

Involvement of Beclin-1 in axonal protection by short-term hyperglycemia against TNF-induced optic nerve damage

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Abstract. Beclin-1 serves a pivotal role in autophagosome formation. A previous study demonstrated that streptozotocin-induced hyperglycemia (HG) ameliorates axonal loss induced by tumor necrosis factor (TNF) with upregulation of autophagy in rats. The aim of present study was to examine whether Beclin-1 is involved in this autophagy machinery. Immunoblot analysis of optic nerves demonstrated that HG upregulated Beclin-1 protein expression when compared with normoglycemia (NG). Intravitreal administration of TNF did not alter the optic nerve Beclin-1 expression in NG nor in HG. Beclin-1 immunoreactivity was revealed to be mainly in astrocytes in optic nerves; however, it was also observed in the neurofilaments of the HG group. Morphometric analysis revealed that HG appeared to have substantial ameliorative effects on axon loss and this ameliorative effect was partially prevented by Beclin-1 small interfering RNA. These results indicated that Beclin-1 may exist in neurons and glia in optic nerves and increased Beclin-1 expression may be at least partially associated with axonal protection by HG.

Introduction

Conflicting relationship has been demonstrated between glaucoma and diabetes mellitus (DM). For example, some previous studies showed that DM increases a risk for development of glaucoma (1,2). Conversely, other studies suggested that DM prevented glaucoma occurrence (3,4). Moreover, a recent study showed that DM is associated with glaucoma, but this association disappeared after adjustment for triglyceride levels (5). In the histological level, previous studies reported that short-term hyperglycemia (HG) preserves retinal structure in a transient high intraocular pressure-induced ischemic rat model (6)

and a common carotid artery occlusion rat model (7). Other study demonstrated that HG condition prevents axon loss in an ocular hypertension rat model (8). Furthermore, a recent study has shown that subconjunctival applied glucose partially preserves retinal ganglion cell (RGC) somata in the transient high intraocular pressure-induced ischemic rat model and transiently increases contrast sensitivity in human subjects with severe primary open-angle glaucoma (9).

Our previous study found that short-term HG ameliorates tumor necrosis factor (TNF)-induced axon loss (10). Since a close relationship between TNF and glaucoma has been implicated (11-16), this TNF-mediated axon loss model may be helpful to clarify the molecular events by which axons are degenerated in RGCs (17). In optic nerves, the short-term HG enhances autophagy machinery (10). Autophagy plays central roles in the pathophysiology of several human diseases (18) and its impaired condition has been linked to neurodegenerative diseases (19-21). Among the autophagy-related (Atg) genes, microtubule-associated protein light chain 3 (LC3)/Atg8 is known as a marker for autophagosomes (22). Beclin-1/Atg6 constitutes Beclin-1 complex which is necessary for autophagic function (23). Up-regulation of Beclin-1 was shown in RGCs after optic nerve transection in rats (24). In addition, increased Beclin-1 protein levels were shown in retinal samples in the rat hypertensive glaucoma model (25) and a monkey hypertensive glaucoma model (26). However, its expression and localization in optic nerve have not yet to be demonstrated. In the present study, we tested whether Beclin-1 is involved in the ameliorative effect of short-term HG against axon loss caused by TNF.

Materials and methods

Animals. The present study used 8-week-old male Wistar rats and was approved by Ethics Committee of the Institute of Experimental Animals of St. Marianna University School of Medicine. The rats were maintained in the controlled rooms (23±1°C; humidity at 55±5%; light on 06:00 to 18:00).

Streptozotocin-induced hyperglycemic (HG) rat model. Single i.p. administration of physiological saline solution (PSS) or 60 mg/kg streptozotocin (STZ; Wako Pure Chemical Industries, Ltd., Osaka, Japan) was carried out for the normoglycemic (NG) rats or the hyperglycemic (HG) rats, respectively. The plasma glucose levels were measured using a glucometer

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(Johnson & Johnson, Tokyo, Japan) 4 days after intraperitoneal injection. We only included the individuals as the HG group when plasma glucose exceeded 250 mg/dl. The plasma glucose levels of HG groups were 441.5 ± 71.3 , 435.0 ± 32.3 , and 432.1 ± 73.3 mg/dl, immunoblot analysis, immunohistochemical analysis, and morphometric analysis studies, respectively.

