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### Ghrelin Modulates Physiologic and Pathologic Retinal Angiogenesis through GHSR-1a

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**PURPOSE.** Vascular degeneration and the ensuing abnormal vascular proliferation are central to proliferative retinopathies. Given the metabolic discordance associated with these diseases, the authors explored the role of ghrelin and its growth hormone secretagogue receptor 1a (GHSR-1a) in proliferative retinopathy.

**METHODS.** In a rat model of oxygen-induced retinopathy (OIR), the contribution of ghrelin and GHSR-1a was investigated using the stable ghrelin analogs [Dap3]-ghrelin and GHRP6 and the GSHR-1a antagonists JMV-2959 and [D-Lys3]-GHRP6. Plasma and retinal levels of ghrelin were analyzed by ELISA, whereas retinal expression and localization of GHSR-1a were examined by immunohistochemistry and Western blot analysis. The angiogenic and vasoprotective properties of ghrelin and its receptor were further confirmed in aortic explants and in models of vaso-obliteration.

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Corresponding author: Sylvain Chemtob, Departments of Pediatrics, Ophthalmology and Pharmacology, Research Center, Sainte-Justine Hospital, Montreal, Quebec, Canada, H3T 1C5; sylvain.chemtob@umontreal.ca. **RESULTS.** Ghrelin is produced locally in the retina, whereas GHSR-1a is abundantly expressed in retinal endothelial cells. Ghrelin levels decrease during the vaso-obliterative phase and rise during the proliferative phase of OIR. Intravitreal delivery of [Dap3]-ghrelin during OIR significantly reduces retinal vessel loss when administered during the hyperoxic phase. Conversely, during the neovascular phase, ghrelin promotes pathologic angiogenesis through the activation of GHSR-1a. These angiogenic effects were confirmed ex vivo in aortic explants.

Conclusions. New roles were disclosed for the ghrelin-GHSR-1a pathway in the preservation of retinal vasculature during the vaso-obliterative phase of OIR and during the angiogenic phase of OIR. These findings suggest that the ghrelin-GHSR-1a pathway can exert opposing effects on retinal vasculature, depending on the phase of retinopathy, and thus holds therapeutic potential for proliferative retinopathies. (*Invest Ophthalmol Vis Sci.* 2011;52:5376–5386) DOI:10.1167/iovs.10-7152

**P**roliferative ischemic retinopathies such as proliferative diabetic retinopathy (PDR) and retinopathy of prematurity (ROP) are leading causes of blindness in the industrialized world. They are both characterized by an initial phase of microvessel loss that yields a hypoxic retina and stimulates a second phase of excessive preretinal blood vessel growth that can ultimately lead to fibrous scar formation and culminate in retinal detachment.<sup>1</sup> In ROP, the vascular degeneration is caused by excessive reactive oxygen species and insufficient levels of physiologic growth factors,<sup>2</sup> whereas hyperglycemia is thought to provoke vessel loss in PDR.<sup>1</sup> In both diseases, tissue hypoxia ensues as a result of loss of functional vasculature and leads to the induction of a number of hypoxia-regulated angiogenic factors that mark the second neovascular phase of retinopathy.

Ghrelin, a gastrointestinal endocrine peptide, regulates food intake and is tightly associated with obesity.<sup>3</sup> It also plays a role in glucose and insulin metabolism.<sup>4,5</sup> Recent studies have shown that the plasma ghrelin level is altered in diabetic patients. Moreover, plasma ghrelin level is also significantly lower in patients with diabetic triopathy compared with patients without diabetes-related vascular complications, making it an interesting candidate to study in the context of proliferative retinopathy.<sup>6</sup>

Of all the contributors to ROP, premature birth remains the greatest risk factor,<sup>7</sup> suggesting that factors present in utero required for normal fetal development may be lacking in infants born prematurely. Such is the case for IGF-1, a polypeptide protein hormone whose fetal plasma levels rise with gestational age and considerably increase during the third trimester of pregnancy but are considerably lower in premature infants born early during the last trimester.<sup>8-10</sup> IGF-1 potentiates the maximal VEGF-induced activation of Akt in endothelial

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cells and therefore contributes to vessel survival,<sup>9</sup> which is essential to prevent the first and instigator phase of ROP.

