Aralia elata inhibits neurodegeneration by downregulating O-GlcNAcylation of NF-κB in diabetic mice

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Received: 2017-03-21 Accepted: 2017-06-12

Abstract

- AIM: To investigate the role of O-GlcNAcylation of nuclear factor-kappa B (NF-κB) in retinal ganglion cell (RGC) death and analysed the effect of Aralia elata (AE) on neurodegeneration in diabetic mice.
- METHODS: C57BL/6 mice with streptozotocin-induced diabetes were fed daily with AE extract or control (CTL) diet at the onset of diabetes mellitus (DM). Two months after injection of streptozotocin or saline, the degree of cell death and the expression of O-GlcNAc transferase (OGT), N-acetyl-b-D-glucosaminidase (OGA), O-GlcNAcylated proteins, and O-GlcNAcylation of NF-κB were examined.
- RESULTS: AE did not affect the metabolic status of diabetic mice. The decrease in the inner retinal thickness (P<0.001 vs CTL, P<0.01 vs DM) and increases in RGCs with terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (P<0.001 vs CTL, P<0.0001 vs DM), glial activation, and active caspase-3 (P<0.0001 vs CTL, P<0.0001 vs DM) were blocked in diabetic retinas of AE extract-fed mice. Expression levels of protein O-GlcNAcylation and OGT were increased in diabetic retinas (P<0.0001 vs CTL), and the level of O-GlcNAcylation of the NF-κB p65 subunit was higher in diabetic retinas than in controls (P<0.0001 vs CTL). AE extract downregulated O-GlcNAcylation of NF-κB and prevented neurodegeneration induced by hyperglycemia (P<0.0001 vs DM).
- CONCLUSION: O-GlcNAcylation of NF-κB is concerned in neuronal degeneration and that AE prevents diabetes-induced RGC apoptosis via downregulation of NF-κB O-GlcNAcylation. Hence, O-GlcNAcylation may be a new object for the treatment of DR, and AE may have therapeutic possibility to prevent diabetes-induced neurodegeneration.
- KEYWORDS: Aralia elata; diabetic retinopathy; neurodegeneration; nuclear factor-kappa B; O-GlcNAc; O-GlcNAc transferase; mice

DOI:10.18240/ijo.2017.08.03


INTRODUCTION

Diabetic retinopathy (DR), a disorder affecting the microvascular structure of the retina, remains a major sight-threatening disease for working adults and a common complication of diabetes[1-2]. In the early stages of the diseases, retinal ganglion cells (RGCs), the major neuronal cells of the retina, and glial cells are compromised as activation of glial cells, which is another feature of retinal neurodegeneration[3]. Moreover, various studies report neuronal apoptosis in diabetic retinas as well as activation of glial cells, which is another feature of retinal neurodegeneration[4,5]. Nowadays, it is evident that considerable damage to retinal neurons is already present at early stages of DR, before any abnormal microcirculatory changes can be detected by fundus examination[7,8]. Nonetheless, the key regulators of neurodegeneration in DR remain uncertain, and further studies are required to identify new therapeutic agents for neuroprotection in the early stages of DR. O-GlcNAcylation is an insignificant protein post-translational modification that participates in addition of a single O-linked β-N-acetylglucosamine (O-GlcNAc) to the hydroxyl groups of serine and/or threonine protein[9,10]. O-GlcNAcylation is concerned in regulating various nuclear or cytoplasmic proteins in a manner similar to protein phosphorylation. But unlike phosphorylation, only two enzymes, O-GlcNAc transferase (OGT) and N-acetyl-β-D-glucosaminidase (OGA) are responsible for the addition of O-GlcNAc to serine/threonine residues and O-GlcNAc elimination[9]. Accumulating evidence reveals the important roles that O-GlcNAcylation participates in several
neurodegeneration in DR. Finally, we analysed the effect of extracts of AE on hyperglycaemia-induced RGC apoptosis and downregulates O-GlcNAcylated proteins in cells, which may play important roles in the pathogenesis of diabetes[12]. Furthermore, the changes of O-GlcNAcsignaling have been involved in the pathogenesis of diabetic complications, including diabetic cardiomyopathy, diabetic nephropathy, and DR[13-16]. In the animal model of DR, O-GlcNAcylated proteins were increased with higher glucose levels in retinal endothelial cells and pericytes, and the OGT-positive cells were located in the ganglion cell layer (GCL), inner nuclear layer (INL), and inner plexiform layer (IPL). Additionally, the number of terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL)-positive RGCs that colocalized with OGT was notably higher in diabetic mice than in control[15,17]. Also, previous studies report that increased expression and O-GlcNAcylation of NF-κB participate in several human diseases, including DR and cancer[12,18].

