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Increase in retinal hypoxia inducible factor-2α, but not hypoxia, early in the progression of diabetes in the rat

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Abstract

Hypoxia and the associated hypoxia-inducible factors (HIFs) may be influential in the progression of diabetic retinopathy. However, little is known of the extent of hypoxia and the levels of HIFs early in the progression of the disease. In the current study, we injected the oxygen-dependent probe pimonidazole (Hypoxyprobe™-1) into diabetic rats, and also performed immunohistochemistry to determine the retinal levels of HIF-1α and HIF-2α. The rats were made diabetic using a single injection of streptozotocin (STZ; 60 mg/kg), with vehicle-injected rats used as non-diabetic controls. The measurements of hypoxia and HIF levels were obtained three weeks following STZ injection, at which time we have previously found significant decreases in retinal blood flow in the same model. In the current experiments, no increases in either HIF-1α or hypoxia were observed in the diabetic rats (compared with controls), and there was even a tendency for hypoxia levels to be decreased (tissue more highly oxygenated). However, we did observe an increase in HIF-2α in the retinas of the diabetic rats. Therefore, we conclude that early diabetes-induced increases in HIF-2α occur independently of hypoxia.

Keywords

hypoxia-inducible factor; HIF-1α; HIF-2α; streptozotocin; diabetes; retina; hypoxia

1. INTRODUCTION

Early stages of diabetic retinopathy are characterized by retinal vascular abnormalities such as microaneurysms, intraretinal hemorrhages, and cotton-wool spots, while later stages of the disease are characterized by retinal neovascularization and the threat of blindness (Yam and Kwok 2007). Although the mechanisms of diabetic retinopathy have not been completely elucidated, retinal hypoxia is often implicated as a central player. Caused by hypoperfusion, leukostasis, and/or capillary dropout, hypoxia in turn may lead to hyperpermeability, neovascularization, and hyperperfusion (Curtis et al., 2009).

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Hypoxia initiates the stabilization of hypoxia inducible factors (HIFs), including HIF-1α and HIF-2α (Arjamaa and Nikinmaa 2006; Holmquist-Mengelbier et al., 2006), which are constitutively expressed and regulated in an oxygen-dependent manner (Lisy and Peet 2008). However, HIF-1α can also be stabilized by stimuli other than hypoxia (Bell et al., 2007). Interestingly, HIF-1α and -2α appear to be activated in different cell populations in the ischemic retina (Mowat et al., 2010).

Upon stabilization, HIF-1α and -2α are transported into the nucleus where they become transcription factors for a variety of downstream targets. One downstream target of HIF-1α is the angiogenic factor vascular endothelial growth factor (VEGF) (Arjamaa and Nikinmaa 2006), which is a target for potential therapy in diabetic retinopathy (Waisbourd et al.,). Downstream targets of HIF-2α are oxidative stress-related genes including catalase-encoding *Cas1*, superoxide dismutase-encoding *SOD1*, and erythropoietin-encoding *EPO* (Warnecke et al., 2004; Ding et al., 2005). In the diabetic retina, the activities of catalase and SOD have been shown to be altered (Obrosova et al., 2006), and EPO appears to play a neural protective role (Zhu et al., 2008).

Evidence for retinal hypoxia has been obtained from diabetic retinopathy patients, with vitreous oxygen tensions of 5.7 mmHg compared with 8.5 mmHg in non-diabetics (Holekamp et al., 2006). In cats that were diabetic for 6–8 years, oxygen microelectrodes placed in the inner retina gave measurements of 7.7 mmHg compared with 16.4 mmHg in controls (Linsenmeier et al., 1998). At a much shorter period of diabetes, mild retinal hypoxia was reported for mice following 5 months of diabetes (de Gooyer et al., 2006), although at a period of 3 months diabetes, we found *less* hypoxia than in controls, for both mice and rats (Wright et al., 2010).

The explanation for our unexpected finding of less hypoxia at 3 months diabetes can only be speculative inasmuch as we have not measured the change in retinal blood flow at that time point. However, in multiple investigations we have found significant (20–45%) reductions in retinal blood flow at approximately 3–5 weeks of diabetes in rats and mice (Lee and Harris 2008; Lee et al., 2008; Wright and Harris 2008; Wright et al., 2009), and therefore the purpose of the current study was to examine the extent of hypoxia and the potential upregulation of HIFs at this early time point when we know that flow is reduced.

2. METHODS

2.1 Animals

Male Wistar rats (Harlan Laboratories) weighing 137 to 158 grams were assigned to i.p. injection of streptozotocin (STZ; 60 mg/kg dissolved in pH 4.5 sodium citrate buffer) or sodium citrate buffer alone. STZ was injected into the animals within 15 min of preparation. The rats received standard chow and water and were housed 1 per cage for 3 weeks following injection. On the day of the experiment, non-fasting blood glucose levels were checked via a tail vein puncture using a One Touch Ultra Glucometer (Milpitas, CA); no insulin was given during the 3-week protocol. The animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

2.2 Measurement of Retinal Hypoxia

Retinal tissue hypoxia was measured using the Hypoxyprobe-1 Omni Kit (Natural Pharmacia International Inc., Burlington, MA), which contains pimonidazole hydrochloride (HCl) and rabbit anti-pimonidazole antisera (primary antibody for immunofluorescent labeling). Pimonidazole remains in hypoxic cells after forming an irreversible adduct with thiol groups in environments with $pO_2 < 10$ mmHg (Raleigh and Koch, C. J., 1990). Using

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the rabbit anti-pimonidazole antisera, immunofluorescent labeling of retinal cross-sections for localization of hypoxia was performed.

2.3 Tissue Preparation for Immunofluorescence Labeling

Three weeks following STZ injection, the rats were injected i.p. with pimonidazole HCl at a dose of 60 mg/kg. Three hours following pimonidazole HCl injection, rats were anaesthetized with Inactin, and eyes were enucleated and placed in phosphate buffered 4% paraformaldehyde (FD Neurotechnologies, Inc., Baltimore, MD) for 3 hours. The eye was cut at the ora serrata, and the lens and anterior portion of the eye were removed. The eyecup (retina, choroid, and sclera) was placed in 20–25% sucrose solution at 4° C overnight for cryoprotection (Chen and Nathans, J., 2007; Nishiguchi et al., 2008), then placed in optimal cutting temperature (O.C.T.) compound and cut at 10 μm thickness and stored at −80°C until labeled.

