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 is also determined the effects and possible mechanisms of ghrelin 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.
Shanghai, China, on the ethical use of animals. Male Sprague-Dawley rats (weighing 100–150 g) were obtained from Shanghai Laboratory Animal Center Co., Ltd. (SLAC; Shanghai, China) and kept on a 12-hour light/dark schedule, with standard chow and drinking water provided ad libitum.

The rat COH model was produced by following a previous procedure. 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. Intraocular 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 whole flat-mounted experiments, 12 for the Western blot experiments, and 6 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–Brn-3a (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 cyanine-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 using an FV1000 confocal laser scanning microscope (Olympus, Tokyo, Japan). To avoid any possible reconstruction stacking artifacts, double labeling was precisely evaluated by sequential scanning using single-layer optical sections at intervals of 1.0 μm.

Western Blot Analyses

Western blot analyses were performed as previously described. Protein samples (20 μg) were separated by using 10% or 15% SDS-PAGE. The primary antibodies included monoclonal mouse anti–β-actin (1:8000) and monoclonal mouse anti-GFAP (1:1000; Sigma), polyclonal goat anti–GHSR-1a (1:1000), polyclonal goat anti-ghrelin (1:1000), polyclonal rabbit anti–phospho-mTOR (1:1000), polyclonal rabbit anti-nmTOR (1:1000), goat anti-caspase3 (1:1000; Santa Cruz Biotechnology), rabbit anti-IC3 (1:1000), rabbit anti-beclin1 (1:1000; Abcam, Cambridge, MA, USA), and rabbit anti–phospho-mTOR (1:1000; Cell Signaling Technology, Danvers, MA, USA). The horseradish peroxidase-conjugated anti-goat or anti-mouse or anti-rabbit IgG (1:8000; Jackson ImmunoResearch Laboratories) were used as the secondary antibodies. The blots were visualized by using the ECL enhanced chemiluminescent reagent (Thermo Scientific, Rockford, IL, USA) and scanned with a FluorChem E imaging system (ProteinSimple, Santa Clara, CA, USA). The density of bands was quantitatively analyzed with National Institutes of Health (Bethesda, MD, USA) image analysis software. The density of each band was expressed as the ratio with the actin band and was normalized to the control bands.

Detection of Apoptotic Cells Using the TUNEL Assay

The TUNEL assay was performed in whole-mounted retinas with Dead End Fluorometric TUNEL System G3250 kit (Promega, Madison, WI, USA), following the manufacturer’s instructions. The whole-mounted retinas were counterstained with DAPI for labeling the nuclei, and scanned in serials at an interval of 1 μm by a 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 fluorescent signals were detected in the sham-operated retinal sections (control, Ctr) (Fig. 1A, a1). Ghrelin-positive fluorescent 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 by 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 significantly increased at G2w (185.4% ± 16.1% 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 (145.9% ± 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/LC-I and beclin1 by Western blot of COH retinas. Figure 4 shows that the ratio of LC3-II/LC-I started to increase at G1w (119.3% ± 2.8% of control, n = 6, P = 0.019), and further 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) (Figs. 4A, 4B). Furthermore, the protein level of beclin1 increased to 132.5% ± 3.0% of the control at G1w (n = 6, P = 0.018), and was maintained at this higher level through G3w (127.3% ± 5.0% of control at G3w, n = 6, P = 0.038), and then declined to control levels at G4w (Figs. 4A, 4C). The changes of apoptosis-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 to total caspase3 increased to 177.4% ± 12.2% of the control (n = 6, P = 0.011) at G2w, and continually increased to 399.8% ± 15.3% of the control (n = 6, P < 0.001) at G4w (Figs. 4A, 4D).

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 apoptosis. To test this possibility, ghrelin was administrated intraperitoneally in COH rats, which did not change the elevated IOP in the operated eyes. Ghrelin treatment increased the survival of RGCs in COH rats (Fig. 5A). The average number of CTB-positive RGCs in retinas obtained from NS-injected COH rats at G3w decreased to 114.6 ± 4.5/field (n = 5, P = 0.001) from the control of 212.2 ± 12.8/field (n = 5), which was reversed by ghrelin treatment to 185.5 ± 10.2/field (n = 5, P = 0.036 versus NS-injected COH retinas).