Aralia elata (AE) generally distributes in several provinces in East Asia, such as China, Japan, and South Korea. The number of patients with diabetes and DR in East Asia is increasing, especially in China. Currently, China currently has 10.6% of its population with diagnosed diabetes and this number has more than doubled from 4.5% in the past 6y, and is catching up with developed counterparts like the USA[19]. The prevalence of DR in diabetic subjects was 9.7% in China[20], 15.9% in Korea[21], and 23.2% in Japan[22]. Furthermore, these rates are also increasing, and so the economic burden of treatment of DR is high in these countries[19]. The water extract of AE has been traditionally used to treat diabetes in Korea, China, and Japan. Moreover, in a previous study, authors report that AE prevents hyperglycaemia-induced RGC apoptosis and downregulates toxicity response element binding protein in DR[23]. Therefore, it would be meaningful for these countries to study whether AE really has the effect of inhibiting the progression of DR.

The current study investigates the role of O-GlcNAcylation in promoting neurodegeneration. We also examined whether increases in O-GlcNAcylation of the nuclear factor-kappa B (NF-κB) contributes to increased apoptosis of RGCs in DR. Finally, we analysed the effect of extracts of AE on neurodegeneration in DR.

**MATERIALS AND METHODS**

**Animals** C57BL/6 mice were provided from the KOATEC (Pyeongtaek, Korea). All animal procedures for this study were in a adherence to the ARVO statement for the Use of Animals in Ophthalmic and Vision Research, and were kept in accordance with the Institutional Animal Care and Use Committee of Gyeongsang National University (Jinju, Korea). To induce diabetes, mice were intraperitoneally injected with streptozotocin (55 mg/kg, STZ, Sigma, St. Louis, MO, USA) dissolved in sodium citrate once a day for 5d. Control mice injected phosphate-buffered saline (PBS). Blood glucose levels were checked every 2wk using a glucometer (Abbott, Alameda, CA, USA) after 8h of fasting. Diabetes was established by blood glucose levels >250 mg/dL at 1wk after the final injection of STZ. Mice were killed 2mo after the last injection of STZ or saline.

**Preparation and Administration of Aralia elata Extract** A freeze-dried powder of AE was obtained from Medvill Co., Ltd. (Seoul, Korea) and prepared as described previously[24]. The AE powder was dissolved and diluted in 0.9% saline and administered to mice at doses of 20 or 100 mg/kg body weight with an oral feeding tube once a day for 7wk after diabetes induction. Finally, the mice were divided into four groups as follows: 1) saline-treated control group; 2) saline-treated diabetic group; 3) 20 mg/kg AE-treated diabetic group; 4) 100 mg/kg AE-treated diabetic group. Each group included 10 mice and a total of 40 mice were used in this study.

**Assessment of Changes in Inner Retinal Thickness** Collected retinas were immersed in 4% paraformaldehyde for 6h, and then washed several times in PBS, cryoprotected in 30% sucrose overnight at 4°C, frozen in liquid nitrogen with O.C.T. compound (Sakura, Tokyo, Japan), and finally cryosectioned on a cryostat (Leica 8400E; Leica, Tokyo, Japan). Samples were stained with haematoxylin and eosin (H&E), and the length (µm) from the GCL to the tip of the INL was measured as inner retinal thickness. The comparison of the inner retinal thickness between diabetic and control groups was performed in four different retinas per group at a distance of about 0.8-1.0 mm from the optic nerve head.