2.4 Immunofluorescence Labeling

Slides were washed twice for 5 min in Triton X-100 with gentle agitation. The slides then were incubated in goat serum for 1.5 hours, followed by incubation with primary antibody mixed in PBS + 1% bovine serum albumin (BSA) overnight at 4° C. Negative controls slides were similarly incubated, but without addition of the primary antibody. Next, the slides were washed twice with Triton X-100 for 5 min with gentle agitation, followed by incubation with secondary antibody mixed in PBS + 1% BSA for 1 hr in the dark. Finally, the slides were washed twice in PBS for 10 min and mounted with Vectashield Hardset mounting medium with DAPI (Vector Laboratories, Inc., Burlingam, CA).

Primary antibodies used were rabbit polyclonal HIF-1α (Lifespan Biosciences, Seattle, WA) at a 1:100 dilution, rabbit polyclonal HIF-2α (Abcam, Cambridge, MA) at a 1:100 dilution, rabbit polyclonal anti-pimonidazole (NPi Inc, Burlington, MA) at a 1:1000 dilution, and a rabbit polyclonal IgG (Abcam, Cambridge, MA) used for an isotype control. The secondary antibody was an FITC goat anti-rabbit IgG-Fc fragment (Jackson Immuno Research Labs, West Grove, PA) at a 1:250 dilution for HIF-1α, a 1:100 dilution for HIF-2α, and a 1:200 dilution for anti-pimonidazole.

2.5 Immunofluorescence Image Analysis

A CoolSNAP ES camera (Photometrics, Tucson, AZ) was attached to a Nikon Eclipse E600FN microscope using an X-Cite 120 Fluorescence illumination system. A 10x objective and a Nikon FITC filter was used for image acquisition using an exposure time of 500 ms. Light intensity measurements were obtained using a radiometer photometer (model ILT 1400-A; International Light Technologies; Peabody, MA) before and after images were obtained from each slide, and then averaged. NIS Elements Basic Research software version 3.0 (Nikon Instruments Inc., Melville, NY) was used for capturing and analyzing the images.

Images obtained from both the central and peripheral retina were analyzed by individual layers (ganglion cell layer, GCL; inner plexiform layer, IPL; inner nuclear layer, INL; outer plexiform layer, OPL; outer nuclear layer, ONL; and photoreceptor layer, PR). In the DAPI counterstained image, a region of interest was drawn around the area to be analyzed, with the identical region of interest used for the matching FITC-conjugated stained image, with a background region of interest collected for subtraction. Following background subtraction from both the sample and isotype control, isotype fluorescence was subtracted from the sample fluorescence, and normalized to the microscope light intensity.

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2.6 Statistics

Data are expressed as means ± standard error of the mean. Statistical t-tests and linear regression analyses were performed with GraphPad Instat version 3.05 software (San Diego, CA), using a criterion of $p<0.05$ as statistically significant.

3. RESULTS

At the beginning of the protocol, the two groups of rats (N=10 control; N=8 STZ) both averaged 146 ± 2 g. However, the STZ rats gained weight more slowly than the controls, with the means significantly different ($p<0.001$) at the end of the 3-week protocol (control: 300 ± 6 g; STZ: 241 ± 3 g). Non-fasting glucose levels averaged 116 ± 3 mg/dl in controls, and above 400 mg/dl in the STZ rats, with the values exceeding the glucose meter maximum (of 600 mg/dl) in one-half of the group. One rat injected with STZ did not become diabetic and was not included in the experimental group.

Hypoxyprobe (pimonidazole) was injected into the rats of both groups, for a measurement of the extent of retinal hypoxia. As shown in Figure 1A, hypoxia tended to be *less* pronounced in all layers of the central retina of the STZ group compared with controls, although the differences did not reach statistical significance. In the peripheral retina (Fig 1B), this tendency (toward an STZ-induced *decrease* in hypoxia) was limited to the ganglion cell and inner plexiform layers.

Cross-sections of the enucleated eyes were stained for both HIF-1α and HIF-2α, as shown in Figures 2 and 3, respectively. Staining for HIF-1α was very similar between control and STZ groups, while there was a tendency for HIF-2α to increase with diabetes, with this tendency reaching statistical significance in the ganglion cell, inner plexiform, and outer plexiform layers of the peripheral retina.

Figure 4 provides sample images of the immunostaining for HIF-1α, HIF-2α, and Hypoxyprobe, with the upper row of images from a control rat, and the lower row from an STZ rat. These examples are reflective of the data in Figures 1–3, with the HIF-2α staining in the STZ rat greater than in the control, despite a decrease in hypoxia (Hypoxyprobe).

In general, there was little correlation between HIF expression and Hypoxyprobe staining, with the one exception being HIF-1α in the central retina of control rats. As shown in Figure 5A, in control rats having Hypoxyprobe staining of less than one-half of the normalized average (i.e., <0.5 on the x-axis), HIF-1α expression was uniformly below average (< 1.0 on the y-axis). In contrast, HIF-1α expression was generally higher than average in layers of increased hypoxia. This correlation was found only for HIF-1α in the central retina of controls ($r^2=0.41$; $p<0.0001$): as shown in Figure 5B, no relationship between these two parameters was found in STZ rats. Additionally, no correlations were found between HIF-2α and Hypoxyprobe in either control or STZ rats.

4. DISCUSSION

In this study, we found that STZ-induced diabetes in the rat increases HIF-2α in the retina, and to our knowledge, this is the first demonstration of this finding. We found the greatest increase in HIF-2α in the peripheral inner retina. Although increases in HIF-2α can occur due to hypoxia, we observed a tendency for the reverse, that is, less hypoxia in the diabetic rat retina. Moreover, we found no increase in HIF-1α in either the central or peripheral retina of the diabetic rats. Taken together, our results suggest a potential hypoxia-independent increase in HIF-2α early in the diabetic retina.

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The tendency for less hypoxia in the diabetic retina may differ from expectations, although we have previously reported statistically significant decreases in hypoxia in the STZ-injected rat following a more lengthy 12-week period of hyperglycemia (Wright et al., 2010). However, inasmuch as we do not have measurements of retinal blood flow at 12 weeks STZ, it is difficult to know how the increase in oxygenation corresponds with any potential changes in perfusion. In the current study, we measured hypoxia and HIFs at a time point (3 weeks) where we have observed a diabetes-induced constriction of retinal arterioles and a significant reduction in retinal blood flow in the rat (Lee et al., 2008). Interestingly, despite a decrease in flow at this time point, we also found a trend toward a decrease in hypoxia in the current study. The extent of "hyperoxygenation" is similar to the level that we observed at 12 weeks of STZ (Wright et al., 2010), which is supported by the oxygen microelectrode results presented by another group (Lau and Linsenmeier 2010).

