Ghrelin Attenuates Retinal Neuronal Autophagy and Apoptosis in an Experimental Rat Glaucoma Model

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PURPOSE. Ghrelin, a natural ligand for the growth hormone secretagogue receptor type 1a (GHSR-1a), may protect retinal neurons against glaucomatous injury. We therefore characterized the underlying mechanism of the ghrelin/GHSR-1a-mediated neuroprotection with a rat chronic intraocular hypertension (COH) model.

METHODS. The rat COH model was produced by blocking episcleral veins. A combination of immunohistochemistry, Western blot, TUNEL assay, and retrograde labeling of retinal ganglion cells (RGCs) was used.

RESULTS. Elevation of intraocular pressure induced a significant increase in ghrelin and GHSR-1a expression in retinal cells, including RGCs and Müller cells. Western blot confirmed that the protein levels of ghrelin exhibited a transient upregulation at week 2 after surgery (G2w), while the GHSR-1a protein levels were maintained at high levels from G2w to G4w. In COH retinas, the ratio of LC3-II/LC-I and beclin1, two autophagy-related proteins, were increased from G1w to G4w, and the cleavage products of caspase3, an apoptotic executioner, was detected from G2w to G4w. Intraperitoneal injection of ghrelin significantly increased the number of surviving RGCs; inhibited the changes of LC3-II/LC-I, beclin1, and the cleavage products of caspase3; and reduced the number of TUNEL-positive cells in COH retinas. Ghrelin treatment also reversed the decreased levels of p-Akt and p-mTOR, upregulated GHSR-1a protein levels, and attenuated glial fibrillary acidic protein levels in COH retinas.

CONCLUSIONS. All these results suggest that ghrelin may provide neuroprotective effect in COH retinas through activating ghrelin/GHSR-1a system, which was mediated by inhibiting retinal autophagy, ganglion cell apoptosis, and Müller cell gliosis.

Keywords: ghrelin, GHSR-1a, chronic intraocular hypertension, autophagy, apoptosis, retina

Ghrelin, a 28-amino-acid peptide, was originally found to be secreted by gastric X/A-like cells. Acylated ghrelin, an activated form of ghrelin, is the natural ligand of the growth hormone secretagogue receptor type 1a (GHSR-1a). Subsequent studies have reported that ghrelin is widely distributed in many types of tissues and plays a pleiotropic role in feeding and energy metabolism, as well as in learning and memory, reward, sleep, and stress. Increasing evidence has also suggested that ghrelin exerts a neuroprotective effect in neurodegenerative diseases, such as Parkinson’s disease, Alzheimer’s disease, and Huntington’s disease. Ghrelin is found in ocular tissues and may be generated directly in rat retinas. The expression of ghrelin has been detected in the posterior epithelium of the iris, ciliary processes of rats, and in retinal Müller cells. Relative to ghrelin, GHSR-1a expression in ocular tissues is more extensive and has been found in the ciliary body, trabecular meshwork, retinal endothelial cells, choroid cells, and Müller cells, suggesting that the ghrelin–GHSR-1a system plays a physiological role in the retina. However, ghrelin may also be involved in many retinal diseases. In the aqueous humor of patients with glaucoma, ghrelin levels are significantly lower than that of control patients. Ghrelin treatment significantly decreases the intraocular pressure in rat and rabbit acute ocular hypertension models, and TUNEL positivity of retinal cells in a rat experimental glaucoma model, by reversing the increased levels of malondialdehyde and nitric oxide synthase-2 in anterior chamber fluid and tissues. These results suggest that the ghrelin–GHSR-1a system may provide a neuroprotective effect in glaucoma. However, the underlying mechanism of ghrelin/GHSR-1a system-mediated neuroprotection in glaucoma is not clearly known. In the present study, we therefore characterized the changes of retinal ghrelin–GHSR-1a expression in a rat chronic intraocular hypertension (COH) model. We also determined the effects and possible mechanisms of ghrelin on neuronal injury in rat retinas with COH.