**Antibodies and Cell Death Assay Kit** Anti-NF-κB (p65 subunit), anti-glial fibrillary acidic protein (GFAP), and OGA antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). A rabbit polyclonal anti-caspase-3 antibody was purchased from Cell Signaling Technology (Danvers, MA, USA). Antibodies against O-GlcNAc were acquired from Thermo Fisher Scientific (Waltham, MA, USA), and antibodies against OGT were purchased from Sigma (St. Louis, MO, USA). Anti-β-actin antibody was purchased from Abcam (Cambridge, UK). Anti-NeuN antibody was purchased from Chemicon (Nuenberg, Germany).

A TUNEL kit (In Situ Cell Death Detection Kit; Roche, Grenzach, Germany) was used to detect apoptosis according to the manufacturer’s guidance. Total numbers of TUNEL-positive RGCs were counted in the GCL (approximately 100 µm) in three random fields per retina from 5-6 retinas per group.

**Western Blot Analysis** Proteins were extracted from four retinas of each group, and 30 µg of protein were subjected to 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membranes were incubated with antibodies against O-GlcNAc, OGT, OGA, anti-caspase-3, and NF-κB (p65 subunit) followed by a horseradish peroxidase-conjugated anti-rabbit secondary.
Immunohistochemistry and Image Capture

Retinal sections probed with antibodies. 10% gels. Proteins were transferred to membranes and then incubated with agarose beads for 2h at 4℃, and centrifuged. The supernatants were incubated with 2 μg of antibody overnight at 4℃. And, samples were incubated with agarose beads for 2h at 4℃. The negative control was prepared with only agarose beads without an antibody. The protein-bead complex was washed and then collected by centrifuge. The complex were boiled in loading buffer to separate the agarose beads and electrophoresed on 10% gels. Proteins were transferred to membranes and then probed with antibodies.

Immunoprecipitation

Protein from retinas were mixed with agarose beads (Santa Cruz Biotechnology), incubated for 1h at 4℃, and centrifuged. The supernatants were incubated with 2 μg of antibody overnight at 4℃. And, samples were incubated with agarose beads for 2h at 4℃. The negative control was prepared with only agarose beads without an antibody. The protein-bead complex was washed and then collected by centrifuge. The complex were boiled in loading buffer to separate the agarose beads and electrophoresed on 10% gels. Proteins were transferred to membranes and then probed with antibodies.

Immunohistochemistry and Image Capture

Retinal sections (10-μm thick) were prepared as described previously.[23,25-26] The sections were incubated with primary antibodies against O-GlcNAc, OGT, OGA, GFAP, and NF-κB (p65 subunit) and followed by an Alexa 488-conjugated goat anti-rabbit secondary IgG (Thermo Fisher Scientific). Blots were reprobed with IgG (Thermo Fisher Scientific). Blots were reprobed with an anti-β-actin antibody to control. All Western blot data are representative of four independent experiments.

Statistical Analysis

Comparisons among experimental groups were operated by one-way ANOVA using Prism 5 (GraphPad Software, San Diego, CA, USA). All data are representatives of four independent experiments, and are presented as mean±standard error. P values <0.05 were considered statistically significant.

RESULTS

The Effect of *Aralia elata* Administration on Body Weight and Blood Glucose Concentration After Induction of Diabetes

Body weights and blood glucose levels are shown in Table 1. Control mice showed a steady increase in body weight during the experiment, whereas diabetic mice exhibited a decrease in body weight. The blood glucose concentrations of diabetic mice significantly and steadily increased, whereas control mice maintained normal glucose concentrations throughout the experiment. AE extract did not significantly affect the body weight of any of the mice. Blood glucose levels of 20 mg/kg AE-treated diabetic mice were maintained at slightly lower concentrations compared with saline-treated diabetic mice except for the 4wk after induction of diabetes.