Intravitreal injection. Intravitreal administration of 10 ng TNF (2 μ l) was carried out into the right eye of rats under anesthetization with a combination of ketamine and xylazine. The left eye was received an intravitreal administration of phosphate-buffered saline (PBS). These intravitreal administrations were carried out 4 days following i.p. injection of PSS or STZ. In the HG group, a simultaneous intravitreal administration of 50 pmol Beclin-1 siRNA (Cell Signaling Technology, Inc., Danvers, MA, USA) with TNF was carried out into the right eyes.

Immunoblot analysis. Thirty-six rats (NG: 18 rats; HG: 18 rats) were euthanatized 1 week after intravitreal administrations for immunoblot analysis. Four mm optic nerves from immediately behind the eye ball were homogenized in protein extraction buffer. Since the optic nerve pieces were small, each sample included two optic nerves. Equal amount of proteins (3 μ g) determined by the Bradford assay was applied and loaded. Then, samples were transferred to PVDF membranes. After blocking, membranes were exposed with the primary antibodies: Anti-Beclin-1 antibody for overnight (1:200; Medical & Biological Laboratories, Co., Ltd., Nagoya, Japan) or anti- β -actin antibody for 2 h (1:500; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). After three times washing, membranes were reacted with the secondary antibodies: Rabbit IgG or mouse IgG. Immunoblots were evaluated with the Amersham ECL detection system (GE Healthcare Life Sciences, Little Chalfont, UK).

Immunohistochemical analysis. Six rats were used for immunohistochemical analysis. One week following intravitreal administration, optic nerve samples were immersed in 10% neutral-buffered formalin. Paraffinized cross sections were made in 2 μ m thick and incubated with 1% bovine serum. The primary antibodies were anti-Beclin-1 antibody (1:100; Medical & Biological Laboratories, Co., Ltd.), glial fibrillary acidic protein (GFAP, a marker of astrocytes; 1:200; Agilent Technologies, Inc., Santa Clara, CA, USA), and neurofilament-L (a marker of neurons; 1:100; Agilent Technologies, Inc.). The secondary antibodies were FITC-labeled and rhodamine-labeled IGG. The slides were mounted in 4',6'-diamidino-2-phenylindole-including medium (Vector Laboratories, Ltd., Peterborough, UK).

Morphometric analysis. Fourteen rats (NG: 4 rats; HG: 10 rats) were euthanatized 2 weeks after intravitreal administration for axon morphometric analysis (10,27). Optic nerve samples were immersed in Karnovsky's solution for overnight. After embedded in plastic blocks, cross thin sections were made beginning 1 mm from the eye ball. The sections were stained with 1% paraphenylen-diamine (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Five images (center and periphery in quadrant per optic nerve) were acquired and quantified using