During the second, proliferative phase of retinopathy, the retinal tissue holds excessively elevated levels of growth factors such as VEGF<sup>11,12</sup> and erythropoietin (Epo).<sup>13,14</sup> Given the permissive role of IGF-1 for VEGF-induced activation of p44/42 MAPK (essential for endothelial cell proliferation), IGF-1 was identified as a key regulator of this second, neovascular phase of ROP.<sup>10</sup> Similar to Epo, IGF-1 thus acts as a double-edged sword in retinopathy: during the early phase of vessel loss, IGF-1 (and Epo) can be protective and reduce the extent of vascular damage; during the second phase, however, both IGF-1 and Epo may augment pathologic vessel growth.<sup>13,14</sup> One modulator of IGF-1 activity is ghrelin, a 28-amino acid blood-borne orexigenic peptide hormone that can dose-dependently stimulate the release of IGF-1<sup>15</sup> and dose-dependently regulate the GH-IGF-1 axis.<sup>16</sup> Ghrelin is predominantly generated in the gut and presents a range of metabolic and cardiovascular functions.<sup>17</sup> It is thought to generate its biological effects through activation of its growth hormone secretagogue receptor-1a (GHSR-1a), a 7 transmembrane G-protein-coupled receptor (GPCR).<sup>16</sup> Importantly, when activated, GHSR-1a exerts many effects-including anti-inflammatory, antiapoptotic,<sup>19</sup> and proangiogenic<sup>20</sup>—that can be considered both beneficial and detrimental in the context of retinopathy. It has been shown that ghrelin is also expressed in the rodent eye with the highest expression level in the retina and iris.<sup>21</sup> Together, this led us to examine the effects of ghrelin and its receptor, GHSR-1a, during different phases of the oxygeninduced ischemic retinopathy (OIR) rat model. Our results demonstrate that ghrelin is produced locally in the retina and, through GHSR-1a, protects retinal vessels against oxygen-induced vaso-obliteration during the early phase of retinopathy. During the second, angiogenic phase, elevated ghrelin levels (through its receptor GHSR-1a) contribute to the aberrant formation of pathologic neovessels.

#### **MATERIALS AND METHODS**

#### Animal Models of Oxygen-Induced Ischemic Retinopathy

Newborn litters of Sprague-Dawley rats (Charles River Laboratory, St. Constant, Quebec, Canada) were used according to a protocol of the Hôpital Sainte-Justine Animal Care Committee. Rats were housed in a controlled 12-hour light/12-hour dark environment. All procedures were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

OIR was induced in the newborn rats with two different oxygenexposure paradigms, as described previously.<sup>22-24</sup> Newborn rats at postnatal day (P) 0 were placed in airtight containers filled with oxygen between 50%  $\pm$  1% and 10%  $\pm$  1% interchanged every 24 hours throughout the first two postnatal weeks.<sup>23</sup> Animals were killed on P17 or P18, allowing maximum neovascularization. For vaso-obliteration protocols, rats were placed in an 80%  $\pm$  5% oxygen environment from P5 to P10. Rats were killed right after the return to room air. Controls were maintained in room air (21% O<sub>2</sub>) throughout the entire development. All rat pup weights were monitored closely to remove runty animals and thus exclude differences in weight gain as possible confounders.<sup>25</sup>

#### Immunohistochemistry Staining

Retinal whole mounts or eye cryosections  $(12-\mu m \text{ thick})$  were prepared for immunohistochemistry and assessed by epifluorescence microscopy (E800; Eclipse; Nikon, Tokyo, Japan). Animals were first perfused with PBS followed by 4% paraffin-formalin aldehyde, and eyes were enucleated for the preparation of retinal whole mount or cryosections. Retinas, after separation from choroids, were first permeabilized with methanol and then stained with TRITC-labeled lectin from *Griffonia simplicifolica* (catalog no. L5264; Sigma-Aldrich, St. Louis, MO) and flat mounted to reveal the retinal vasculature system. Eye cryosections were rinsed with PBS and then blocked in PBS with 0.5% Triton X-100 and 3% BSA. The sections were stained with affinity-purified polyclonal antibody against GHSR-1a (Ab 373) developed in our laboratory, cellular retinaldehyde-binding protein (CRALBP; Affinity BioReagents, Golden, CO), and glial fibrillary acidic protein (GFAP 100  $\mu$ g; Oncogene, Cambridge, MA) followed by AlexaFluor secondary antibody against respective species for the detection of specific cell types in the retina.

#### **Cell Cultures**

Porcine brain microvascular endothelial cells (PBMECs; Cell Systems, Kirkland, WA), rat Müller cells (kindly provided by Adriana DiPolo, University of Montreal), and astrocytes (Lonza, Walkersville, MD) were suspended in endothelial basal medium-2 (EBM-2) with a supplemental kit, Dulbecco's modified Eagle's medium, and acidic bold-basal medium with a supplemental kit, respectively, containing 10% fetal bovine serum (FBS), seeded in 75-cm<sup>2</sup> flasks and placed in a humidified atmosphere at 37°C until a confluent monolayer of cells was observed. Confluent endothelial cells were then harvested and resuspended in cell lysate buffer for Western blot analysis.