The amount of pimonidazole binding can potentially be influenced by factors other than hypoxia alone, with factors such as pH, sulfhydryl concentrations, and nitroreductase concentrations having been given previous consideration (Kleiter et al., 2006). However, we are unaware that any of these factors would be altered between our control and diabetic groups to the extent that would influence our interpretations. With respect to one of these factors, pimonidazole binding in anoxic V79 cells is reduced when pH is substantially lowered by a full pH unit, to a value of 6.4 rather than 7.4 (Kleiter et al., 2006). To our knowledge there are no reports of intraretinal pH measurements in hyperglycemic vs normoglycemic rats in the initial few weeks of chemically induced hyperglycemia. However, intraretinal pH has been measured in diabetic cats, with the inner retina tending to be 0.07–0.08 pH units more acidic than in normoglycemic cats (Budzynski et al., 2005). Therefore, unless the change toward acidity is much more substantial in our rat model, we predict that pH probably has little influence on our hypoxia assay. Finally, our pimonidazole data suggesting a decrease in retinal oxygenation in early diabetes has been corroborated for the time point of 12 weeks STZ by pO_2 microelectrode measurements (Lau and Linsenmeier 2010), in a protocol that would be insensitive to the factors listed above.

When speculating the cause and effect between vasoconstriction and changes in oxygenation, it could have been predicted that the early diabetes-induced decreases in retinal blood flow would lead to hypoxia. However, data from our lab seem to suggest that *hyperoxygenation*, instead, may lead to vasoconstriction and a decrease in retinal blood flow. Certainly, there is ample evidence that increases in oxygenation induce vasoconstriction. In addition, the scenario of elevated oxygenation, and/or decreased oxygen consumption in the diabetic retina, has been postulated previously to produce a decrease in retinal blood flow (Small et al., 1987; Rimmer and Linsenmeier 1993).

The hypoxia-independent increase in HIF-2α may need further investigation. Elevated levels of reactive oxygen species (ROS) could be hypothesized to stabilize HIF-2α independent of hypoxia, as occurs for HIF-1α (Bell et al., 2007). However, if this is the case, the potential ROS-induced increase in HIFs may be transient, since increases in ROS in the diabetic retina continue through 6 weeks to 6 months (Kowluru et al., 2006; Obrosova et al., 2006; Kanwar et al., 2007; Zheng et al., 2007; Gubitosi-Klug et al., 2008), whereas the increase in HIFs may or may not be sustained over that period. HIF-1α has been reported to increase in rats 2 weeks following STZ injection (Poulaki et al., 2004) but not after 3 weeks (current study), or at 4 or 12 weeks STZ (Wright et al., 2010). HIF-2α might follow a similar but slightly different pattern compared to HIF-1α, with increases following 3 weeks of STZ (current study) but not after 4 or 12 weeks STZ (Wright et al., 2010).

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In conclusion, we find an early increase in HIF-2α in the diabetic rat retina at a time when we observe no increase in either hypoxia or HIF-1α. The stimulus for the increase in HIF-2α, and the possible signaling consequences of the increase, have yet to be determined.

- The study investigated retinal hypoxia and HIFs at 3 weeks STZ-induced diabetes in rats.
- No increases in hypoxia or HIF-1α were observed in the diabetic retina at this time point.
- HIF-2α was increased in the diabetic retina, with the levels independent of hypoxia.

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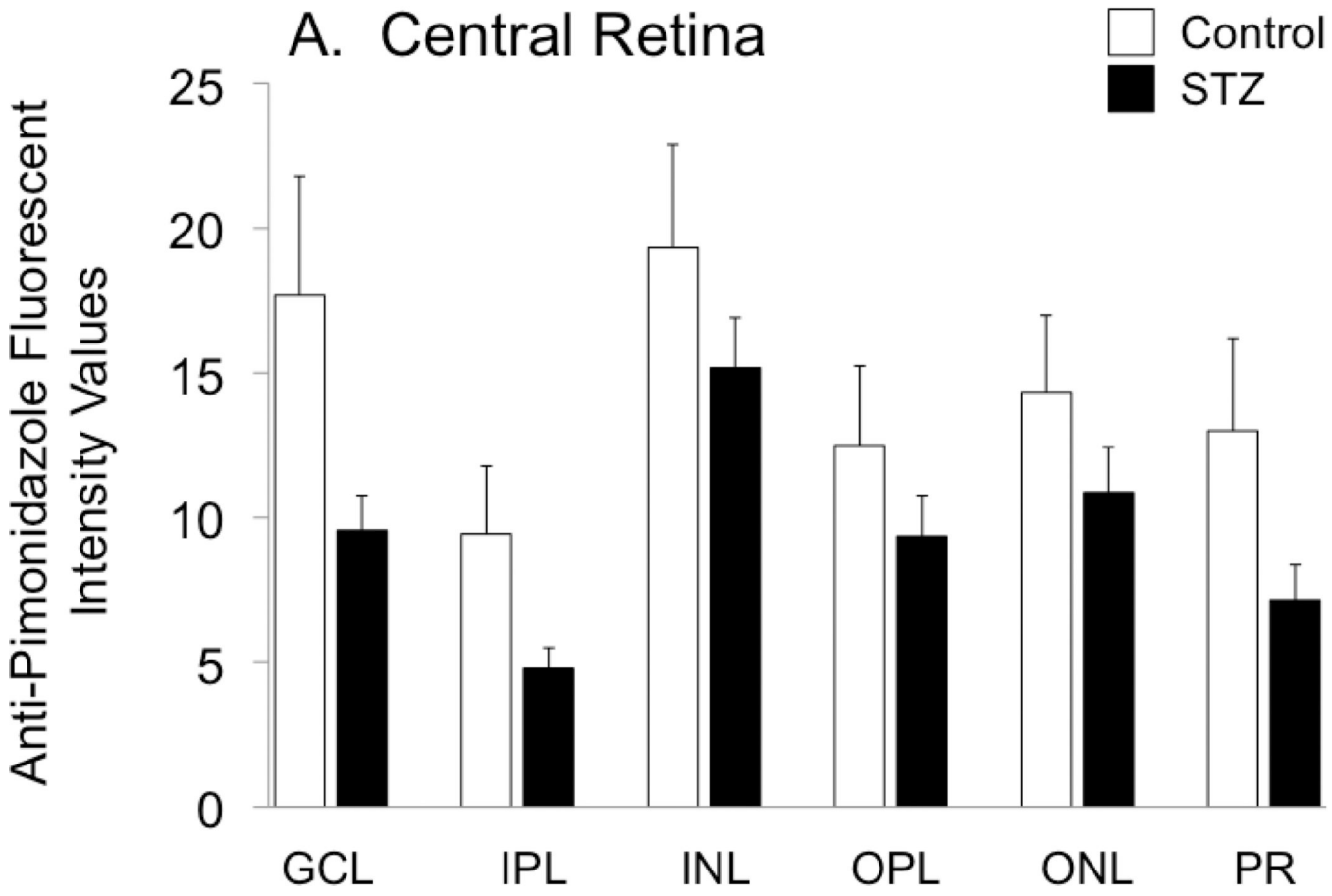