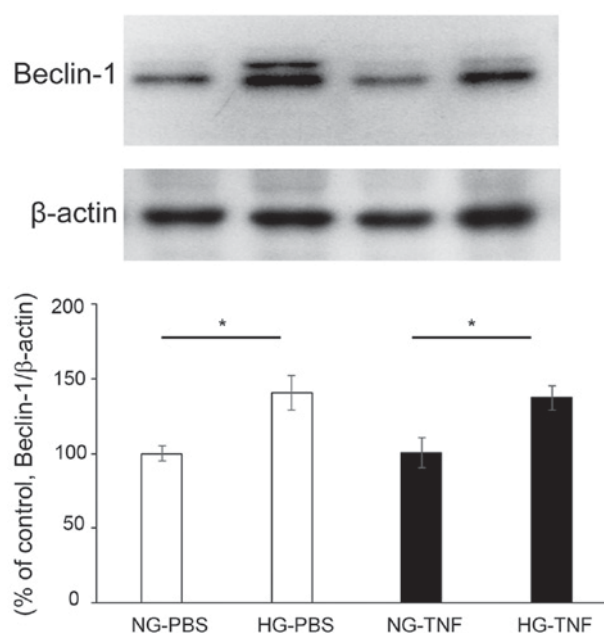


Figure 1. Beclin-1 protein expression in the optic nerves 1 week following intravitreal administration. Normalization was conducted using β -actin levels. Values are expressed as a percentage of the control and are presented as the mean \pm standard error of the mean (n=8-9). *P<0.05, as indicated. HG, hyperglycemia; TNF, tumor necrosis factor; NG, normoglycemia.

an image-processing software (Aphelion, ADCIS, Hérouville Saint-Clair, France). The average of axon number in each optic nerve was expressed as the number per mm².

Statistical analysis. Data are presented as mean \pm standard error of the mean. Differences among groups were analyzed using one-way analysis of variance with Dunnett's post hoc test. JMP v12.0.1 software (SAS Institute, Inc., Cary, NC, USA) was used for statistical analyses. P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of TNF and HG on Beclin-1 levels in Optic Nerve. The current study found that HG condition markedly increases Beclin-1 protein levels in optic nerves (Fig. 1). These significant increments were seen in both PBS-treated and TNF-treated eyes (Fig. 1). However, intravitreal administration of TNF did not alter the Beclin-1 expression in both NG and HG conditions.

Localization of Beclin-1 in optic nerve. In cross sections, immunohistochemical study revealed abundant colocalization of Beclin-1 and GFAP in the optic nerves in NG group (Fig. 2A-C). Although immunoreactivity pattern of Beclin-1 is different from that of neurofilament (Fig. 2D-F), some immunoreactivities of Beclin-1 were apparently colocalized with those of neurofilament in the HG group (Fig. 2G-L). These findings suggest that Beclin-1 is present mainly in glial cells, but partially in neurofilament, and that the expression of Beclin-1 may be upregulated by HG.

Effect of HG and Beclin-1 siRNA on Axonal Loss induced by TNF. Consistent with our previous findings (10), the current study

