Insulin Increases Retinal Hemorrhage in Mild Oxygen-Induced Retinopathy in the Rat: Inhibition by Riluzole

Min-Heui Yoo,[1] *Young Hee Yoon,*[2] *Hyewon Chung,*[2] *Kyung Sook Cho,*[1] *and Jae-Young Koh*[1,3]

Purpose. Although hyperglycemia is likely the main stimulus for VEGF induction in diabetic retinopathy (DR), a switch from oral hypoglycemic therapy to parenteral insulin injection, despite producing better glucose control, sometimes paradoxically aggravates DR. The induction of VEGF by insulin, as observed in certain conditions, may be a plausible mechanism for this phenomenon. In the present study, to determine the role of insulin in proliferative diabetic retinopathy, the authors examined whether insulin treatment affected the outcome of oxygen-induced retinopathy (OIR) in rats and whether the anti–amyotrophic lateral sclerosis (ALS) drug riluzole with protein kinase C–inhibiting activity can attenuate the effects of insulin.

Methods. To examine in vivo the effects of insulin, mild OIR was produced in 7-day-old rat pups by raising them with a nursing mother in a 55% oxygen environment for 5 days. After that, rat pups were injected daily with subcutaneous saline or insulin (4 U/d) with or without additional riluzole injection (10 mg/kg/d, intraperitoneally) for 5 days in room air.

Results. Insulin treatment substantially increased VEGF levels, extraretinal vessel formation, matrix metalloproteinase activity, and the extent of retinal hemorrhage in rat pups with mild OIR compared with saline controls. Riluzole substantially reduced all these changes induced by insulin.

Conclusions. In the present study, OIR was used as a surrogate model for DR because the core pathology and the VEGF-mediated mechanism are shared by both conditions. As in human DR, in rat pups with mild OIR, insulin treatment aggravated retinal hemorrhage, which was blocked by riluzole. Riluzole is a Food and Drug Administration–approved anti-ALS drug with a favorable adverse effect profile. It may be useful as an anti-VEGF treatment in DR, especially in reducing the retinal hemorrhage that often occurs shortly after the switch from oral hypoglycemics to parenteral insulin. (*Invest Ophthalmol Vis Sci.* 2007;48:5671–5676) DOI:10.1167/iovs.07-0395

From the Departments of [2]Ophthalmology and [3]Neurology, and the [1]NRL Neural Injury Research Center, University of Ulsan College of Medicine, Seoul, Korea.

Supported by Korean Ministry of Health and Welfare Research Grant 02-PJ1-PG1-CH02-0003 (YHY) and by the National Research Laboratory program of Korean Ministry of Science and Technology (J-YK).

Submitted for publication April 3, 2007; revised July 19, 2007; accepted October 19, 2007.

Disclosure: **M.-H. Yoo**, None; **Y.H. Yoon**, None; **H. Chung**, None; **K.S. Cho**, None; **J.-Y. Koh**, None

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Corresponding author: Jae-Young Koh, Department of Neurology, NRL Neural Injury Research Center, University of Ulsan College of Medicine, 388–1 Poongnap-Dong Songpa-Gu, Seoul, 138-736, Korea; jkko@amc.seoul.kr.

Upregulation of vascular endothelial growth factor (VEGF) likely plays a pivotal role in retinal neovascularization in diabetes mellitus, retinopathy of prematurity, and age-related macular degeneration.[1,2] VEGF is induced in the retina by hypoxia and hyperglycemia, which are frequent accompaniments of diabetes.[3–8] However, paradoxically, diabetic retinopathy is often aggravated acutely after switching from oral hypoglycemics to insulin injection, despite the achievement of better glucose control. Interestingly, several studies have reported a possible link between insulin-like growth factor (IGF)-1 receptor and VEGF-dependent retinal neovascularization.[9–12]. Because IGF-1 receptor and insulin receptor share signaling cascades, it seems possible that the transient aggravation of diabetic retinopathy by insulin injection may also involve VEGF upregulation. In fact, Doronzo et al.[13] reported that insulin enhanced VEGF protein expression and secretion in cells from lean Zucker +/*fa* rats.

In an earlier study, we demonstrated that riluzole reduced the proliferation of human umbilical vein endothelial cells (HUVECs) by VEGF and the neovascularization in oxygen-induced retinopathy (OIR) of the rat, a model for proliferative retinal vasculopathy in humans.[14] Riluzole has been approved for human use by the Food and Drug Administration (FDA) in patients with amyotrophic lateral sclerosis (ALS) and, thus far, has a favorable adverse effect profile.[15,16] In the previous experiments, we also found that the reduction of endothelial cell proliferation by riluzole was mediated by the inhibition of protein kinase C (PKC) βII in HUVEC culture.[14,17] These effects were confirmed in vivo in OIR in the rat. OIR changes[18–20]—abnormal blood vessel growth, formation of blood vessel tufts, extraretinal neovascularization, central vasoconstriction, retinal hemorrhage, and vascular tortuosity—were all reduced with riluzole treatment.[18,21] In the present study, we examined whether insulin treatment would alter OIR in rat pups and whether riluzole would prevent it.