#### Western Blot Analysis

Protein was extracted from either retina tissue homogenates or cell cultures, as mentioned. Bradford protein assay<sup>26</sup> was used to determine the total protein concentration. Approximately 50 µg total protein extract was loaded on SDS-polyacrylamide gels and run at 100 V, followed by transfer onto a polyscreen PVDF transfer membrane (PerkinElmer, Waltham, MA) by electroblotting, as previously described.<sup>27</sup> Solution of 5% skim milk (MP Biomedicals, Irvine, CA) in TBS/Tween-20 buffer (137 mmol NaCl, 20 mmol Tris-HCl, pH 7.4, 0.1% Tween-20) was used to block for 1 hour at room temperature. Specific primary antibody against IGF-1 (Millipore, Billerica, MA), VEGF (Santa Cruz Biotechnology, Santa Cruz, CA), IGF-1R (Chemicon, Temecula, CA), GHSR-1a (Ab 373, developed in our laboratory), or β-actin (Sigma) was added to the membrane, followed by an anti-rabbit-IgG or antimouse- IgG HRP-horseradish peroxidase-conjugated secondary antibody (dilution 1:5000) dissolved in 2% BSA in TBS-T buffer. Details regarding washing and film development procedures were as described.<sup>28</sup> All the Western blot analyses were repeated three times with different samples.

### Intravitreal Injections and Developmental Growth Assessment

[Dap<sub>3</sub>]-Ghrelin (81 nM final intravitreal concentration; Peptide International; IC50 8.1 nM29), JMV-2959 (56 nM independently or 560 nM when together with [Dap<sub>3</sub>]-Ghrelin; Aeterna Zentaris; IC<sub>50</sub> 5.6 nM), GHRP-6 (24 nM; Europeptides; IC50 2.4 nM30), [D-Lys3] GHRP-6 (24 nM independently or 240 nM when together with GHRP-6; Europeptides;  $IC_{50}$  32  $\mu$ M<sup>31</sup>) or control (0.9% saline) was injected intravitreally into the rat pups using a 10-µL Hamilton syringe with a glass-pulled capillary. The injection was delivered through the sclera, and the tip of the needle was inserted at a 45° angle into the vitreous space. When accessing the role of Ghrelin developmentally, animals were injected at P1 (0.5  $\mu$ L) and P4 (1  $\mu$ L) and were killed at P6 to quantify vasculature growth. During the neovascularization phase of OIR, animals were injected at P14 (2 µL) and P16 (2 µL) before sacrifice at P18 for quantification of neovascularization. In case of vaso-obliteration, injections were performed at P7 and P9 during the hyperoxic exposure, and animals were killed at P10 right after removal from the oxygen chamber.

## Quantification of Vaso-obliteration and Retinal Neovascularization

The retinal vasculature scoring system has been described previously<sup>32</sup> (also shown in detail in Supplementary Fig. S2, http://www.iovs.org/

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lookup/suppl/doi:10.1167/iovs.10-7152/-/DCSupplemental). Quantifications of the vaso-obliterative zone and neovascular vessel formation were carried out as previously described.<sup>33,34</sup> Partial images of the G. simplicifolica lectin (GS lectin)-stained retinal flatmounts were taken at  $\times 4$  magnification with an epifluorescence microscope (E800; Eclipse; Nikon) and were imported into image editing software (Photoshop; Adobe, San Jose, CA). Retinal segments were then merged to produce an image of the entire retina by the automated photomerge function (Photoshop, version CS3; Adobe). Total retinal areas and the avascular zones were traced in an imaging program (ImagePro Plus 4.5; Media Cybernetics, Silver Spring, MD), and the number of pixels was calculated. The calculated number of pixels in the affected areas was then converted to area in unit of square millimeters (ImagePro Plus 4.5; Media Cybernetics). The affected area was identified as previously described.<sup>33,35</sup> Vessels were identified with the red staining from GS lectin. Vessel densities were expressed as the ratio of pixel numbers of vascularized area to the total pixels of the entire image (ImagePro Plus 4.5; Media Cybernetics). Vessel tortuosity was graded on a scale of 0 to 3, with 0 indicating the absence of curved vessels and 3 indicating massive number of curved vessels.

#### **Thymidine Incorporation**

As described previously,<sup>36</sup> PBMECs were seeded in 24-well plates with a concentration of 15,000 cells/well and were treated with either  $10^{-8}$  M Dap-ghrelin,  $10^{-8}$  M JMV-2959, or  $10^{-8}$  M Dapghrelin plus  $10^{-7}$  M JMV-2959 for 24 hours. After starving with serum-free medium for 4 hours, 50 µL of 1 µCi/mL [3H] thymidine was added to each well and incorporated for 6 hours. After incorporation, ice-cold 5% trichloroacetic acid was used to wash each well four times. Cells were then lysed with 200 µL of 0.1% Triton/ 0.1 N NaOH and were transferred to counting vials, together with 4 mL scintillation fluid. The  $\beta$  radiation signals were then counted on a multipurpose scintillation counter (LS 6500; Beckman Coulter, Hialeah, FL).