Ghrelin injection consistently inhibited RGC apoptosis in COH retinas assayed by the TUNEL staining technique in whole flat-mounted retinas. Figure 5B shows that sparse TUNEL-positive signals were detected in retinal sections obtained from sham-operated eyes (Ctr) (a1, a3), but a significant increase in the number of TUNEL-positive signals was observed in COH retinas at G3w (b1–b3). Ghrelin injection significantly reduced the number of TUNEL-positive signals in COH retinas at G3w (c1–c5). The average total number of TUNEL-positive cells in whole flat-mounted retinas increased from a control value of 75.4 ± 11.2 (n = 6) to 161.0 ± 22.9 (n = 6, P = 0.005) in COH retinas, while it decreased to 99.0 ± 10.4 after ghrelin treatment of COH retinas (n = 6, P = 0.017 versus NS-injected retinas, and P = 0.796 versus control). Furthermore, the protein levels of LC3-II/LC3-I and beclin1 in retinas obtained from ghrelin-injected rats at G3w were significantly reduced (n = 6, P = 0.017 and P = 0.021 versus NS-injected retinas, respectively) (Figs. 5C–5E). The increase in cleavage products 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.

It has been previously reported that the Akt/mTOR signal pathway negatively regulates autophagy and is implicated in cell survival. Both Akt and mTOR activities decreased in COH retinas, as shown by decreased ratios of p-Akt/Akt (67.3% ± 7.2% of control, n = 6, P = 0.005) and p-mTOR/TOR (63.4% ± 2.5% of control, n = 6, P = 0.008), but ghrelin treatment reversed the decreased ratios of p-Akt/Akt and p-mTOR/TOR to control levels (Figs. 6A–6C).

Previous studies have suggested that Müller cell reactivation (gliosis), as evidenced by elevated expression of GFAP, contributes to RGC apoptosis in glaucoma. Because GHSR-1a was expressed in Müller cells (Fig. 2), we investigated whether ghrelin injection affected GFAP expression in COH retinas. Consistent with our previous report, Figure 6 shows that GFAP expression was significantly increased in NS-injected COH retinas at G3w (399.5% ± 4.9% of control, n = 6, P < 0.001). Ghrelin injection partially reduced the GFAP protein level to 266.8% ± 15.1% of the control values (n = 6, P = 0.006 versus NS-injected retinas and P = 0.003 versus control) (Figs. 6D, 6F). Additionally, ghrelin injection induced an increase in GHSR-1a expression in COH retinas at G3w, with an average of 161.9% ± 2.5% of the control values (n = 6, P = 0.019 versus NS-injected retinas and P < 0.001 versus control) (Figs. 6D, 6E).

DISCUSSION

Glaucoma is a retinal neurodegenerative disease with characteristic optic nerve head damage and progressive visual field loss that is associated with RGC death. Currently, there is no approved neuroprotective treatment for glaucoma. Previous studies have reported that activation of the ghrelin–GHSR-1a system may be an innovative palliative and neuroprotective
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.\(^{15,35}\) 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,\(^{16}\) 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 endfoot 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.\(^{16}\) However, previous studies have reported that ghrelin levels in glaucoma patients decrease in the aqueous humor, but are unchanged in serum,\(^{36}\) 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.\(^{37,38}\) It should be noted that Rocha-Sousa et al.\(^{18}\) 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.\(^{17,18}\) Ghrelin injection dilates the vessels\(^{39}\) and reduces the mean arterial pressure in patients with chronic heart failure.\(^{40}\) Ghrelin may also relax both the iris sphincter and dilator muscles in eyes.\(^{17,41}\) However, in the present study, intraper-
Itral 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 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 lipiddated 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).
activity of ghrelin has been reported in numerous of pathophysiological conditions, such as ischemia, inflammation, and nutrient deprivation.50–53 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.22 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.24 Activated Müller cells may contribute to RGC apoptosis by releasing several harmful factors, such as TNF-α, IL-1, and nitric oxide.54,55 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.22 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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**References**

Neuroprotective Role of Ghrelin in Glaucoma