Materials and Methods

Animals and Oxygen-Induced Retinopathy

Sprague–Dawley rats were obtained from Charles River Laboratories (St. Constant, Quebec, Canada). Experiments were performed in accordance with the Guideline for Care and Use of Laboratory Animals (University of Ulsan, Seoul, Korea) and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. From postnatal day (P) 5 to P12, rat pups were raised in a 55% ± 5% oxygen chamber with their mothers for feeding. The accuracy of oxygen tension in the chamber was monitored twice daily with an oxygen analyzer (Hudson Ventronics, Temecula, CA). After exposure to hyperoxia, rat pups were raised for another 5 days (P12-P17) in a room-air environment.[21,22] Control rat pups were raised in room air throughout the period (P5-P17). Saline or insulin (4 U/d) was injected subcutaneously once daily from P12 to P16. Riluzole (10 mg/kg/d) was injected intraperitoneally daily. To maintain the amount of blood glucose, rats were supplied with 5% dextrose in saline at least four times a day. The blood glucose of rats was monitored four times a day with a blood glucose test meter (Arkray, Kyoto, Japan).

Copyright © Association for Research in Vision and Ophthalmology

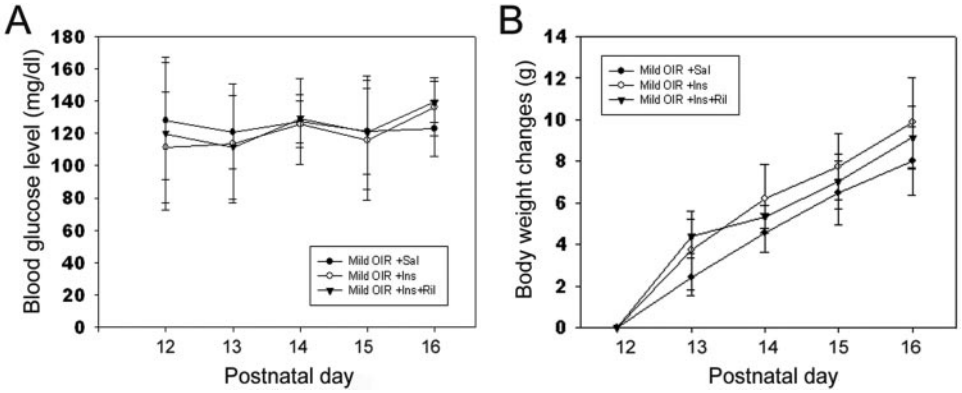

FIGURE 1. Blood glucose levels and body weight changes in experimental groups. (**A**) Bars denote daily mean blood glucose levels (mean ± SEM, n = 17–29) in saline control, insulin-treated, and insulin plus riluzole–treated rat pups. Blood glucose levels were measured four times a day for 5 days of drug administration. (**B**) Data (n = 17–29) represent body weight changes (g) from initial values of saline control, insulin-treated, and insulin plus riluzole–treated rat pups at indicated days after birth. Drugs were administered from P12 to P17. No significant differences were noted between groups.

Fluorescein Dextran Angiography of the Retinal Blood Vessel

At P17, all rat pups were anesthetized with intraperitoneal injections of 80 mg/kg ketamine and 15 mg/kg xylazine. After thoracotomy, the left ventricle was perfused with 0.5 mL of 20 mg/mL fluorescein isothiocyanate-labeled dextran (2,000,000 MWt) dissolved in ultra-pure water. Eyes were placed in 4% paraformaldehyde in PBS, and retinas were removed under a dissecting microscope. Retinas were flat-mounted on slides using gelatin and overlaid with coverslips, and the assembly was sealed with nail polish. Fluorescein angiography was obtained using a fluorescence microscope (BX60; Olympus, Tokyo, Japan) and a digital camera (C-2000Z; Olympus).

Retinopathy Scoring

The retinopathy scoring system for animal models,[23–25] modified from the International Criteria for Retinopathy of Prematurity system for humans,[26] was used to quantify the severity of retinopathy. Blood vessel growth, extraretinal neovascularization, central vasoconstriction, and vessel tortuosity were given scores from 0 to 3. Blood vessel tufts were scored from 0 to 4. Hemorrhage was scored from 0 to 1. Hence, the maximum possible score was 17. Retinopathy was scored by two independent evaluators masked to conditions.

Retinal Section and Hematoxylin and Eosin Staining

Rats were killed with xylazine and ketamine. The eyes were frozen in liquid nitrogen, and 10-μm-thick sections were made through the cornea parallel to the optic disc using a cryostat (Leica Instruments GmbH, Darmstadt, Germany). Tissue sections were stained with hematoxylin and eosin; 50-μm sections were used for counting the number of nuclei beyond the inner limiting membrane in a masked fashion.[21,26]

Immunoprecipitation, Western Blotting, and Enzyme-Linked Immunosorbent Assay

Retinas of rat pups were isolated, lysed, and centrifuged. Aliquots of supernatant were incubated with VEGF antibody (Santa Cruz Biotechnology, Santa Cruz, CA) overnight and then were precipitated (Protein A beads; Upstate Biotechnology, Lake Placid, NY) at 4°C for 1 hour. Immunoprecipitates were washed three times with lysis buffer, followed by immunoblot analysis. Samples were separated using 10% SDS-PAGE and were transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). Membranes were incubated overnight at 4°C with VEGF antibody (Santa Cruz Biotechnology). Substrate (SuperSignal West Dura; Pierce Biotechnology, Rockford, IL) for chemiluminescence was used to visualize immunoreactive bands.[14]