FIGURE 1. The ghrelin receptor GHSR-1a is robustly expressed in the retina. (a) Retinal cross-sections stained with anti-GHSR-1a revealed the total distribution of the receptor in the rat retina. Scale bar, 30 µm. (b-d) Colocalization revealed GHSR-1a expression in both endothelial cells (lectin; b) and Müller cells (CRALBP; c) but not in astrocytes (GFAP; d). Scale bar, 30 µm. (e) Representative image of Western blot analysis indicating GHSR-1a receptor expression in various retinal cell types, confirming the predominant vascular expression of this receptor. (f) Densitometry quantification of relative GHSR-1a expression in various neural cell types (n = 3).

#### **Aortic Explants Microvascular Sprouting**

Aortas from adult Sprague-Dawley rats were dissected and cut into 1-mm-thick rings. After they were washed in PBS, aortic rings were placed in growth factor-reduced extracellular matrix (Matrigel; BD Biosciences, Franklin Lakes, NJ) in 24-well tissue culture plates and were cultured for 6 days in EBM-2 media with EGM supplemental kit and 10% FBS containing either vehicle (saline), Dap-ghrelin  $(10^{-8} \text{ M})$ , JMV-2959  $(10^{-8} \text{ M})$ , or Dap-ghrelin + JMV-2959  $(10^{-8} \text{ M} \text{ and } 10^{-7} \text{ M})$ , respectively). Microphotographs of individual explants with their microvascular sproutings were taken on day 6. Imaging software (ImagePro Plus 4.5; Media Cybernetics) was used to calculate the area covered by endothelial outgrowth.

#### ELISA of Ghrelin in Retina and Plasma

Frozen retina from one eye was homogenized in 150  $\mu$ L acetonitrile-1% trifluoroacetic acid in water mixture (70:30 vol/vol) using a micro dual tissue grinder (Kimble Kontes size 19; Fisher Scientific, Pittsburgh, PA). The homogenate was then centrifuged at 17,000*g* for 10 minutes at 4°C, and the supernatant was collected in an Eppendorf tube. The supernatant was delipidated with hexane (Fisher Scientific) by vortexing. The hexane phase was then discarded, and the acetonitrile extract was evaporated to dryness (Savant SpeedVac Concentrator; Thermo Scientific, Pittsburgh, PA). The residue was resuspended in 30  $\mu$ L matrix solution of the ghrelin ELISA kit (Millipore). Plasma samples collected in EDTA and protease inhibitor (Pefabloc 1 mg/mL; Roche Applied Science, Indianapolis, IN) were first acidified with 1 N HCl

(100  $\mu$ L/mL plasma) and centrifuged (3000g, 5 minutes at 4°C). Ghrelin levels were determined on acidified plasma samples according to the manufacturer's instructions on the ELISA kit (Millipore).

#### **Statistical Analysis**

Data are presented as mean  $\pm$  SEM. Comparisons were made between groups by either Student's *t*-test (two groups only) or one-way analysis of variance followed by post hoc Bonferroni's test for comparison among means. *P* < 0.05 was considered statistically significant.

#### RESULTS

### The Ghrelin Receptor GHSR-1a Is Robustly Expressed in the Retina

To establish the contribution of ghrelin-GHSR-1a in retinal angiogenesis, we first investigated the expression of its highaffinity GPCR GHSR-1a in the retina. Immunohistochemical analysis on retinal cryosections reveals robust expression of GHSR-1a in the retina and choroid, with vascular structures showing the most intense staining (Fig. 1a). Colocalization with the endothelial cell (EC) marker *G. simplicifolica* lectin (GS lectin) confirmed the prominent expression of GHSR-1a in retinal ECs (Fig. 1b). Expression of GHSR-1a also overlaps with the Müller cell marker CRALBP (Fig. 1c), whereas other glia, such as astrocytes, are devoid of GHSR-1a (Fig. 1d). These



FIGURE 2. Ghrelin is generated directly in the retina and is modulated in OIR. (a) Retinal expression of ghrelin (mRNA) from P7 rats exposed to high oxygen for 48 hours (P5-P7) and age-matched animals raised in room air (mean ± SEM; \*\*P < 0.01 compared with normoxia control; n = 4). (b) Expression of ghrelin mRNA in P18 OIR rats (50%/ 10% alternating oxygen; PO-P14) and age matched in room air raised animals (mean  $\pm$  SEM; \*P < 0.05compared with normoxia control; n = 4). (c-e) Colocalization revealed Ghrelin expression in Müller cells (specifically in Muller cell end-feet) (CRALBP; c) but not in endothelial cells (lectin; d) or astrocytes (GFAP; e). Scale bar, 30  $\mu$ m.