METHODS

Animals and Rat COH Model

All animal procedures were carried out in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the guidelines of Fudan University.
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The detailed animal numbers used in each experiment are described. Three episcleral veins of the right eyes were carefully separated, ligated, and cauterized by using an OPMI VISU 140 microscope (Carl Zeiss, Oberkochen, Germany). In sham-operated control eyes, the surgery was performed by using a similar procedure except for the occlusion of veins. Intracocular pressure (IOP) was measured with a TonoLab rebound tonometer (Icare, Finland). All measurements were performed between 9:00 and 11:00 AM to avoid possible circadian differences. The average value of five consecutive measurements with a deviation < 5% was used in the analyses. The IOP of the right eye was measured immediately before the surgery as a baseline, after surgery (G0d), on the day following surgery (G1d), and then weekly afterward (G1w-G4w). This study used a total of 111 rats, including 29 rats as controls (11 for the whole flat-mounted experiments, 12 for the Western blot experiments, and 6 for the vertical slices) and 82 rats for the COH model (22 for the whole flat-mounted experiments, 36 for the Western blot experiments, and 24 for the vertical slices). The detailed animal numbers used in each experiment are listed in the Results section.

Ghrelin Administration

Rats with COH were injected intraperitoneally with ghrelin (40 μg/kg; Phoenix Pharmaceuticals, Inc., Burlingame, CA, USA), once daily for 7 days from G0d (immediately after surgery). COH rats receiving injections of an equal volume of normal saline (NS) were used as controls.

Immunohistochemistry

Immunohistochemistry was performed as previously described. For immunofluorescence double labeling, the sections were incubated with a cocktail of two primary antibodies overnight at 4°C. The primary antibodies used in the present study included polyclonal goat or rabbit anti-GHSR-1a (1:50; Santa Cruz Biotechnology, Santa Cruz, CA, USA, or Sigma-Aldrich Corp., St. Louis, MO, USA), polyclonal goat anti-ghrelin (1:50), monoclonal mouse anti-β-actin (1:100; Santa Cruz Biotechnology), and monoclonal mouse anti-glial fibrillary acidic protein (anti-GFAP, 1:500; Sigma). Binding of the primary antibodies was visualized by incubating with Cy3-conjugated donkey anti-mouse IgG and/or Alexa Fluor 488-conjugated donkey anti-rabbit or anti-goat IgG (1:800; Jackson ImmunoResearch Laboratories, West Grove, PA, USA). The samples were mounted with antifade mounting medium with 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA, USA) and the images were captured by using an Olympus confocal laser scanning microscope (Fluoview 1000; Olympus). All TUNEL-positive signals that merged with DAPI staining in each retina were counted for analyses. The images were acquired and exported with FV10-ASW Viewer 1.7 software (Olympus, Tokyo, Japan) and assembled with Adobe Photoshop 8.0.1 (Adobe Systems, Inc., San Jose, CA, USA).

Labeling and Quantification of RGCs

RGCs were retrogradely labeled by injecting cholera toxin subunit B (CTB; Sigma) into the superior colliculus bilaterally. The labeled RGCs were visualized by immunohistochemistry using anti-CTB antibody (1:4000; Sigma) in whole flat-mounted retinas. The images were captured by using an Olympus confocal laser scanning microscope through an ×40 objective (Fluoview 1000; Olympus). The number of CTB-positive RGCs was counted as previously described. Two fields, one from the central region and the other from the peripheral region, were selected at each of four angles of the retina (0°, 90°, 180°, and 270°). A total of eight fields were chosen from each retina, and the numbers of CTB-positive cells were counted.

Statistical Analysis

All data are expressed as the mean ± SEM. Statistical analyses were performed by using GraphPad Prism software (version 5.0; GraphPad Software, San Diego, CA, USA). A 1-way analysis of variance with Bonferroni’s post hoc test (multiple comparisons) was used as appropriate. A value of P < 0.05 was considered significant.

Results

Changes in the Expression of Ghrelin and GHSR-1a in COH Retinas

Similar to our previous reports, the rat COH model was successfully produced, with the average IOP of the operated eyes maintained at levels from 17.5 ± 0.2 mm Hg at G0d (n = 82) to 14.3 ± 0.1 mm Hg at G4w (n = 12), which was significantly higher than that of unoperated eyes (9.7 ± 0.1...
mm Hg, n = 82) and sham-operated eyes (9.4 ± 0.1 mm Hg, n = 29) (P all < 0.001).