Tissues containing retina and vitreous were isolated from the enucleated eyeballs and placed in 300 μL lysis buffer (20 mM Tris-Cl [pH 7.4]), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 μM Na_3VO_4, 1 μg/mL leupeptin, and 1 mM phenylmethylsulfonyl fluoride) and were sonicated. The lysate was centrifuged at 14,000 rpm for 15 minutes at 4°C, and VEGF levels in the supernatant were determined with the rat VEGF ELISA kit (Quantikine; R&D Systems, Minneapolis, MN) in accordance with the manufacturer's protocol.

Immunohistochemical Staining

Mouse anti-VEGF (Santa Cruz Biotechnology) was used as the primary antibody. Sections were fixed with 4% paraformaldehyde for 3 hours, treated with 0.3% H_2O_2 for 30 minutes, blocked with 3% goat serum, and incubated overnight with primary antibody diluted at 1:200 in PBS. For fluorescence staining, FITC-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA) was used. A fluorescence microscope (BX60; Olympus) was used to detect VEGF with FITC.

In Situ Zymography

An assay kit (EnzCheck Gelatinase Assay Kit; Molecular Probes, Eugene, OR) was used for in situ zymography according to the provided protocol. Sections were incubated with 40 μg DQ-gelatin-fluorescein isothiocyanate (Molecular Probes), examined under the fluorescence microscope, and photographed.

Statistical Analyses

All data are presented as mean ± SD. To compare multiple sets of data, one-way ANOVA with post hoc Fisher least significant difference test was used. For paired data sets, a two tailed t-test was used. $P < 0.05$ was considered to represent a significant difference.

RESULTS

To test the effect of insulin on retinopathy, rat pups were injected subcutaneously with saline or 4 U human insulin once a day. To prevent lowering of blood glucose levels, insulin-injected rats were also supplemented with intravenous glucose four times a day. With this protocol, average daily blood glucose levels, measured four times a day, were not significantly different among the saline, insulin, and insulin plus riluzole groups (Fig. 1A), nor were body weight changes (Fig. 1B).

Aggravation by Insulin of Intraretinal Hemorrhage in Mild OIR of the Rat

To test the possibility that insulin increases VEGF levels in retina and, hence, may aggravate proliferative retinal vasculopathy, mild OIR was produced in rat pups as described in Materials and Methods (Fig. 2). Pathologic changes of OIR included blood vessel growth, retinal hemorrhage, vascular tuft formation, and vessel tortuosity (Fig. 2, white arrow). Compared with those with the full-blown OIR (raised in 75% O_2),[14] rats with mild OIR exhibited less severe pathologic changes in all the areas of retinopathy. Insulin treatment mark-