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results were confirmed by Western blot on candidate retinal cell populations, with retinal ECs showing the highest levels of expression (Figs. 1e, 1f).

## Ghrelin Is Generated Directly in the Retina and Is Modulated in OIR

In light of the pronounced expression of GHSR-1a in the retina, we next sought to determine whether ghrelin itself was produced in the retina. Classically, ghrelin is reported to be produced in the gut, where it stimulates gastrointestinal motility and appetite and is also known to be produced in the arcuate nucleus, where it can trigger the secretion of growth hormone (GH) from the anterior pituitary gland.<sup>17</sup> By real-time PCR, our

data demonstrate that ghrelin is also produced in the retina (Fig. 2). Of importance in the context of OIR, the retinal expression of ghrelin mRNA decreases by half during the vaso-obliterative phase (Fig. 2a; P < 0.01) and is induced twofold during the vasoproliferative phase (Fig. 2b; P < 0.05).

To determine the origin of ghrelin in the retina, we performed immunohistochemical analysis of ghrelin on retinal sagittal sections. Colocalization of ghrelin with the Müller cell marker CRALBP in the innermost retinal layer reveals a Müller cell expression pattern, predominantly in the endfeet (Fig. 2c). Neither retinal ECs (stained by GS lectin) nor other glia such as astrocytes (stained by GFAP) express ghrelin (Figs. 1d, 1e). This expression pattern, taken with that of GHSR-1a (Fig. 1),



FIGURE 3. Ghrelin protects retinal endothelial cells against hyperoxiainduced vaso-obliteration through GHSR-1a. (a-c) Representative images of retinal whole mounts stained with G. simplicifolica lectin from P10 animals after intravitreal injection with vehicle (saline; a), [Dap3]-Ghrelin (81 nM; b), or GHRP-6 (24 nM; c) during hyperoxic exposure. Red tracings: borders of vaso-obliteration zone. Scale bar, 1 mm. (d) Quantification of vaso-obliteration area after intravitreal administration of GHSR-1a agonist confirm the vasoprotective effects of the agonist (mean  $\pm$  SD; \*\*\*P < 0.001 compared with vehicle control; n = 6). (e-l) Representative high-magnification images of G. simplicifolica lectinstained retinal whole mounts from P10 animals after intravitreal injection with vehicle (f), [Dap3]-Ghrelin (81 nM; g) JMV-2959 (56 nM; h), or both (81 nM [Dap3]-Ghrelin and 560 nM JMV-2959; (i), as well as GHRP-6 (24 nM; j), [D-Lys3]-GHRP-6 (24 nM; k), or both (24 nM GHRP-6 and 240 nM [D-Lys3]-GHRP-6; 1), after hyperoxic exposure. Normoxic vehicle controls (e). Scale bar, 200 µm. Vessels were revealed by red fluorescence detected by fluorescence microscopy.

suggests a paracrine loop between Müller cells and vessels with respect to the actions of ghrelin.

#### Stimulation of GHSR-1a Protects Retinal Endothelial Cells against Hyperoxia-Induced Vaso-obliteration

In light of the angiogenic properties of ghrelin,<sup>20</sup> we studied the ability of this pathway to preserve the retinal vasculature in a rat model of oxygen-induced vaso-obliteration (80% O<sub>2</sub> from P5 to P10). Rats receiving intravitreal injection of either of the stable ghrelin analogs, [Dap3]-ghrelin (81 nM final intravitreal concentration) or GHRP6 (24 nM final intravitreal concentration), at P7 and P9 show a marked reduction in vaso-obliterated area by one-half and three-quarters, respectively (P < 0.001compared with vehicle control; Figs. 3a-d). Moreover, analysis of retinal vascular density in regions directly adjacent to the vaso-obliterated zones (Supplementary Fig. S1, http://www. iovs.org/lookup/suppl/doi:10.1167/iovs.10-7152/-/DCSupplemental) reveals that either of the two ghrelin analogs tested ([Dap3]ghrelin or GHRP6) preserves 30% to 40% more vasculature, as reflected by measuring vascular density (P < 0.0001 compared with vehicle-injected hyperoxia control) (Figs. 3g, 3j, 3m). Consistent with a vasoprotective role for GHSR-1a, inhibition of this receptor with the specific antagonists JMV-2959 (560 nM final intravitreal concentration) or [D-Lys3] GHRP-6 (240 nM final intravitreal concentration) significantly reduces the protective effect of the agonists (Figs. 3i, 3l, 3m). The vasoprotective effects of ghrelin analogs are likely mediated through a GHSR-1adependent upregulation of IGF-1 and VEGF, as revealed by Western blot analysis (Fig. 3n). Levels of both factors rise after ghrelin stimulation in a GHSR-1a- dependent manner (Figs. 30, 3p). These data suggest that ghrelin (acting through GHSR-1a) can preserve