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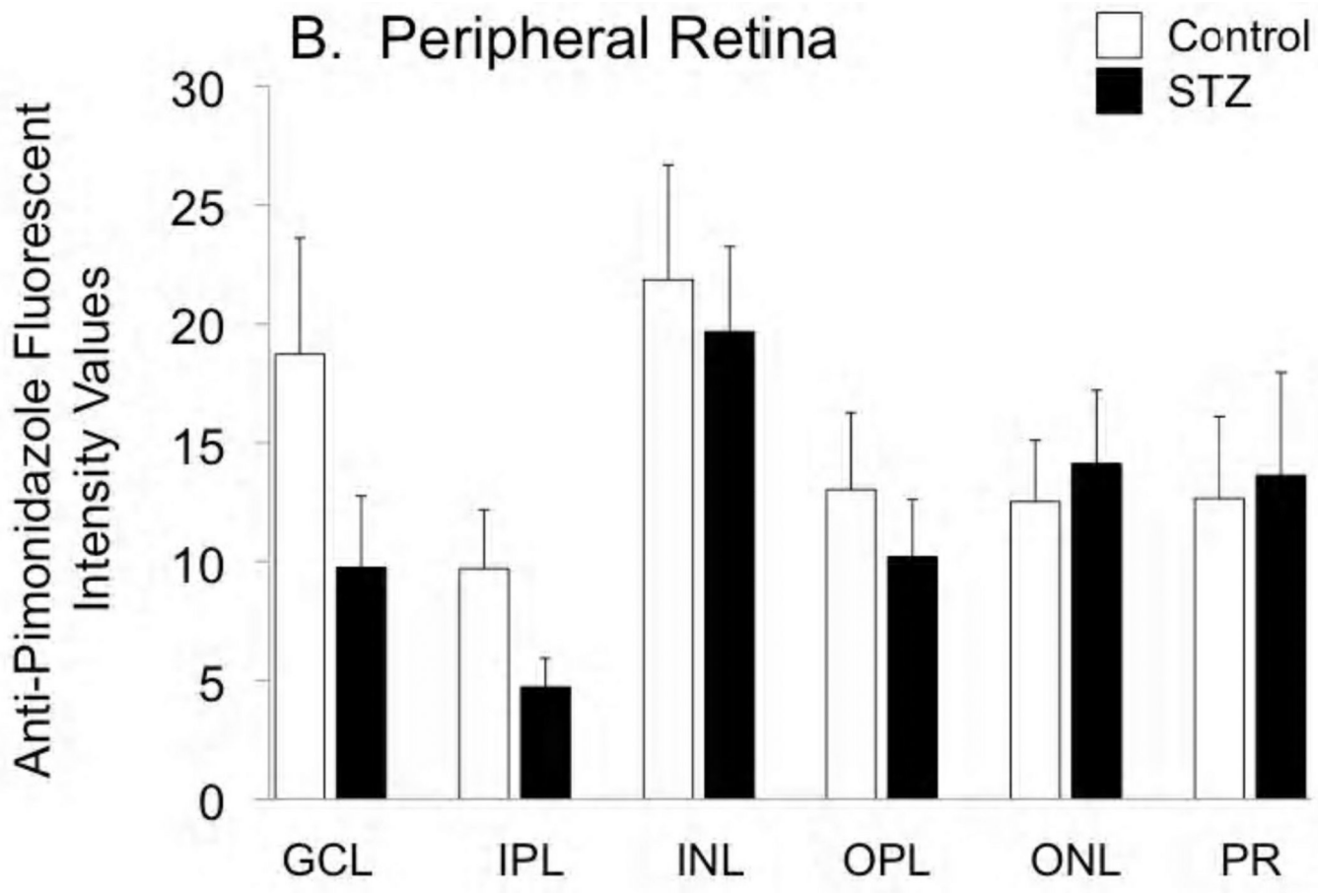

Figure 1.
Fluorescent staining intensities for Hypoxyprobe (anti-pimonidazole antibody) in the central retina (A) and peripheral retina (B) of control and STZ diabetic rats. Layers are abbreviated GCL (ganglion cell layer), IPL (inner plexiform layer), INL (inner nuclear layer), OPL (outer plexiform layer), ONL (outer nuclear layer), and PR (photoreceptor layer).