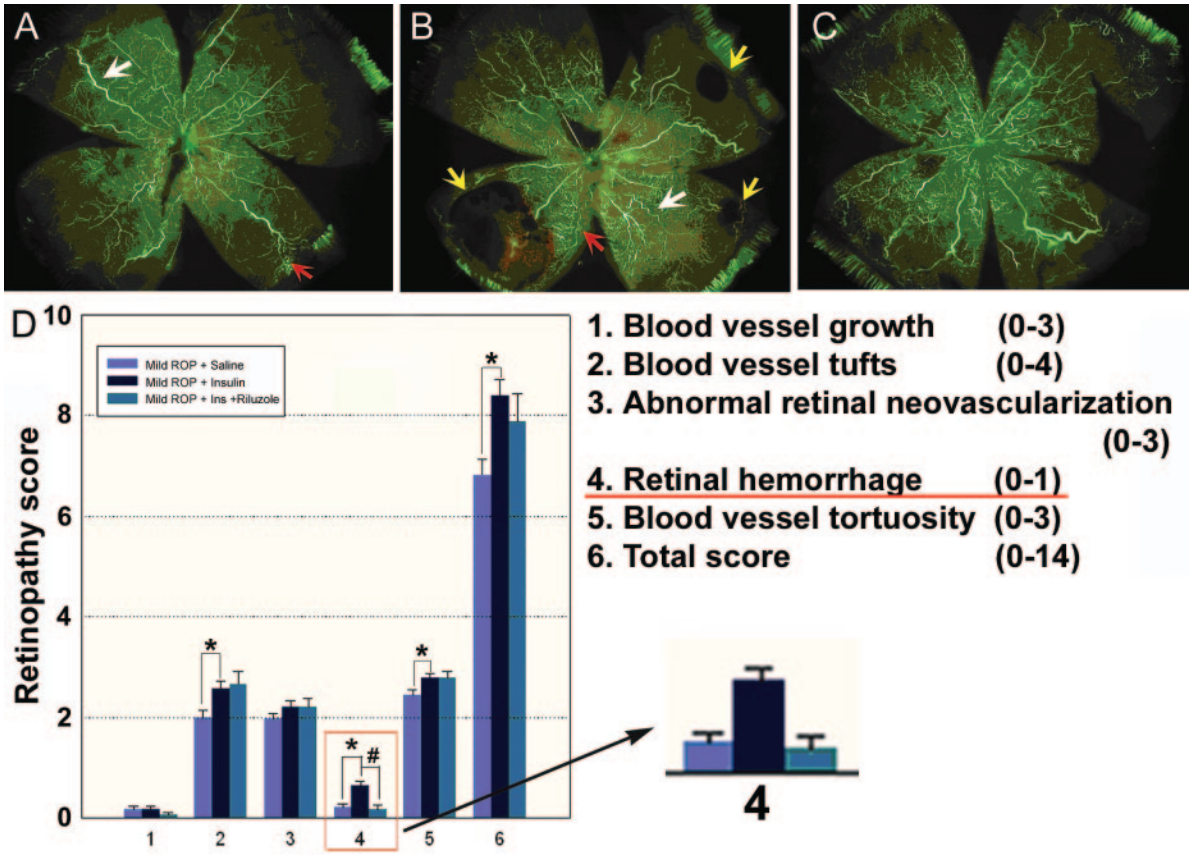

FIGURE 2. Insulin increases retinal hemorrhage in mild OIR: inhibition with riluzole. (**A–C**) Fluorescein angiograms of retinal whole mounts obtained from rats with mild OIR, saline control (**A**), insulin-treated (4 U/d) (**B**), and insulin plus riluzole (10 mg/kg/d)-treated. Dark areas represent hemorrhages. (**D**) Bars denote retinopathy scores in rats subjected to the mild OIR protocol, treated with saline, insulin, or insulin plus riluzole. Numbers denote pathologic changes as denoted. Of retinopathy parameters, retinal hemorrhage was most markedly increased by the insulin treatment. Cotreatment with riluzole substantially reduced the extent of retinal hemorrhage. *Yellow arrows*: retinal hemorrhage. *Red arrows*: vessel tuft formation. *White arrows*: vessel tortuosity. *Difference from saline ($P < 0.05$). #Differences from insulin ($P < 0.05$).

edly and specifically increased retinal hemorrhage in mild OIR (Fig. 2B). Hemorrhage was scored as all (1) or none (0); however, the marked differences in retinal hemorrhage were only weakly reflected in the total retinopathy scores (Fig. 2D). The aggravation of retinal hemorrhage by insulin was markedly reduced by the coadministration of riluzole (Fig. 2C).

Reduction of VEGF Induction by Insulin in the Eyes of Mild POP

VEGF upregulation in proliferative retinopathy results in the formation of abnormal vessels, which are prone to leakage and hemorrhage. Given that insulin induced VEGF in cell cultures (not shown), it seemed possible that the VEGF upregulation by insulin also underlay the increase of retinal hemorrhage in mild OIR of the rat. To test this possibility, we performed immunohistochemical staining of retina using a monoclonal anti–VEGF antibody. Of the retinal layers, the ganglion cell layer and inner plexiform layer showed pronounced levels of VEGF from immunostaining (Fig. 3). Consistent with the culture results, insulin treatment increased the levels of VEGF in these retinal layers (Fig. 3B), whose effect was reduced by riluzole (Fig. 3C). In addition, immunoblots and quantification confirmed that the VEGF induction by insulin was reduced by riluzole (Figs. 3D, 3E). Furthermore, VEGF levels estimated with ELISA were substantially increased in the insulin group (Fig. 3F). Again, riluzole reduced the insulin effect.

Reduction of Extraretinal Vessel Formation in Mild OIR Treated with Insulin

Extraretinal vessels are likely the main source for the retinal hemorrhage. To determine whether insulin treatment specifically increased extraretinal vessel formation, the retinal sections were stained with hematoxylin and eosin, and the endothelial cells above the internal limiting membrane were counted (Fig. 4). Compared with the control (Fig. 4A), insulin treatment significantly increased the number of extraretinal endothelial cells (Fig. 4B). Riluzole reduced this change (Fig. 4C).

The results of cell counting are presented in Figure 4D, which confirms the eyeball impression that insulin increased extraretinal neovascularization in mild OIR, whereas riluzole markedly reduced the insulin-induced change.

Inhibition of MMP Activation by Insulin in Mild OIR Model

One of the events downstream of insulin and VEGF signaling was MMP activation,[27] which has been observed in eyes with proliferative retinopathy (Fig. 5). MMP activation may further compromise the integrity of new blood vessels (Fig. 5B) and thus may contribute to edema formation and hemorrhage. The fact that insulin treatment specifically increased retinal hemorrhage in the eyes of insulin-treated OIR rats suggests the involvement of the MMP activation. To examine this possibility, in situ zymography was performed, which showed that insulin