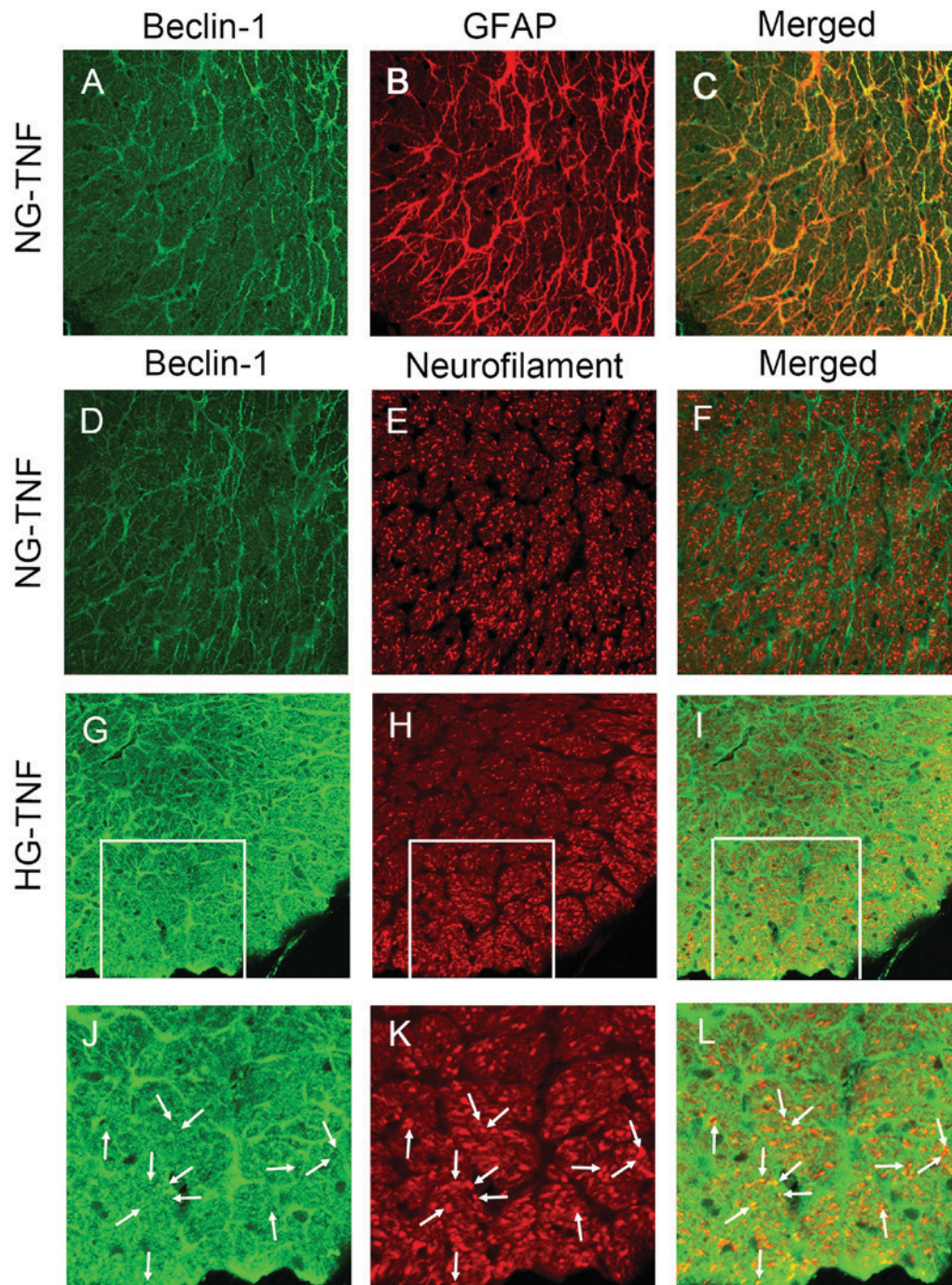


Figure 2. Immunohistochemical analysis of optic nerve cross sections. Substantial colocalization of Beclin-1 and GFAP was seen in optic nerves in the (A-C) NG-TNF group. Double-staining for Beclin-1 and neurofilaments revealed different staining patterns in the (D-F) NG-TNF group. (G-I) Double-staining for Beclin-1 and neurofilaments in the HG-TNF group. (J-L) High-magnification images of the inset area in the HG group. Beclin-1 immuno-positive dots were colocalized with neurofilament-positive dots (arrows). (A-I) Magnification, x40; and (J-L) Magnification, x80. HG, hyperglycemia; TNF, tumor necrosis factor; NG, normoglycemia; GFAP, glial fibrillary acidic protein.

showed that STZ-induced HG condition appeared a significant ameliorative effect on axonal loss caused by TNF (Fig. 3B, C, E). Since we found a significant upregulation of Beclin-1 protein level in the HG group, we examined whether Beclin-1 siRNA alters this protective effect. Noticeable degenerative changes were seen in the HG-TNF with Beclin-1 siRNA treatment group (Fig. 3D). Although no significant difference in the axon number was seen in between the HG-TNF group and the HG-TNF with Beclin-1 siRNA treatment group, no significant difference was also seen in between the NG-TNF group and the HG-TNF with

Beclin-1 siRNA treatment group (Fig. 3E). These observations suggested that the protective effect of HG was only partially suppressed by Beclin-1 siRNA.