FIGURE 3. (Continued) (m) Quantification of vasculature density in hyperoxia-exposed (vaso-obliterated) retina confirm the protection of the ghrelin-GHSR1a pathway (mean  $\pm$ SD; \*\*P < 0.01, \*\*\*P < 0.0001 compared with hyperoxia vehicle control; n = 5-12). (n) Western blot of retinal protein extract demonstrated the induction of both IGF-1 and VEGF after intravitreal administration of the stable Ghrelin analog ([Dap3]-Ghrelin; 81 nM) or GHSR-1a agonist GHRP-6 (24 nM). The receptor antagonists (JMV-2959; 560 nM) and ([Lys3]-GHRP-6; 240nM) blocked this induction. Densitometry quantification of IGF-1 (o) and VEGF (p) expression levels after the administration of GHSR-1a agonists and antagonists (mean  $\pm$  SEM; \*P < 0.05, \*\*P < 0.01 compared with vehicle-injected control; compilation of n = 5-12 animals).



FIGURE 4. Ghrelin promotes retinal endothelial cell growth through GHSR-1a. (a) Quantification of PB-MEC proliferation by thymidine incorporation after treatment with [Dap3]-Ghrelin (10<sup>-8</sup>M), JMV-2959  $(10^{-8}M)$ , or both  $(10^{-8}M \text{ [Dap3]-}$ Ghrelin and  $10^{\text{[minus7}}M \text{ JMV-2959}$ ; mean  $\pm$  SD; \*\*\*P < 0.001 compared with vehicle control; n = 3). (**b**-**f**) Representative microphotograph (b-e) and quantification (f) of aortic explants sprouting treated with [Dap3]-Ghrelin  $(10^{-8} \text{ M})$ , JMV-2959  $(10^{-8} \text{ M})$ , or both  $(10^{-8} \text{ M of } [\text{Dap3}]\text{-}\text{Ghrelin and } 10^{-7} \text{ M}$ JMV-2959; mean  $\pm$  SD; \*\*\*P < 0.001compared with vehicle control; n =6). Scale bar, 1 mm. (g-n) Representative G. simplicifolica lectin-stained retinal whole mounts indicating the proangiogenic effects of ghrelin-GHSR-1a during retinal development (g-m) and quantification (n) of P6 retinal vascular density after intravitreal administration at P4 of [Dap3]-Ghrelin (81 nM; h) JMV-2959 (56 nM; i) or both (81 nM [Dap3]-Ghrelin and 560 nM JMV-2959; (j), as well as GHRP-6 (24 nM; k), [D-Lys3]-GHRP-6 (24 nM; 1), or both (24 nM GHRP and 240 nM [D-Lys3]-GHRP-6; **m**) (mean  $\pm$  SD; \*\*\**P* < 0.001 compared with vehicle control; n =6-20). Vessels were revealed by red fluorescence detected by fluorescence microscopy. Scale bar, 200  $\mu m.$ 

the retinal vasculature during the hyperoxic phase of the OIR model.

# Ghrelin Promotes Endothelial Cell Growth through GHSR-1a

To investigate whether ghrelin (through GHSR-1a) promotes retinal endothelial cell proliferation, we added to PBMECs in culture the stable ghrelin analog [Dap3]-ghrelin  $(10^{-8}M)$  and assessed proliferation in a radioactive-thymidine incorporation assay. Treatment with [Dap3]-ghrelin leads to a 10-fold increase (P < 0.0001) in the proliferation of primary rat neuromicrovascular endothelial cells (Fig. 4a). Antagonism of GHSR-1a with JMV-2959 ( $10^{-8}M$ ) completely blocks the increase in PBMEC proliferation, with levels of cell division dropping beneath vehicle control. Similarly, in the aortic explant model of 3-dimensional vascular sprouting, exposure to [Dap3]-ghrelin ( $10^{-8}M$ ) led to an approximately 3.5-fold induction of vascular sprout formation (P < 0.001) (Figs. 4b, 4c, 4f). Addition of the JMV-2959 antagonist ( $10^{-7}M$ ) effectively abrogated this effect (Figs. 4d-f).