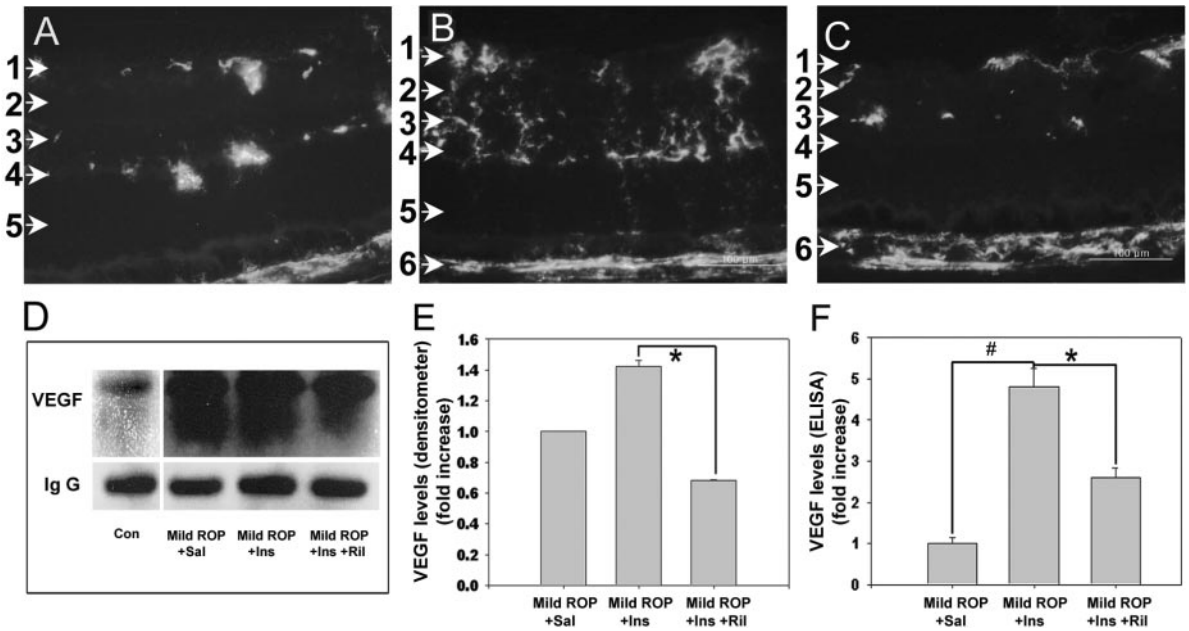

FIGURE 3. Insulin induces VEGF in mild OIR. (**A–C**) Fluorescence photomicrographs of retinas immunohistochemically stained for VEGF. Rats were subjected to the mild OIR protocol and received saline (**A**), insulin (**B**), or insulin plus riluzole (**C**). Insulin treatment increased VEGF immunoreactivity in the ganglion cell layer and the inner nuclear layer (**B**). Cotreatment with riluzole reduced VEGF induction by insulin in both layers (**C**). *Arrows*: 1, ganglion cell layer; 2, inner plexiform layer; 3, inner nuclear layer; 4, outer plexiform layer; 5, outer nuclear layer; 6, pigment epithelium. Scale bar, 100 μm. (**D**) Immunoblots for VEGF of anti–VEGF antibody immunoprecipitates. Conditions are the same as previously described. (**E**) Densitometric quantification of immunoblots for VEGF ($n = 3$). (**F**) VEGF levels in vitreoretinal tissues as estimated with ELISA ($n = 3$). *Difference from insulin. #Difference from saline ($P < 0.05$).

treatment significantly increased MMP activity in the vitreoretinal preparations of the mild OIR rats (compare Figs. 5A and 5B). Again, riluzole cotreatment substantially reduced the increase of MMP activity by insulin (Fig. 5C). Quantification of the MMP activity with densitometric analysis confirmed these changes (Fig. 5D).

DISCUSSION

The core findings of the present study are that insulin increases intraretinal hemorrhage and extraretinal neovascularization in the mild OIR model by increasing VEGF expression and that these effects are ameliorated by riluzole. The VEGF-mediated