Discussion

Opposite autophagic status was demonstrated in skeletal muscle between in the glucose-infusion HG rat and the STZ-induced HG rat (28). In STZ-induced HG rats, low insulin level prevented the m-TOR signaling, thereby leading

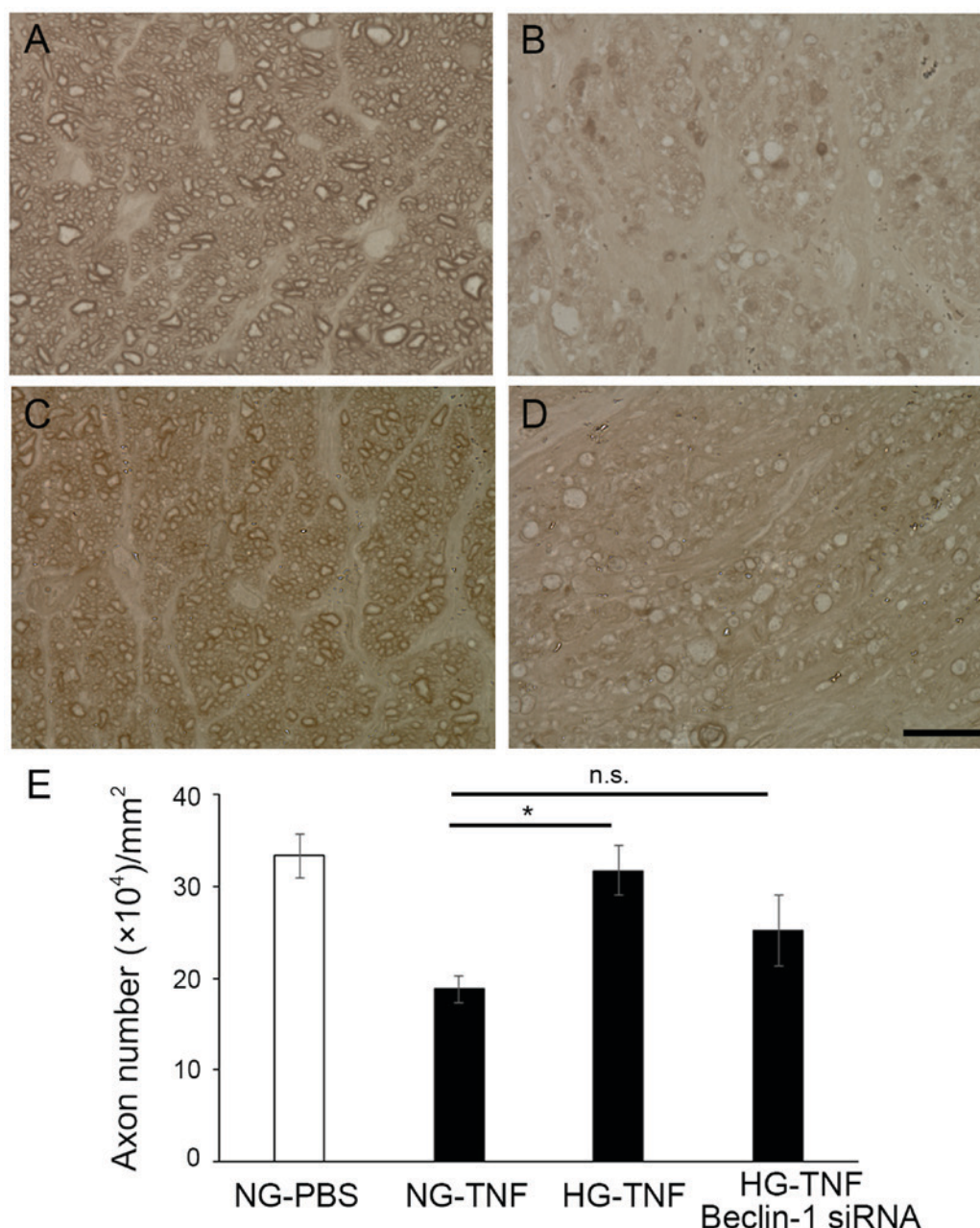


Figure 3. Beclin-1 siRNA only partially suppresses the axonal-protective effects of short-term HG. Histological results of cross sectioned optic nerves in the (A) NG-PBS, (B) NG-TNF, (C) HG-TNF, or (D) HG-TNF + 50 pmol Beclin-1 siRNA groups. Scale bar=10 μ m; magnification, x100. (E) Comparison of axon numbers as shown by computer-assisted image analysis (Aphelion imaging software). Five different areas of 1,446.5 μ m² each (totaling 7,232.5 μ m²) from each eye were used for analysis (n=4-5 per group). *P<0.05, as indicated. HG, hyperglycemia; TNF, tumor necrosis factor; NG, normoglycemia; siRNA, small interfering RNA; n.s., not significant.

to enhancement of autophagy (28). Consistent with this finding, our previous study found enhanced autophagy in optic nerve in the STZ-induced HG rats (10). In addition, a recent study demonstrated enhanced autophagy in hippocampus with ischemia in the STZ-induced HG rats (29). In the current study, remarkable increase in Beclin-1 expression was found in the optic nerve in the STZ-induced HG rats. It has been shown that Beclin-1 plays an essential role for the formation of autophagosomes in a HT22 hippocampal cells (30). That study also demonstrated that Beclin-1 is necessary for upregulation of LC3-II (30). Moreover, some recent studies demonstrated increased Beclin-1 protein levels in the hippocampus in STZ-induced HG rats (31) and in the retina in STZ-induced

HG mice (32). Therefore, upregulation of Beclin-1 can be observed in several types of neuronal tissue as well as optic nerve under STZ-induced HG condition. Thus, we next examined the localization of Beclin-1 in optic nerve.

Although LC3 immunoreactivity exists in nerve fiber in optic nerve (10), Beclin-1 immunoreactivity is present mainly in glia in optic nerve. Consistently, a previous study indicated that Beclin-1 expression was found in the primary astrocytes (33). On the other hand, because it was reported that Beclin-1 presents RGC bodies (24), and we observed partial colocalization of Beclin-1 and neurofilaments, it is likely that Beclin-1 also exists in neurons. In addition, it was shown that Beclin-1 mainly expressed in neuronal cells and scarcely