In the group administered with 40 mg/kg AE, blood glucose levels were slightly lower than in the saline-treated diabetic group, especially the difference was statistically significant in the 2wk after induction of diabetes (Table 1).

Diabetic Retinal Neurodegeneration and Neuroprotective Effects of *Aralia Elata*

We examined RGC apoptosis, glial activation, changes in inner retinal thickness, and activation of caspase-3. Next, we investigated whether treatment with AE extract protected against these changes. TUNEL-positive cells were mainly located in the GCL of diabetic mice, with some in the INL and OPL (Figure 1A). The numbers of TUNEL-positive cells were increased in the GCL of the diabetic group compared with the control (P<0.0001) (Figure 1B). Treatment with AE extract decreased the number of TUNEL-positive apoptotic cells in the diabetic retinas (Figure 1A, 1B).

Moreover, glial activation was noted in diabetic retinas, which was prevented by treatment with AE extract (Figure 1C). Inner retinal thickness was thinner in diabetic retinas than in control retinas (Figure 2). AE treatment increased the inner retinal thickness compared with the untreated diabetic group (Figure 2).

The level of active caspase-3 was significantly increased in diabetic retinas compared with that in controls. This increase was blocked by treatment with AE extract (P<0.001 vs diabetic group) (Figure 3).

Changes in Protein O-GlcNAcylation, O-GlcNAc Transferase, N-acetyl-β-D-glucosaminidase and Effects of *Aralia Elata*

O-GlcNAcylation of retinal proteins was notably increased in the Western blot analysis (Figure 4A). Quantification showed that the levels of OGT were higher in diabetic retinas than in controls (Figure 4B). However, there were no significant changes in OGA expression between control and diabetic groups (Figure 4C). In the AE-treated diabetic groups, O-GlcNAcylation of proteins and OGT were decreased compared to the saline-treated diabetic group (Figure 4A, 4B). On the other hand, treatment with AE extract exerted no apparent effect on the protein levels of OGA (Figure 4C).

<table>
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<tr>
<th>Time</th>
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<td>Week 0</td>
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</tr>
<tr>
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AE: *Aralia elata*: AE20: 20 mg/kg AE; AE100: 100 mg/kg AE; DM: Diabetes mellitus; *P*<0.05 vs CTL; *P*<0.05 vs DM.
Immunohistochemical studies showed a high concentration of proteins with O-GlcNAcylation localized in the GCL (and INL) of diabetic mice (Figure 4D). Treatment with AE extract inhibited these changes.

**Relationship Between Retinal Ganglion Cell Death and O-GlcNAc Transferase Expression and Effects of *Aralia Elata***

To determine whether OGT affects RGC death in DR, triple immunofluorescence staining was performed for OGT, NeuN, and TUNEL. Immunoreactivity for OGT was markedly increased in the GCL of diabetic retinas compared to control retinas, whereas treatment with AE extract attenuated these changes (Figure 5A). Interestingly, most OGT-positive cells colocalized with TUNEL and NeuN staining in the retinal GCL of both diabetic and control mice (white arrows, Figure 5A). Furthermore, the total number of OGT- and TUNEL-positive RGCs was greater in diabetic retinas compared with controls, but AE treatment reduced RGC death in the diabetic retinas ($P<0.0001$ vs untreated diabetic group) (Figure 5B).
Aralia Elata Suppresses NF-κB Expression and Decreases Levels of O-GlcNAc-modified NF-κB in DR

The levels of NF-κB (p65 subunit) were increased in diabetic retinas compared with controls in Western blot analysis ($P<0.0001$) (Figure 6A, 6B). However, AE extract treatment reduced levels of NF-κB in DR ($P<0.0001$). Furthermore, we found that NF-κB immunoreactivity was colocalized with TUNEL, and nuclear translocation of NF-κB was significantly increased in the GCL of DR (Figure 6C, boxed area). Interestingly, NF-κB colocalization with TUNEL was notably reduced in the GCL of diabetic mice treated with AE compared with untreated mice (Figure 6C).