Changes in ghrelin expression were then examined in COH retinas. As shown in Figure 1, weak ghrelin-positive fluorescence signals were detected in the sham-operated retinal sections (control, Ctr) (Fig. 1A, a1). Ghrelin-positive fluorescence signals increased at 2 and 3 weeks (G2w and G3w), and slightly declined at G4w (Fig. 1A, b1–e1). There was no fluorescent signal in the negative control sections (Fig. 1A, f1), in which PBS was used instead of anti-ghrelin IgG. Double labeling revealed that ghrelin was not colocalized with Brn-3a, an RGC marker (Fig. 1A, a3–e3). In contrast, many ghrelin-positive cells were doubly labeled with GFAP, a Müller cell marker, especially in the end-feet of the cells (Fig. 1B, a3–e3).

We further examined changes of GHSR-1a expression in COH retinas. Figure 2A shows that GHSR-1a expression was weak in sham-operated retinal sections (Ctr), but it was increased in COH rat retinal sections at G2w and G3w (Fig. 2A, c1, d1). Double immunostaining revealed the colocalization of GHSR-1a with Brn-3a, suggesting that GHSR-1a was expressed in RGCs (Fig. 2A, a3–e3), while GHSR-1a was sparsely colocalized with GFAP in the end-feet of Müller cells (Fig. 2B, a3–e3).

Western blot confirmed that the protein levels of ghrelin and GHSR-1a changed as a function of time in COH retinas. Figure 3A shows that the protein level of ghrelin was slightly declined at G4w (Fig. 3C). GHSR-1a increased at G2w (185.4% of control, n = 6, P = 0.001), and then declined to control levels (Fig. 3B). The GHSR-1a protein level started to increase at G2w (147.8% ± 1.1% of control, n = 6, P < 0.001), maintained a high level at G3w (143.9% ± 1.5% of control, n = 6, P < 0.001), and then slightly declined at G4w (Fig. 3C).

IOP Elevation Induces Retinal Neuronal Autophagy and Apoptosis

We tested whether IOP elevation induces retinal neuronal autophagy and apoptosis. Because LC3-II and beclin1 are typical biochemical markers for autophagy, we first detected changes of LC3-II/LC1 and beclin1 by Western blot of COH retinas. Figure 4 shows that the ratio of LC3-II/LC1 started to increase at G1w (119.3% ± 2.8% of control, n = 6, P = 0.019), but was maintained at this higher level at G4w (164.1% ± 7.5% of control, n = 6, P < 0.001) (Figs. 4A, 4B). Furthermore, the protein level of beclin1 increased to 132.6% ± 1.5% of the control (n = 6, P < 0.001) at G2w, with a peak at G3w (169.5% ± 3.0% of control, n = 6, P < 0.001), and then was maintained at this higher level at G4w (161.4% ± 7.9% of control, n = 6, P < 0.001) (Figs. 4A, 4B). The changes of autophagy-related proteins were also examined. The cleavage product of caspase3 revealed a significant increase in COH retinas. The average ratio of the cleavage product of caspase3 in COH retinas was partially reversed by ghrelin injection, with the average ratio of cleaved caspase3 to total caspase3 reduced to 168.8% ± 18.9% of the control values at G3w (n = 6, P = 0.005 vs. 263.6% ± 9.3% of control in NS-injected retinas, n = 6) (Fig. 5F). These results suggest that ghrelin protected RGCs from injury induced by IOP elevation by inhibiting autophagy and apoptosis.

Ghrelin Increases the Survival of RGCs by Reducing IOP Elevation-Induced Retinal Neuronal Autophagy and Apoptosis