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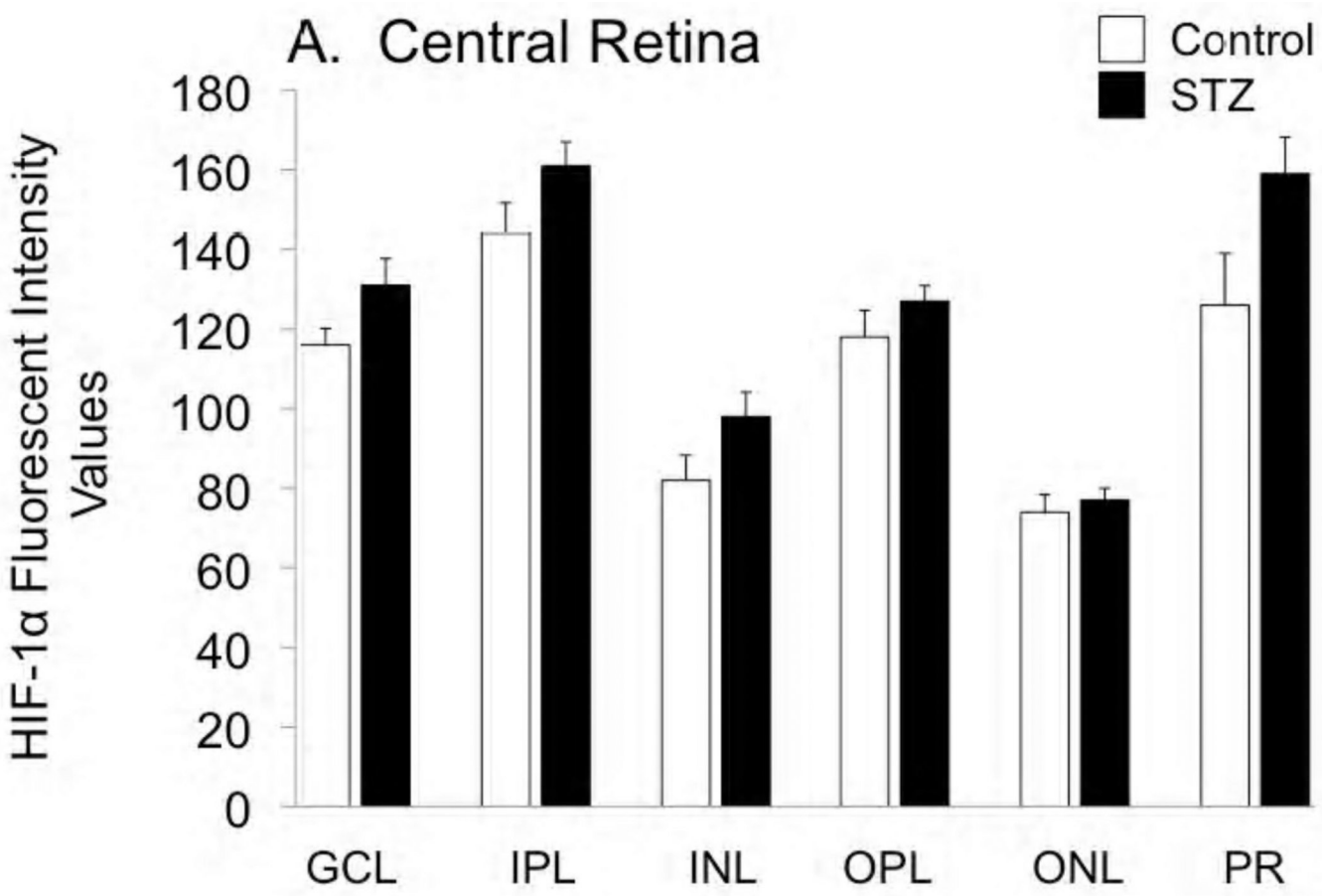

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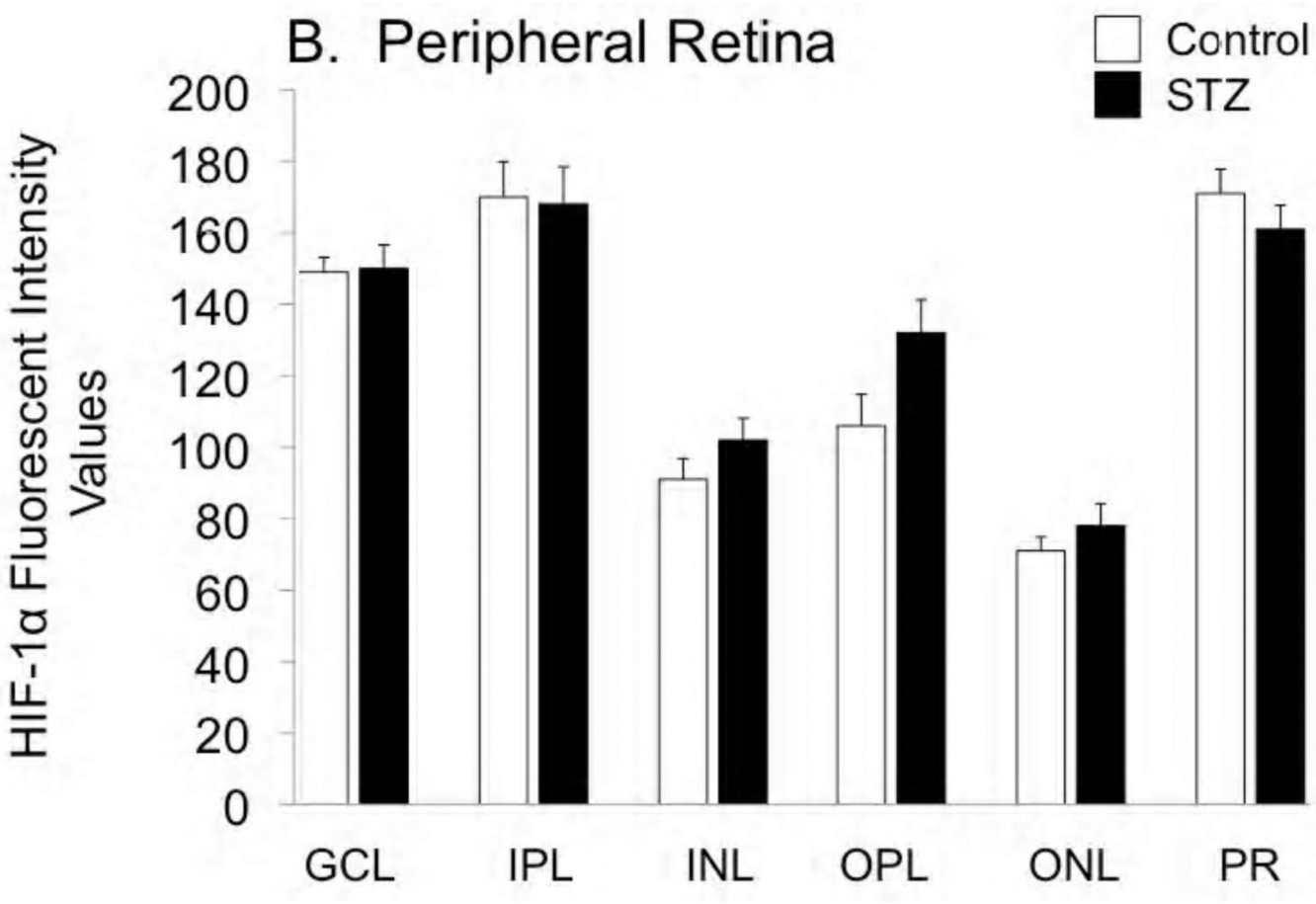

Figure 2.