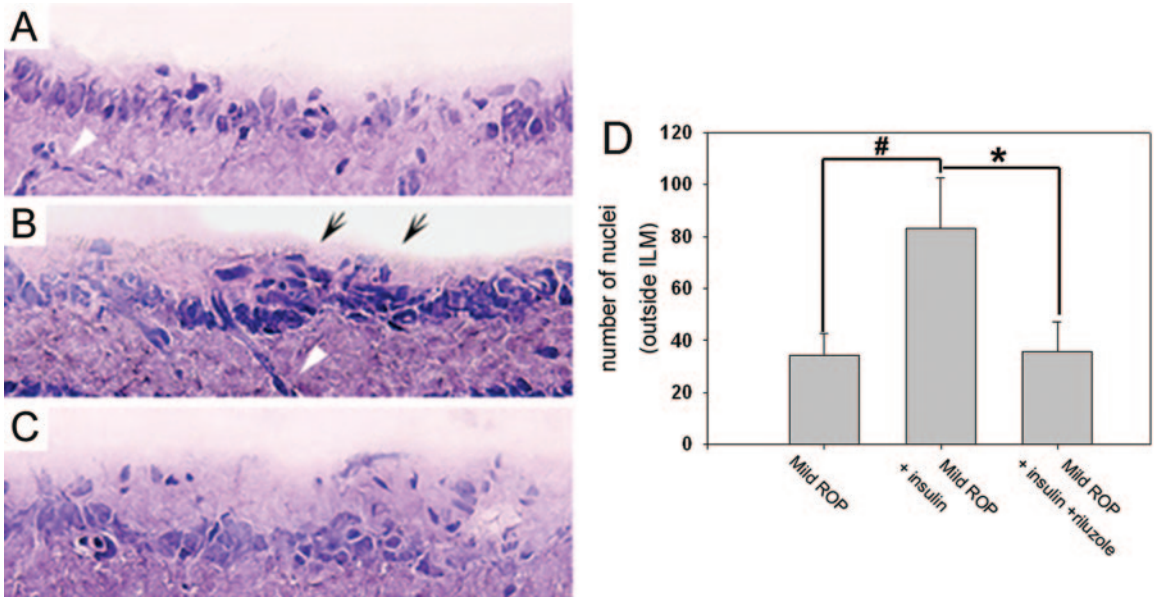

FIGURE 4. Insulin increases extraretinal vessel formation in mild OIR: inhibition by riluzole. (**A–C**) Hematoxylin and eosin–stained retinal sections of eyes that underwent the mild OIR protocol. Rats were injected with saline (**A**), insulin (**B**), or insulin plus riluzole (**C**). Insulin increased the extraretinal vessel formation above the internal limiting membrane (ILM), and cotreatment with riluzole inhibited it. *Arrows*: extraretinal neovascularization. *Arrowheads*: enlarged intraretinal vessel profiles. (**D**) Number of nuclei ($n = 16$) outside the internal limiting membrane in 15 consecutive retinal sections of mild OIR eyes, treated with saline (*left*), insulin (*middle*), or insulin plus riluzole (*right*). #Difference from saline ($P < 0.01$). *Difference from insulin ($P < 0.01$).

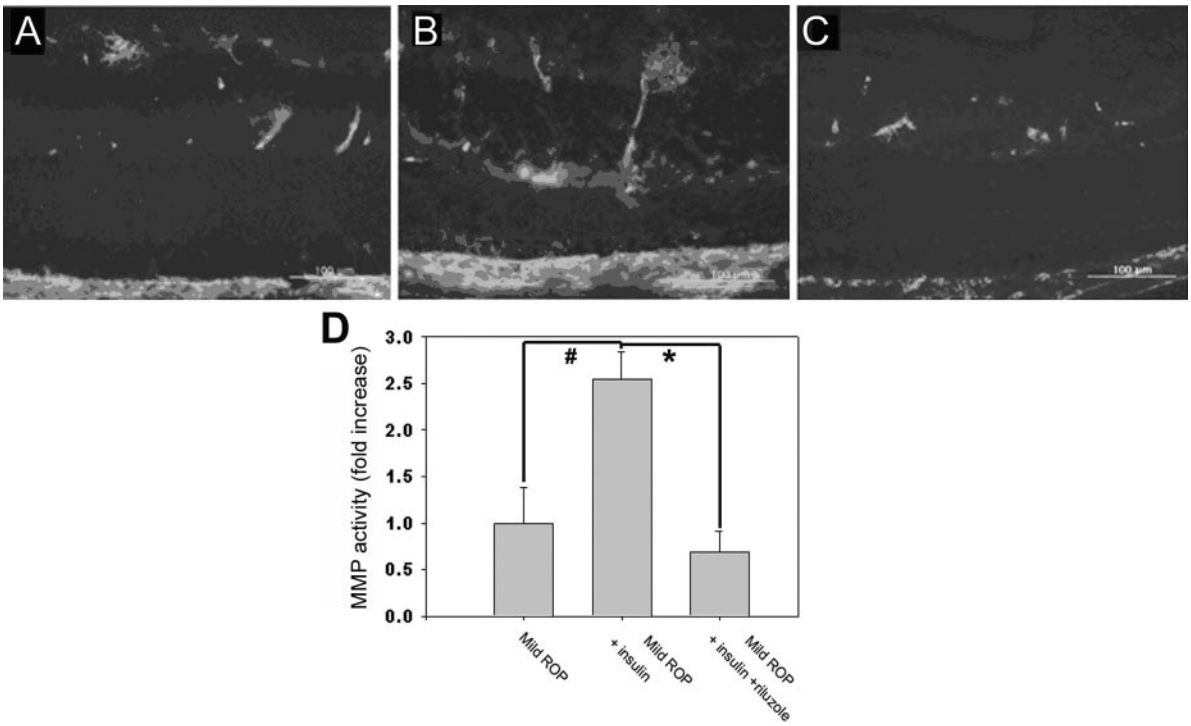

FIGURE 5. Insulin increases MMP activity in the retinas of OIR rats: inhibition by riluzole. In situ zymography of retinas of rats subjected to the mild OIR protocol and treated with saline (**A**), insulin (**B**), or insulin plus riluzole (**C**). Insulin treatment increased MMP activity (**B**) in the ganglion cell and inner nuclear layers, where VEGF upregulation was noted. Cotreatment with riluzole inhibited the increase of MMP activity (**C**). Scale bar, 100 μm. (**D**) Densitometric quantification of MMP activity ($n = 3 \sim 4$). *Difference from insulin ($P < 0.05$).

mechanism may contribute to acute aggravation of diabetic retinopathy, which occurs often at the time of switch from oral hypoglycemics to parenteral insulin treatment. Because riluzole is effective in reducing VEGF expression and retinal hemorrhage after the insulin treatment, it may help reduce the aggravation of retinopathy in patients with diabetes.