Because the expressions of ghrelin and GHSR-1a in COH retinas increased and GHSR-1a was mainly localized to RGCs, it is possible that the neuroprotective effects of ghrelin were mediated by reducing retinal neuronal autophagy and apopto-
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FIGURE 1. Ghrelin expression in Müller cells and RGCs of COH rats. (A) Immunofluorescence labeling showing ghrelin (green) expression profiles in rat retinal vertical slices taken from sham-operated retina (Ctr) (a1), and those obtained at different postoperational times (G1w, G2w, G3w, and G4w) (b1–e1). f1 is a negative control staining, in which PBS was used as primary antibody instead of anti-ghrelin IgG. a2–f2 are corresponding Brn3a (red) and DAPI (blue) images. a3–f3 are merged images. (B) Immunofluorescence labeling showing ghrelin (green) expression profiles in rat retinal vertical slices taken from sham-operated retina (Ctr) (a1), and those obtained at different postoperational times (G1w, G2w, G3w, and G4w) (b1–e1), and a negative control staining (f1). a2–f2 are corresponding GFAP (red) and DAPI (blue) images. a3–f3 are merged images. Note that ghrelin is colocalized with GFAP, but not with Brn-3a. Scale bar: 10 μm, for all images. INL, inner nuclear layer; ONL, outer nuclear layer.
FIGURE 2. GHSR-1a expression in Müller cells and RGCs of rats with COH. (A) Immunofluorescence labeling showing GHSR-1a (green) expression profiles in rat retinal vertical slices taken from sham-operated retina (Ctr) (a1), and those obtained at different postoperational times (G1w, G2w, G3w, and G4w) (b1–e1). a2–e2 are corresponding Brn-3a (red) and DAPI (blue) images. a3–e3 are merged images. (B) Immunofluorescence labeling showing GHSR-1a (green) expression profiles in rat retinal vertical slices taken from sham-operated retina (Ctr) (a1), and those obtained at different postoperational times (G1w, G2w, G3w, and G4w) (b1–e1). a2–e2 are corresponding GFAP (red) and DAPI (blue) images. a3–e3 are merged images. Note that GHSR-1a is colocalized with both Brn-3a and GFAP. Scale bar: 10 μm, for all images.
strategy in the treatment of neurodegenerative diseases in the central nervous system. Our present results suggested that the ghrelin–GHRS-1a system is also a potential candidate for protection of RGCs during glaucoma.

Consistent with a previous report, our results revealed that ghrelin was mainly expressed in Müller cells in normal retinas. Elevated expression of ghrelin was observed in almost the entire COH retina, including the ganglion cell layer (GCL), the inner plexiform layer (IPL), and the outer plexiform layer (OPL) in COH retinas (Fig. 1). Furthermore, the expression of ghrelin in Müller cells was also increased, especially in the end-foot of the cells (Fig. 1). Although GHRS-1a expression was also increased in COH retinas, it was localized to RGCs (Fig. 2). These results suggest that the endogenous ghrelin–GHRS-1a system was activated, accompanied by elevation of the IOP, with ghrelin functioning in an autocrine or paracrine manner. Consistent with this possibility, it has previously been reported that ghrelin is generated locally in the retina. However, previous studies have reported that ghrelin levels in glaucoma patients decrease in the aqueous humor, but are unchanged in serum, which is inconsistent with our finding that the protein levels of ghrelin and GHSR-1a significantly increased in COH retinas. As an explanation for this inconsistency, it is possible that ghrelin is synthesized and released locally, and it is also possible that it has a short half-life because of its short length. It should be noted that Rocha-Sousa et al. have reported that ghrelin and GHSR-1 are expressed in the anterior segment of the eye, including ciliary body processes and trabecular meshwork, which may be responsible for a hypotensive effect in acute ocular hypertension animal models.

Some evidence suggests that the protective effect of ghrelin in glaucoma may result from its hypotensive effect. For example, subconjunctival injection of ghrelin decreases the IOP levels in rats and rabbits with acute ocular hypertension. Ghrelin injection dilates the vessels and reduces the mean arterial pressure in patients with chronic heart failure. Ghrelin may also relax both the iris sphincter and dilator muscles in eyes. However, in the present study, intraper-
Internal injection of ghrelin did not significantly change the elevated IOP in COH eyes, and a recent study has reported that after the IOP is increased, intraperitoneal treatment of ghrelin every day for 14 days does not significantly change the IOP in a rat experimental glaucoma model. We speculate that different delivery methods and/or delivery times, such as before or after the IOP elevation, may have influenced the effect of ghrelin on the IOP in COH eyes. This issue should be addressed in future studies.

A major finding of the present study was that ghrelin may provide retinal neuronal protection by inhibiting neuronal autophagy and apoptosis in COH retinas, which is supported by the following evidence. First, ghrelin treatment rescued the IOP elevation-induced increase in protein levels of the autophagy-related proteins LC3-II/I and beclin1 (Fig. 5). During physiological conditions, autophagy plays an important role in maintaining cellular homeostasis. However, impaired autophagy could contribute to neurodegenerative disorders. Usually, beclin1 and LC3-II are considered biomarkers of ongoing autophagy; they are involved in the nucleation and assembly of the isolation membrane and/or phagophore elongation step during autophagosome formation. LC3-II, a lipidated form of LC3, is generated from the modification of LC3-I (a cytosolic form of LC3) and is the only protein marker that is reliably associated with completed autophagosomes. Additionally, we found that ghrelin treatment rescued the decreased activity of the Akt/mTOR signaling pathway in COH retinas, further confirming the involvement of autophagy inhibition in the effects of ghrelin (Fig. 6). Second, ghrelin treatment significantly reduced the IOP elevation-induced increase in the protein levels of the cleaved caspase3, and the number of TUNEL-positive cells in COH retinas (Fig. 5).