Fluorescent immunostaining for HIF-1α in the central retina (A) and peripheral retina (B) of control and STZ diabetic rats. Layers are abbreviated GCL (ganglion cell layer), IPL (inner plexiform layer), INL (inner nuclear layer), OPL (outer plexiform layer), ONL (outer nuclear layer), and PR (photoreceptor layer).

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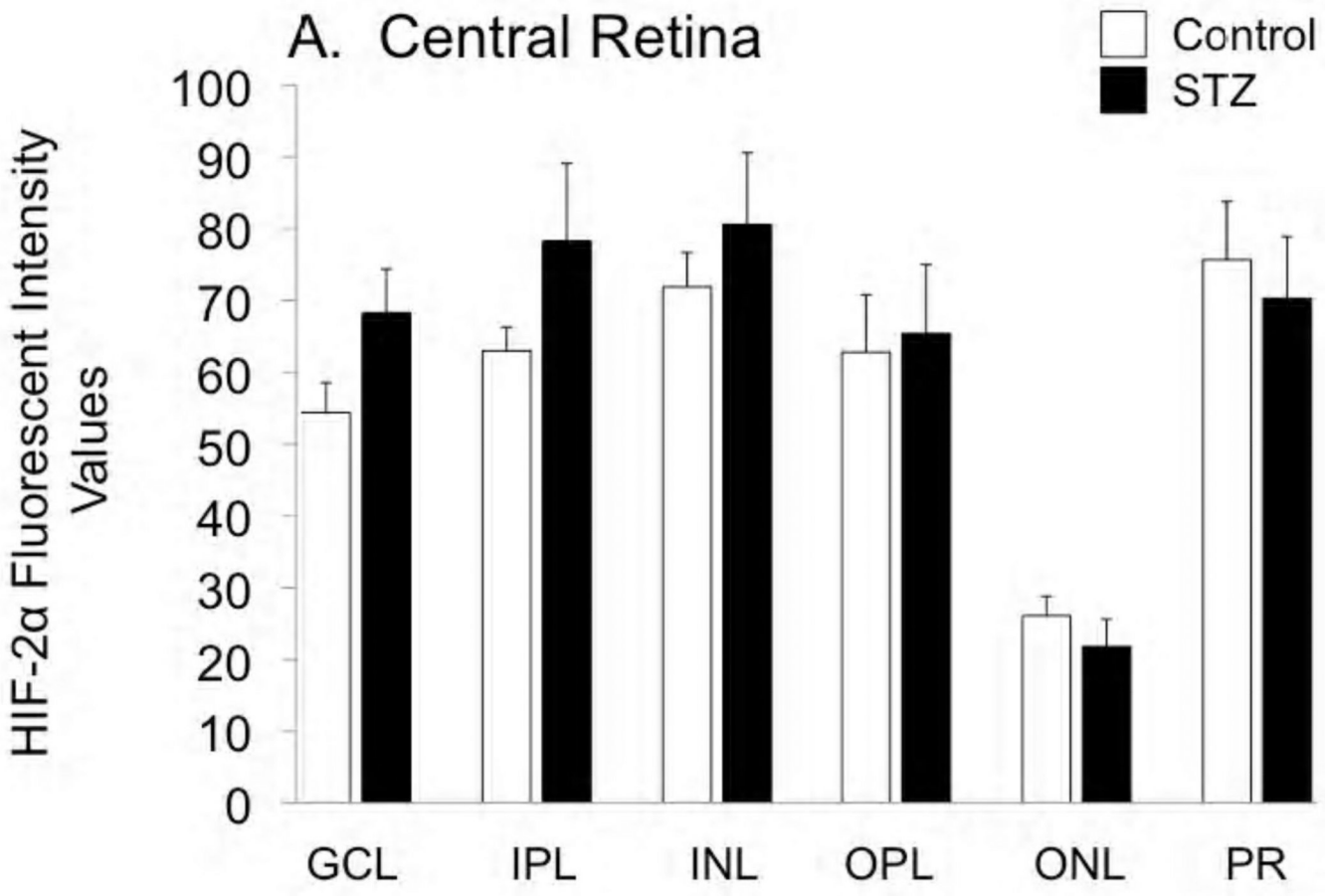