The rat model of OIR is usually produced by exposing rat pups to a severely hyperoxic (75% ± 5%) environment.[18–20] Such OIR eyes exhibit abnormal blood vessel growth, blood vessel tufts, extraretinal neovascularization, central vasoconstriction, retinal hemorrhage, and vascular tortuosity. However, to examine the possibility that insulin aggravates OIR, we must turn to a mild OIR model by raising pups in a modestly hyperoxic (55% ± 5%) environment.[28] In this model, abnormalities are milder in severity but represent all changes except for central vasoconstriction. Interestingly, the retinopathy aggravating effect of insulin was limited to retinal hemorrhage; other pathologic changes were scored similarly to those in saline controls. Hemorrhage is scored as all or none; hence, the apparently dramatic increase in retinal hemorrhage is not well represented in the total OIR score. However, clinically, retinal hemorrhage is the key pathologic event that compromises vision in proliferative retinopathy; thus, this effect may be important in certain clinical situations.

Although mechanisms of increases in retinal hemorrhage in insulin-treated eyes are unknown, the upregulation of VEGF is likely involved. In fact, insulin treatment increased levels of VEGF in certain layers of retina in mild OIR rat pups. VEGF induces abnormal new vessels, which are more prone to leakage and hemorrhage. In particular, insulin increased the formation of extraretinal vessels that are likely the source of extensive retinal hemorrhage in OIR. In addition, insulin increased MMP activity in the retina, which may further increase the chance of retinal hemorrhage.

The present study showed that riluzole effectively reduced VEGF induction and extraretinal vessel formation and increased MMP activity and retinal hemorrhage in insulin-treated mild OIR rat pups. Riluzole is a benzthiazole compound that inhibits glutamatergic transmission[29,30] and neuronal cell death.[31,32] With its proven neuroprotective efficacy, the FDA in 1996 approved riluzole for the treatment of ALS.[26] Although initially considered as an antiexcitotoxic drug, riluzole does not directly act on glutamate receptors. Rather, it decreases the release of neurotransmitters such as glutamate and acetylcholine from presynaptic terminals.[29,30] In addition to its inhibitory effect on glutamate release, riluzole inhibits inward currents through voltage-gated Na channels and voltage-gated calcium channels.[33,34] All these effects may contribute to its neuroprotective effect in ALS.

Recently, we have demonstrated that riluzole inhibits PKC in cortical cell cultures and reduces VEGF-mediated endothelial cell proliferation in vitro and in vivo, also likely by inhibiting PKC, particularly the β-II isoform. Regardless of the mechanism, riluzole is highly effective in reducing all pathologic changes in full OIR[14] and retinal hemorrhage in insulin-treated mild OIR. Given that riluzole is routinely administered to ALS patients for prolonged periods and is not known to cause any serious adverse effects, it is now regarded as safe for long-term use. Considering its favorable adverse effect profile, riluzole should be considered a potential therapeutic agent for proliferative retinopathy including diabetic retinopathy.

References

1. Kim BS, Chen J, Weinstein T, Noiri E, Goligorsk MS. VEGF expression in hypoxia and hyperglycemia: reciprocal effect on branching angiogenesis in epithelial-endothelial co-cultures. *J Am Soc Nephrol.* 2002;13:2027–2036.