expressed in GFAP-positive astrocytes in mouse cerebral cortex slices (34). It was also shown that the colocalization of Beclin-1 and neuronal cells was observed in rat brain slices (35). Therefore, the distribution of Beclin-1 may vary depending on the type of neurons, but it may be present both in neurons and glia. It was assumed that intravitreal administration of siRNA downregulates the protein level of optic nerve which exists in neurofilaments (27). Our current morphometric analysis showed that the intravitreal administration of Beclin-1 siRNA failed to abolish the protective effect of HG. It is reasonable to speculate that intravitreal administration of Beclin-1 siRNA may affect the Beclin-1 expression in RGC bodies and their axons but not glial cells in optic nerve. Because it is difficult to distinguish the change in Beclin-1 protein level between intraaxon and glia in optic nerve after intravitreal injection of siRNA, this method (i.e., local knockdown) may have a limitation to address the role of protein which exists both in axon and glia. Nonetheless, since no significant difference in the axon number was seen in between the NG-TNF group and the HG-TNF with Beclin-1 siRNA treatment group, one hypothesis posits that Beclin-1 inside axons can play some roles for protective effect of HG. A protective role of Beclin-1 has been shown in a mouse neurodegeneration model (36). Further studies will be necessary to elucidate the detail role of Beclin-1 in axonal degeneration.

In conclusion, our findings suggest that Beclin-1 exists both in neurons and glia in optic nerve and enhanced Beclin-1 may be at least partially associated with axonal protection by HG induction.

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Availability of data and materials

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

Authors' contributions

KS, YK and CT performed experiments. KS, YK, CT and HT conceived and designed the research, and analyzed the data. KS, YK and HT wrote and revised the article. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of the Institute of Experimental Animals of St. Marianna University Graduate School of Medicine (approval no: 1610004).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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