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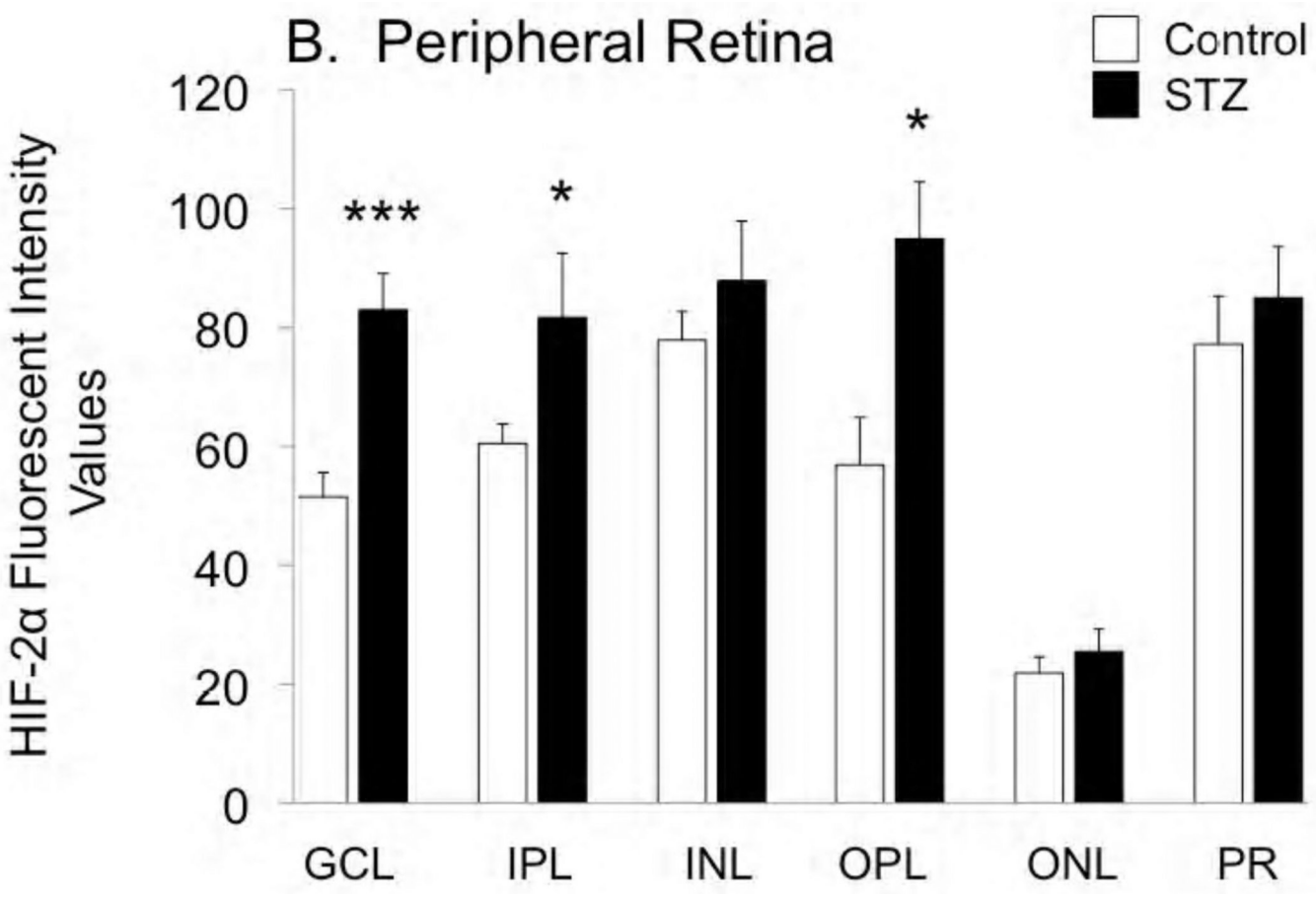

Figure 3.

Fluorescent immunostaining for HIF-2α in the central retina (A) and peripheral retina (B) of control and STZ diabetic rats. Layers are abbreviated GCL (ganglion cell layer), IPL (inner plexiform layer), INL (inner nuclear layer), OPL (outer plexiform layer), ONL (outer nuclear layer), and PR (photoreceptor layer). *$p<0.05$ and ***$p<0.001$ vs controls.

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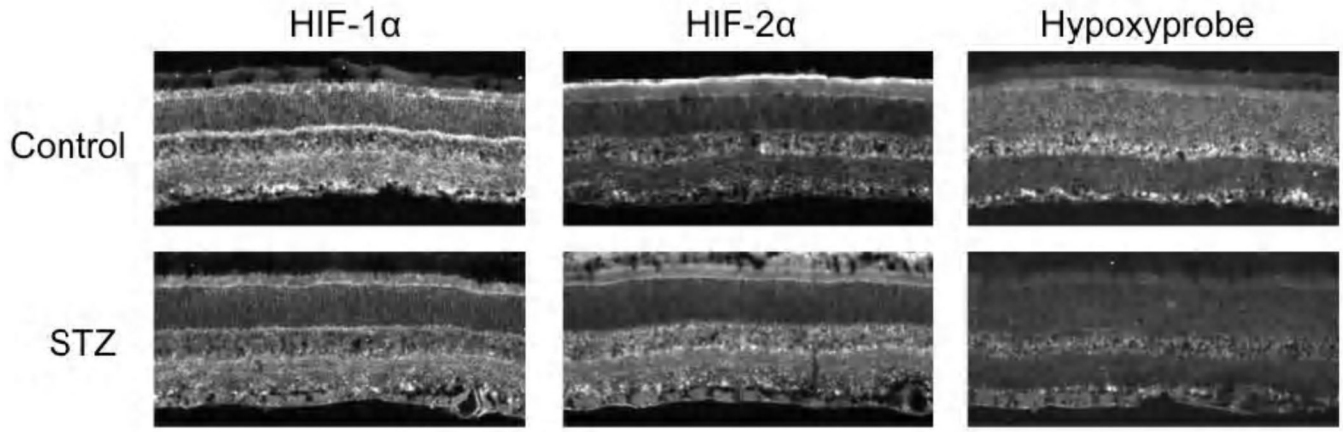

Figure 4.
Example images of HIF-1α, HIF-2α, and Hypoxyprobe staining in the central retinas of one control and one STZ rat. In each frame, the ganglion cell layer is at the bottom, and the photoreceptor layer is toward the top.