2. Witmer AN, Vrensen GF, Van Noorden CJ, Schlingemann RO. Vascular endothelial growth factors and angiogenesis in eye disease. *Prog Retin Eye Res*. 2003;22:1–29.
3. Hammes HP, Lin J, Bretzel RG, Brownlee M, Breier G. Upregulation of the vascular endothelial growth factor/vascular endothelial growth factor receptor system in experimental background diabetic retinopathy of the rat. *Diabetes*. 1998;47:401–406.
4. Lorenzi M, Cagliero E. Pathobiology of endothelial and other vascular cells in diabetes mellitus: call for data. *Diabetes*. 40;6:653–659.
5. Murata T, Nagai R, Ishibashi T, Inomuta H, Ikeda K, Horiuchi S. The relationship between accumulation of advanced glycation end products and expression of vascular endothelial growth factor in human diabetic retinas. *Diabetologia*. 1997;40:764–769.
6. Tanaka Y, Katoh S, Hori S, Miura M, Yamashita H. Vascular endothelial growth factor in diabetic retinopathy. *Lancet*. 1997;24:349:1520.
7. Marsh S, Nakhoul FM, Skorecki K, et al. Hypoxic induction of vascular endothelial growth factor is markedly decreased in diabetic individuals who do not develop retinopathy. *Diabetes Care*. 2000;23:1375–1380.
8. Pe'er J, Shweiki D, Itin A, Hemo I, Gnessin H, Keshet E. Hypoxia-induced expression of vascular endothelial growth factor by retinal cells is a common factor in neovascularizing ocular diseases. *Lab Invest*. 1995;72:638–645.
9. Lu M, Amano S, Miyamoto K, et al. Insulin-induced vascular endothelial growth factor expression in retina. *Invest Ophthalmol Vis Sci*. 1999;40:3281–3286.
10. Ruberte J, Ayuso E, Navarro M, et al. Increased ocular levels of IGF-1 in transgenic mice lead to diabetes-like eye disease. *J Clin Invest*. 2004;113:1149–1157.
11. Paques M, Massin P, Gaudric A. Growth factors and diabetic retinopathy. *Diabetes Metab*. 1997;23:125–130.
12. Kondo T, Vicent D, Suzuma K, et al. Knockout of insulin and IGF-1 receptors on vascular endothelial cells protects against retinal neovascularization. *J Clin Invest*. 2003;111:1835–1842.
13. Doronzo G, Russo I, Mattiello L, Anfossi G, Bosia A, Trovati M. Insulin activates vascular endothelial growth factor in vascular smooth muscle cells: influence of nitric oxide and of insulin resistance. *Eur J Clin Invest*. 2004;34:664–673.
14. Yoo MH, Hyun HJ, Koh JY, Yoon YH. Riluzole inhibits VEGF-induced endothelial cell proliferation in vitro and hyperoxia-induced abnormal vessel formation in vivo. *Invest Ophthalmol Vis Sci*. 2005;46:4780–4787.
15. Groeneveld GJ, Van Kan HJM, Kalmijn S, et al. Riluzole serum concentrations in patients with ALS. *Neurology*. 2003;61:1141–1143.
16. Borras-Blasco J, Plaza-Macias I, Navarro-Ruiz A, Peris-Marti J, Anton-Cano A. Riluzole as a treatment for amyotrophic lateral sclerosis. *Rev Neurol*. 1998;27:1021–1027.
17. Xia P, Aiello LP, Ishii H, et al. Characterization of vascular endothelial growth factor's effect on the activation of protein kinase C, its isoforms, and endothelial cell growth. *J Clin Invest*. 1996;98:2018–2026.
18. Gole GA, Gannon BJ, Goodger AM. Oxygen induced retinopathy: the kitten model re-examined. *Aust J Ophthalmol*. 1982;10:223–232.
19. Ventresca MR, Gonder JR, Tanswell AK. Oxygen-induced proliferative retinopathy in the newborn rat. *Can J Ophthalmol*. 1990;25:186–189.
20. Ricci B. Oxygen-induced retinopathy in the rat model. *Doc Ophthalmol*. 1990;74:171–177.
21. Higgins RD, Yu K, Sanders RJ, Nandgaonkar BN, Rotschild T, Rifkin DB. Diltiazem reduces retinal neovascularization in a mouse model of oxygen induced retinopathy. *Curr Eye Res*. 1999;18:20–27.
22. Beauchamp MH, Marrache AM, Hou X, et al. Platelet-activating factor in vasoobliteration of oxygen-induced retinopathy. *Invest Ophthalmol Vis Sci*. 2002;43:3327–3337.
23. Phelps DL, Rosenbaum AL. Effects of marginal hypoxemia on recovery from oxygen-induced retinopathy in the kitten model. *Pediatrics*. 1984;73:1–6.
24. Phelps DL. Retinopathy of prematurity: an estimate of vision loss in the United States—1979. *Pediatrics*. 1981;67:924–925.
25. Smith LE, Wesolowski E, McLellan A, et al Oxygen-induced retinopathy in the mouse. *Invest Ophthalmol Vis Sci*. 1994;35:101–111.
26. An international classification of retinopathy of prematurity. *Pediatrics*. 1984;74:127–133.
27. Ayala JE, Boustead JN, Chapman SC, et al. Insulin-mediated activation of activator protein-1 through the mitogen-activated protein kinase pathway stimulates collagenase-1 gene transcription in the MES 13 mesangial cell line. *J Mol Endocrinol*. 2004;33:263–280.
28. Keshet E. Preventing pathological regression of blood vessels. *J Clin Invest*. 2003;112:27–29.
29. Meininger V, Lacomblez L, Salachas F. What has changed with riluzole? *J Neurol*. 2000;247:VI19-VI22.
30. Doble A. Effects of riluzole in glutamatergic neurotransmission in the mammalian central nervous system, and other pharmacological effects. *Rev Contemp Pharmacother*. 1997;8:213–225.
31. Schiefer J, Landwehrmeyer GB, Lüesse HG, et al. Riluzole prolongs survival time and alters nuclear inclusion formation in a transgenic mouse model of Huntington's disease. *Movement Disord*. 2002;17:748–757.
32. Ettaiche M, Fillacier K, Widmann C, Heurteaux C, Lazdunski M. Riluzole improves functional recovery after ischemia in the rat retina. *Invest Ophthalmol Vis Sci*. 1999;40:729–736.
33. Cheramy A, Barbeito L, Godeheu G, Glowinski J. Riluzole inhibits the release of glutamate in the caudate nucleus of the cat in vivo. *Neurosci Lett*. 1992;147:209–212.
34. Jehle T, Bauer J, Blauth E, et al. Effects of riluzole on electrically evoked neurotransmitter release. *Br J Pharmacol*. 2000;130:1227–1234.