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

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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

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. 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 (Arkay, Kyoto, Japan).
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 MW) 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 cover slips, 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, modified from the International Criteria for Retinopathy of Prematurity system for humans, 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.

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.

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 mM Na3VO4, 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% H2O2 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 QD-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 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% O2,14 rats with mild OIR exhibited less severe pathologic changes in all the areas of retinopathy. Insulin treatment mark-
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, 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
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
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% \textsuperscript{2}) environment.\textsuperscript{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% \textsuperscript{2}) environment.\textsuperscript{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\textsuperscript{29,30} and neuronal cell death.\textsuperscript{31,32} With its proven neuroprotective efficacy, the FDA in 1996 approved riluzole for the treatment of ALS.\textsuperscript{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.\textsuperscript{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.\textsuperscript{55–54} 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 \( \beta \)-II isoform. Regardless of the mechanism, riluzole is highly effective in reducing all pathologic changes in full OIR\textsuperscript{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**