**Figure 5.** Ghrelin treatment increases the survival of RGCs by inhibiting autophagy and apoptosis in glaucoma rats. (A) Images of two representative regions, central and peripheral ones, taken from sham-operated (Ctr) (a1, b1), vehicle (NS)-injected (a2, b2), and ghrelin-injected (a3, b3) whole flat-mounted retinas at 3 week (G3w) of postoperational time. An area located at less than 1.0 mm (commonly between 200 and 830 μm) distant from the optic nerve head (ONH) was defined as the central region, and that located between 3.0 and 3.63 mm distant from the ONH as the peripheral region. RGCs were retrogradely labeled by CTB (red). Scale bar: 50 μm, for all images. (B) Microphotographs showing representative confocal images of TUNEL signals (green) in whole flat-mounted retinas taken from sham-operated (Ctr) (a1, a3), vehicle (NS)-injected (b1, b3), and ghrelin-injected (c1, c3) rat retinas at 3 week (G3w) of postoperative time. Scale bar: 10 μm, for all images. (C) Representative immunoblots showing the changes of LC3-II/LC3-I, beclin1, and caspase3 expression in sham-operated (Ctr), vehicle (NS)-injected, or ghrelin-injected retinas at 3 week (G3w) of postoperative time. The loading samples in each group were from two different rat retinas. (D–F) Bar chart summarizing the average densitometric quantification of immunoreactive bands of LC3-II/LC3-I ratio (D), beclin1 (E), and cleavage product of caspase3 to caspase3 ratio (F) under different conditions as shown in (A). All the data are normalized to control. n = 6 for all groups, *P < 0.05 and **P < 0.001 versus Ctr; #P < 0.05 versus G3w+NS.
Apoptosis is the culmination of a complex series of molecular events, including activation of caspases in injured cells. Caspase3 plays a central role in the execution phase of cell apoptosis, which is activated by both intrinsic and extrinsic apoptotic pathways. Caspase3 zymogen has no activity until it is cleaved into 17-kDa and 12-kDa subunits, which are considered to be markers of apoptosis. The anti-apoptotic activity of ghrelin has been reported in numerous of pathophysiological conditions, such as ischemia, inflammation, and nutrient deprivation. In an experimental glaucoma model, ghrelin treatment reduces the TUNEL positivity in COH retinas by inhibiting the levels of oxidation products in anterior chamber fluid, suggesting that the anti-apoptotic effect of ghrelin is mediated by acting on the anterior segment of the eye. It should be noted that ghrelin treatment did not completely rescue the IOP elevation-induced increase in protein levels of the cleaved caspase3, suggesting that neuronal apoptosis in COH retinas involves multiple paths. Our results also showed that ghrelin treatment induced an increase in protein levels of GHSR-1a (Fig. 6). Because GHSR-1a is localized to RGCs, it is possible that the elevated expression of GHSR-1a may provide a direct protective role for RGCs against apoptosis during ghrelin treatment. Consistent with this possibility, we observed that ghrelin treatment increased the survival of RGCs in COH retinas (Fig. 5).

In COH retinas, Müller cells may undergo gliosis. Activated Müller cells may contribute to RGC apoptosis by releasing several harmful factors, such as TNF-α, IL-1, and nitric oxide. Our results revealed that ghrelin treatment partially attenuated the GFAP protein levels in COH retinas, suggesting that neuroprotection of the ghrelin-GHRS-1a system is mediated by inhibition of Müller cell gliosis, which is consistent with a report by Can et al. Based on past reports and the present study, there is ample reason to further study the detailed mechanisms involved in the ghrelin-GHRS-1a system-induced suppression of Müller cell gliosis.

**Conclusions**

Ghrelin and GHSR-1a were upregulated in RGCs and Müller cells in COH retinas. Ghrelin treatment provided neuroprotective effects by suppressing the elevated autophagy and apoptosis, and partially inhibiting Müller cell gliosis in COH retinas.

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