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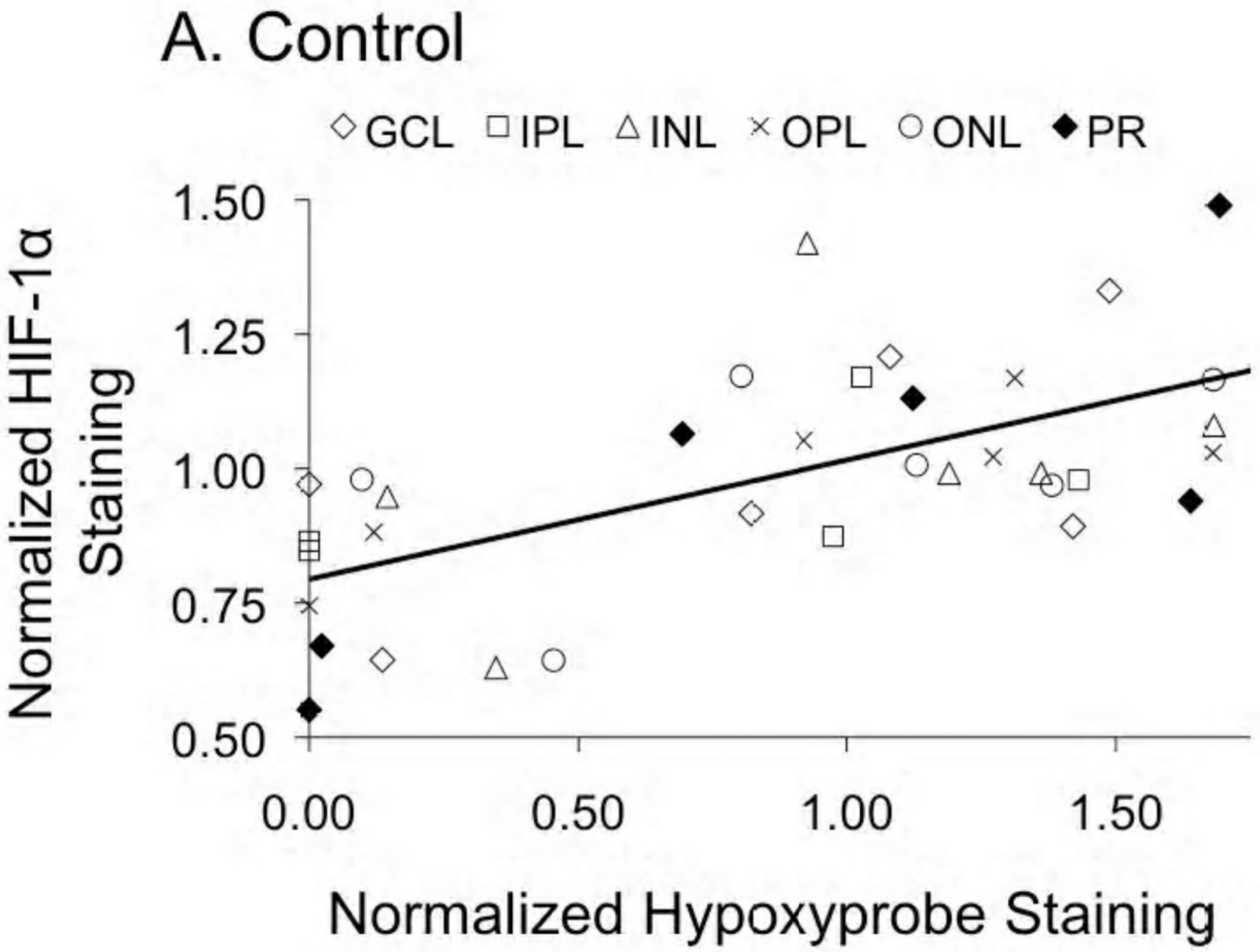

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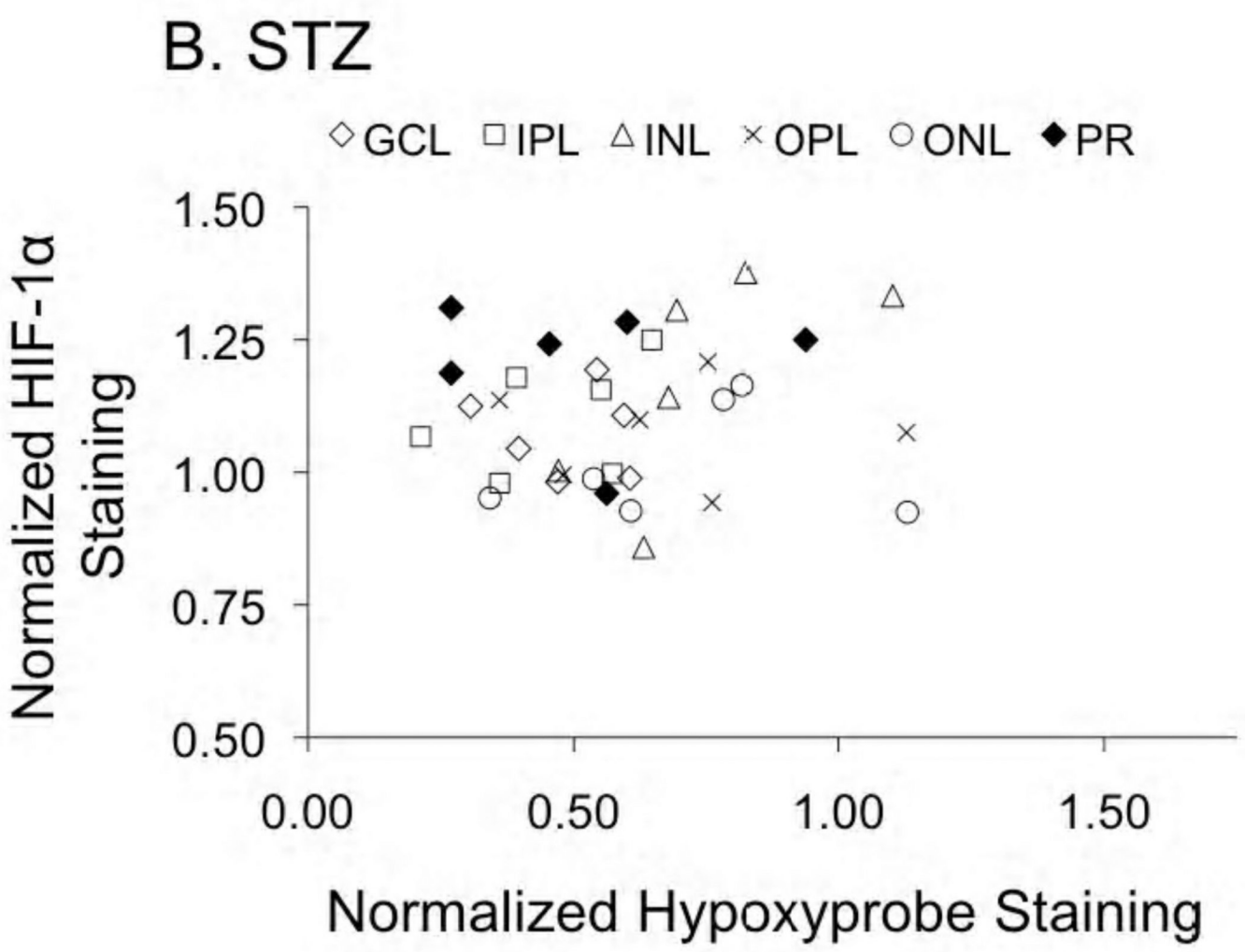

Figure 5.

HIF-1α and Hypoxyprobe staining presented as normalized to the control average. Panel A provides the relationship (r^2=0.41; $p<0.0001$) in control rats; no relationship between the two parameters was found in the STZ rats (panel B). Data are provided for the ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL), outer nuclear layer (ONL), and photoreceptor layer (PR).