Finally, we assessed O-GlcNAcylation of NF-κB in DR using co-immunoprecipitation assays (Figure 7A). As expected, O-GlcNAcylation of the p65 subunit of NF-κB was greater in the diabetic retinas than in control retinas (Figure 7A). However, AE treatment reduced the amount of NF-κB O-GlcNAcylation in diabetic retinas ($P<0.0001$ vs untreated diabetic group) (Figure 7).

**DISCUSSION**

In the present study, we suggest that O-GlcNAcylation of NF-κB is involved in RGC death and that AE treatment prevents diabetes-induced RGC apoptosis via downregulation of NF-κB O-GlcNAcylation in DR. One of the earlier signs in DR is the abnormalities of capillary circulation with leakage of retinal vessels in the inner retina [27]. Until recently, considerable attention was given to protection of retinal circulations, with less understanding given to neuroprotection in DR [3]. Nonetheless, various studies showed that retinal neuronal changes occur before clinically detectable microvascular abnormalities [28].

Several factors have been involved in the pathogenesis of DR, including increases of vascular endothelial growth factor [29], tumour necrosis factor (TNF)-α [30], advanced
glycation end products\textsuperscript{[31]}, inflammation\textsuperscript{[32]}, and several polyl pathways\textsuperscript{[33]}. Among them, NF-kB plays crucial roles in the induction of vascular permeability, angiogenesis, and neurodegeneration in DR\textsuperscript{[3,34]}. Numerous studies showed that NF-kB is activated through multiple pathways in DR. First, hyperglycemia-induced oxidative stress leads to the activation of NF-kB\textsuperscript{[35]}. Next, TNF-\alpha-mediated NF-kB activation is concerned in diabetes-related leukostasis, inflammation, and apoptosis\textsuperscript{[36]}. Specifically, TNF-\alpha mediates phosphorylation of the p65 subunit of NF-kB at Ser536, which was shown to be reciprocally applied by O-GlcNAc\textsuperscript{[37]}. Altered O-GlcNAcylation of NF-kB leads to an increased nuclear translocation of RelA.

**Figure 5 Effect of AE on OGT and RGC death in diabetic mice**

A: Representative triple staining for OGT, TUNEL, and NeuN in the retinas of control and diabetic mice with or without AE. The white arrows indicate TUNEL-positive cells that were stained for OGT and NeuN in the retinas of control and diabetic mice. Scale bar, 50 µm. Quantification of TUNEL-positive RGCs that double-labelled for OGT in the retinas of control or diabetic mice with or without AE (B). \textsuperscript{a}P<0.0001 vs CTL; \textsuperscript{b}P<0.0001 vs DM. AE: *Aralia elata*; AE20: 20 mg/kg AE; AE100: 100 mg/kg AE; CTL: Control; DM: Diabetes mellitus; GCL: Ganglion cell layer; IPL: Inner plexiform layer; INL: Inner nuclear layer; OGT: O-GlcNAc transferase; OPL: Outer plexiform layer; ON: Outer nuclear layer; RGC: Retinal ganglion cell.
and increases NF-κB transcriptional activity[38]. Previous study also reports that O-GlcNAcylation of NF-κB is important for its nuclear translocation and acceleration of cancer metastasis[18], and hyperglycemia-induced activation and RGC death in DR[17]. Consistent with these studies, our data show that OGT protein expression was increased and related to RGC death in diabetic retinas (especially, in the GCL). Moreover, we suggested that NF-κB underwent O-GlcNAcylation, and that increased O-GlcNAcylation and translocation of the p65 subunit contributed to RGC death.

