line apoptosis induced by hydrostatic pressure, Brain Res. 1086 (2006) 191–200.
[11] G. Tezel, M.B. Wax, Hypoxia-inducible factor 1 alpha in the glaucomatous retinal and optic nerve head, Arch. Ophthalmol. 122 (2004) 1348–1356.
[12] A. Caccamo, S. Oddo, L.M. Billings, K.N. Green, H. Martinez-Coria, A. Fisher, F.M. LaFerla, M1 receptors play a central role in modulating AD-like pathology in transgenic mice, Neuron 49 (2006) 671–682.
[13] Y. Qiu, H.Z. Chen, X.J. Wu, Z.J. Jin, 6beta-acetoxy nortropane regulated processing of amyloid precursor protein in CHOm1 cells and rat brain, Eur. J. Pharmacol. 468 (2003) 1–8.
[14] Y. Qiu, X.J. Wu, H.Z. Chen, Simultaneous changes in secretory amyloid precursor protein and beta-amyloid peptide release from rat hippocampus by activation of muscarinic receptors, Neurosci. Lett. 352 (2003) 41–44.
[15] R. Postina, A closer look at alpha-secretase, Curr. Alzheimer Res. 5 (2008) 179–186.
[16] A. Miki, Y. Otori, T. Morimoto, M. Okada, Y. Tano, Protective effect of donepezil on retinal ganglion cells in vitro and in vivo, Curr. Eye Res. 31 (2006) 69–77.

[17] W. Zhou, X. Zhu, L. Zhu, Y.Y. Cui, H. Wang, H. Qi, Q.S. Ren, H.Z. Chen, Neuroprotection of muscarinic receptor agonist pilocarpine against glutamate-induced apoptosis in retinal neurons, Cell. Mol. Neurobiol. 28 (2008) 263–275.
[18] R.R. Krishnamoorthy, P. Agarwal, G. Prasanna, K. Vopat, W. Lambert, H.J. Sheedlo, I.H. Pang, D. Shade, R.J. Wordinger, T. Yorio, A.F. Clark, N. Agarwal, Characterization of a transformed rat retinal ganglion cell line, Brain Res. Mol. Brain Res. 86 (2001) 1–12.
[19] A.R. Borenstein, Y. Wu, J.A. Mortimer, G.D. Schellenberg, W.C. McCormick, J.D. Bowen, S. McCurry, E.B. Larson, Developmental and vascular risk factors for Alzheimer's disease, Neurobiol. Aging 26 (2005) 325–334.
[20] I. Skoog, D. Gustafson, Update on hypertension and Alzheimer's disease, Neurol. Res. 28 (2006) 605–611.
[21] X. Sun, G. He, H. Qing, W. Zhou, F. Dobie, F. Cai, M. Staufenbiel, L.E. Huang, W. Song, Hypoxia facilitates Alzheimer's disease pathogenesis by up-regulating BACE1 gene expression, Proc. Natl. Acad. Sci. USA 103 (2006) 18727–18732.
[22] R. Wang, Y.W. Zhang, X. Zhang, R. Liu, X. Zhang, S. Hong, K. Xia, J. Xia, Z. Zhang, H. Xu, Transcriptional regulation of APH-1A and increased gamma-secretase cleavage of APP and Notch by HIF-1 and hypoxia, FASEB J. 20 (2006) 1275–1277.
[23] X. Zhang, K. Zhou, R. Wang, J. Cui, S.A. Lipton, F.F. Liao, H. Xu, Y.W. Zhang, Hypoxia-inducible factor 1alpha (HIF-1alpha)-mediated hypoxia increases BACE1 expression and beta-amyloid generation, J. Biol. Chem. 282 (2007) 10873–10880.
[24] Y. Wen, O. Onyewuchi, S. Yang, R. Liu, J.W. Simpkins, Increased beta-secretase activity and expression in rats following transient cerebral ischemia, Brain Res. 1009 (2004) 1–8.
[25] A.J. Marshall, M. Rattray, P.F. Vaughan, Chronic hypoxia in the human neuroblastoma SH-SY5Y causes reduced expression of the putative alpha-secretases, ADAM10 and TACE, without altering their mRNA levels, Brain Res. 1099 (2006) 18–24.