We next investigated the effects of ghrelin analogs on developmental retinal angiogenesis in rats, where the vascular plexus forms predominantly during the first postnatal week of life.<sup>37</sup> Rats were injected intravitreally at P1 and P4 with one of the two stable ghrelin analogs, [Dap3]-ghrelin (81 nM) or GHRP6 (24 nM). Both treatments double vascular density at the vascular front (Figs. 4h, 4k, 4n; P < 0.001 compared with vehicle control). Supplementary Figure S2 (http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-7152//DCSupplemental) shows representative retinal flatmounts as well as the areas sampled. Addition of the GHSR-1a antagonists JMV-2959 (560 nM) or [D-Lys3] GHRP-6 (560 nM) block this increase in retinal vascular density (Figs. 4j, 4m, 4n). Together, these data reveal that ghrelin, through GHSR-1a, is a potent modulator of developmental retinal vessel growth.

# Ghrelin Levels Rise in the Neovascular Phase of Oxygen-Induced Retinopathy

The effect of ghrelin on retinal vascular growth prompted us to determine whether ghrelin levels increase during phases of active vessel growth in the second phase of OIR. We analyzed both systemic and retinal levels of ghrelin in rats subjected to OIR (exposure to 50% oxygen from P1 to P14 [vaso-obliterative phase] followed by room air exposure for 3 days [ischemic vasoproliferative phase]). Circulating ghrelin levels are 35-fold higher in OIR (Fig. 5a; P < 0.05), whereas retinal levels are threefold higher (P < 0.001) than in age-matched animals (Fig. 5b). It is likely that the large increase in plasma ghrelin is systemic given that retinal levels only rise threefold (as deter-

mined by quantitative PCR). This expression profile, along with that observed during the vaso-obliterative phase (Fig. 2a), is consistent with the vasoprotective effects of ghrelin against oxygen-induced stress and, on the other hand, the proangiogenic actions of ghrelin during the proliferative phase of OIR (see below).

#### Inhibition of GHSR-1a Attenuates Pathologic Retinal Neovascularization

As retinal levels of ghrelin increased during the proliferative phase of OIR (Fig. 5b) and ghrelin receptor was abundantly expressed in the rat retina during the progression of retinopathy (Fig. 1), we sought to determine whether their interplay could contribute to pathologic vessel formation. Intravitreal administration at P14 of either one of the two GHSR-1a antagonists, JMV-2959 (56 nM; Figs. 6e, 6f) or [D-Lys3] GHRP-6 (24 nM; Figs. 6g, 6h), results in reduced neovessel formation compared with vehicle-treated retinas (Figs. 6c, 6d). This was quantified by counting the area of neovascular tufts present at P18. Both GHSR-1a antagonists reduce neovascular formation by about two-thirds compared with vehicle controls (Fig. 6i; P < 0.001). Moreover, the extent of tortuous vessel in peripheral vascular fronts (Supplementary Fig. S3a, http://www. iovs.org/lookup/suppl/doi:10.1167/iovs.10-7152/-/DCSupplemental) is reduced by more than 90% with both treatments (Fig. 6j; P <0.001).

The overall severity of pathologic neovessel growth was assessed by a previously established total retinopathy score<sup>38,39</sup> (Supplementary Fig. S3, http://www.iovs.org/lookup/suppl/ doi:10.1167/iovs.10-7152/-/DCSupplemental). This score determines the severity of proliferative disease based on the presence of extraretinal neovascularization, blood vessel tuft formation, and extent of blood vessel tortuosity. Pharmacologic inhibition of the GHSR-1a receptor reduces the proliferative retinopathy score significantly compared with vehicle controls (Figs. 5k, 5l).

#### DISCUSSION

To date, therapeutic options to counter proliferative retinopathies are limited to invasive interventions such as cryotherapy, laser photocoagulation, and vitrectomy, all of which target the second proliferative phase of retinopathy.<sup>1,2</sup> One of the primary triggers for this pathologic retinal angiogenesis is a profound imbalance between metabolic demand and available supply, leading to an overall hypoxic retinal tissue.<sup>39</sup> To reinstate energetic equilibrium in avascular retinal areas, the ischemic tissue mounts a compensatory vasoproliferative response to rebuild a capillary network in an attempt to reperfuse the



**FIGURE 5.** Ghrelin levels rise in the neovascular phase of OIR. (a) Plasma and (b) retinal ghrelin expression level in P17 hyperoxia-exposed animals compared with normoxia control reveal a robust induction during the neovascular phase of OIR (mean  $\pm$  SD; \*P < 0.05, \*\*\*P < 0.0001; n = 3-8).