Neurodegeneration of retina is a crucial component of DR and is typically accomplished by a decreased number of RGCs, a thinning inner retina, and an increased numbers of apoptotic cells[28]. Our current study revealed a notable decrease in inner retinal thickness, a marked increase in the number of TUNEL-positive cells, and increased glial activation in the diabetic retinas, consistent with the findings of earlier investigations. Importantly, AE treatment reversed these changes in the diabetic mice. Additionally, we found that AE reduced levels of NF-κBO-GlcNAcylation, which is known to play an important role in RGC apoptosis in DR[17]. Consistent with this, some studies show that inhibiting O-GlcNAcylation in retinal vascular endothelial cells protects the vascular integrity and reduces the expression of vascular endothelial growth factor.

Figure 6 Effect of AE administration on levels of NF-κB  Representative Western blot and quantification of NF-κB (A, B), and immunofluorescent studies for TUNEL, NF-κB, and DAPI (nuclear counterstain) in the retinas of control or diabetic mice with or without AE treatment (C). The boxed area shows colocalization of NF-κB with TUNEL and nuclear translocation of the NF-κB p65 subunit in the GCL of diabetic retinas. Band intensity was normalized to β-actin. *P<0.0001 vs CTL; †P<0.0001 vs DM. AE: Aralia elata; AE20: 20 mg/kg AE; AE100: 100 mg/kg AE; CTL: Control; DM: Diabetes mellitus, Scale bar: 50 μm.
Neuroprotection by *Aralia elata* in diabetic mice

![Figure 7 Effect of AE treatment on NF-κB O-GlcNAcylation in diabetic retinas](image)

in vitro^[39]$, and O-GlcNAcylation and nuclear translocation of p65 subunit of NF-κB increases its transcriptional activities^[18]$. Unfortunately, more specific OGT inhibitors generally do not work well in most living cells or animals and also affect O-GlcNAcylation of many proteins^[40]$. Therefore, inhibition of NF-κB O-GlcNAcylation by AE administration represents a promising target for successful neuroprotection. In addition, it is well known that AE exhibits numerous biological activities, including cytoprotective, anti-inflammatory, antioxidative, antiviral, and antidiabetic properties^[24,41]$. Indeed, the AE extract used in this study contained phenolic compounds [*i.e.*, 3, 4-dihydroxybenzoic acid (DHBA), chlorogenic acid, and caffeic acid] as revealed by high-performance liquid chromatography analysis^[42]$. Ban et al^[43]$ reported that DHBA safeguards against amyloid beta protein-induced neuronal cell death, and some reports show that chlorogenic acid and caffeic acid have neuroprotective actions^[44]$. Our previous study showed that AE prevents hyperglycemia-induced RGC apoptosis and downregulates toxicity response element binding protein in DR^[23]$. Consequently, AE may have therapeutic potential to regulate O-GlcNAcylation of proteins and prevent diabetes-induced retinal neurodegeneration in DR.

Taken together, our findings indicate that O-GlcNAcylation of NF-κB contributes to neuronal degeneration and that AE treatment prevents diabetes-induced RGC apoptosis via downregulation of NF-κB O-GlcNAcylation. Thus, O-GlcNAcylation may be a new target for treatment of DR, and AE may have therapeutic abilities to prevent diabetes and neurodegeneration in DR. However, much more work is needed to understand the mechanisms of O-GlcNAcylation and its relationship with other pathogenesis in DR.

ACKNOWLEDGEMENTS

**Foundations:** Supported by the Basic Science Research Program Through the National Research Foundation (NRF) of Korea Funded by the Ministry of Science, ICT, and Future Planning 2014049413, NRF-2015R1A5A2008833 and NRF-2015R1C1A1A02037702.

**Conflicts of Interest:** Kim SJ, None; Kim MJ, None; Choi MY, None; Kim YS, None; Yoo JM, None; Hong EK, None; Ju S, None; Choi WS, None.

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