FIGURE 6. Pharmacologic inhibition of GHSR-1a prevents pathologic retinal neovascularization and improves retinopathy. (a-h) Representative images of G. simplicifolica lectinstained retinal whole mounts from vehicle-injected normoxic controls (a, b) and vehicle-injected (saline; c, d), JMV-2959 (56 nM; e, f), and [D-Lys3] GHRP-6-injected (24 nM; g, h) rats during the neovascular phase (P18) of OIR. Top: 500 µm. Bottom: 1 mm. Vessels were revealed as red fluorescence detected by fluorescence microscopy. (i-k) Calculated total vessel buds (i), vessel tortuosity area (j), and retinopathy score (k) of OIR retinal vasculatures after intravitreal injection of vehicle (saline), JMV-2959 (56 nM), or [D-Lys3] GHRP-6 (24 nM; mean  $\pm$  SD; \*P < 0.05 compared with vehicle control: n = 6-16). (1) Summary table indicating the subscores of each parameter investigated.

ischemic tissue. However, uncontrolled overexpression of angiogenic factors leads to excessive sprouting of pathologic (and nonfunctional) neovessels instead of functional capillaries. An attractive approach to reduce the metabolic strain in proliferative retinopathies would be to devise vasoprotective strategies to preserve the original retinal vasculature from decay and thus maintain adequate tissue perfusion with oxygen and nutrients. This approach would reduce the ischemic stimulus and thus avert the tissue hypoxia that prompts pathologic retinal vessel growth.

In the present study, we report that the ghrelin receptor GHSR-1a is abundantly expressed by vascular endothelial cells throughout the retina and, when activated by either ghrelin or its stable analogues, leads to a profound vasoprotection of the retinal vasculature against hyperoxic stress. Ghrelin and its analogs may, therefore, be promising to prevent vascular dropout in early retinopathy, before ischemic disease develops and pathologic angiogenesis occurs. We report that within the retina itself, ghrelin effectively induces IGF-1 and VEGF production, a paradigm that is analogous to what occurs in the arcuate nucleus, where ghrelin triggers the secretion of GH from the anterior pituitary gland.<sup>17</sup> The vasoprotective effects of ghrelin are, therefore, likely mediated through the induction of IGF-1 (GH-IGF-1 axis) and VEGF, both well documented to be protective toward retinal vascular beds.9,10,12,40-43 Notwithstanding the systemic contribution of ghrelin, our study also demonstrates that ghrelin is produced locally in the retina, and the dynamics of its expression pattern are consistent with both a vasoprotective role and one that contributes to exacerbating pathologic neovascularization. Future studies will have to determine the relative contribution of systemic versus local production.

In OIR retinas, ghrelin levels rise robustly during the second phase, when pathologic vessel formation is observed. Our results demonstrate that during this second, proliferative phase, ghrelin contributes to the destructive preretinal angiogenesis, as pharmacologic inhibition of GHSR-1a significantly improves outcome by reducing destructive neovascularization. These findings are consistent with both the previously described role of ghrelin in promoting angiogenesis<sup>20</sup> and our findings that ghrelin robustly induces retinal angiogenesis during retinal vascular development and in ex vivo experiments. Such a dual action of ghrelin is caused by the concentration difference in different stages of OIR (i.e., decreased during the vaso-obliteration phase and increased during the neovascularization phase). Based on these findings, ghrelin agonists, while beneficial in the early phase of retinopathy, would likely have detrimental effects once pathologic angiogenesis has commenced. Our results demonstrate that during these proliferative stages the opposite approach, suppression of ghrelin-GHRHS-1a signaling, yields therapeutic benefits. The different effects on the pathology outcome of the same molecule during the two phases of proliferative retinopathy are not exclusive to ghrelin but can be observed with Epo and VEGF; administration during the early phases attenuates disease whereas administration during the late neovascular phase aggravates the pathology.<sup>9,13,41,42,44,45</sup> As with Epo, with which local production supersedes plasma contribution during ischemic retinal disease,<sup>46</sup> the heightened expression of ghrelin in the retina during OIR suggests a similar paradigm for this peptide.

In summary, our data point to a novel role for the ghrelin-GHSR-1a pathway in proliferative retinopathies. Our findings reveal that agonists of GHSR-1a given during the first vasodegenerative phase protect against vaso-obliteration and help maintain retinal vascular integrity, thus preventing proliferative retinopathy. Conversely, GHSR-1a antagonists are used during the second phase of proliferative retinopathy because they reduce pathologic neovascular formation directly. A careful dosing and timing regimen will, therefore, be needed to ensure the appropriateness of any therapeutic intervention in the ghrelin-GHSR-1a pathway during retinopathy. Both the novel angiogenic and the vasoprotective properties of the ghrelin-GHSR-1a axis identified in this study may provide an interesting target for investigation beyond the eye, such as in diseases like cancer and stroke, in which vascular growth is also perturbed.

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