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The Anti-Inflammatory Effects of Glucagon-Like Peptide Receptor Agonist Lixisenatide on the Retinal Nuclear and Nerve Fiber Layers in an Animal Model of Early Type 2 Diabetes

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This study explored the anti-inflammatory effects of a glucagon-like peptide-1 receptor agonist (GLP-1RA), known as lixisenatide, on the eyes of early type 2 diabetic mice. Diabetic (*db/db*) mice were divided into three groups: GLP-1RA [lixisenatide (LIX)], insulin (INS) with controlled hyperglycemia based on the glucose concentration of lixisenatide, and diabetic control (D-CON). Nondiabetic control mice (*db/dm*) were also characterized for comparison. After 8 weeks of treatment, mRNA levels of inflammatory markers, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling, immunohistochemical staining; Western blot of glial fibrillary acidic protein (GFAP) and thioredoxin-interacting protein; and retinal thickness were assessed in the central and peripheral neurosensory retina. LIX showed decreased immunohistochemical staining for both thioredoxin-interacting protein and GFAP in the central and peripheral neurosensory retina compared with D-CON and INS, and decreased expression of these proteins in the neurosensory retina and immunohistochemical staining in the optic nerve head for GFAP compared with D-CON. The inner nuclear layer in the peripheral retina in LIX was only thinner than those of D-CON and INS. In an early type 2 diabetic mouse model, lixisenatide treatment showed superior anti-inflammatory effects on the retina and optic nerve head independent of hyperglycemia. Thus, the neuroprotective effects of lixisenatide treatment in the peripheral inner nuclear layer should be evaluated in early type 2 diabetic retinopathy. (*Am J Pathol* 2020, 190: 1080–1094; <https://doi.org/10.1016/j.ajpath.2020.01.011>)

Type 2 diabetes (T2D) is caused by β -cell dysfunction in the pancreas in response to the increased insulin demand of nutrient overload or obesity-associated insulin resistance.¹ The role of β -cells in the pathogenesis of diabetes has remained controversial. However, many studies have shown that hyperglycemia and inflammatory cytokines cause endoplasmic reticulum stress, the accumulation of reactive oxygen species, and eventually, β -cell dysfunction, and apoptosis.² Moreover, oxidative stress plays an important role in diabetic vascular complications.³

Diabetic retinopathy (DR), one such diabetic vascular complications and the most common cause of visual deterioration in adult populations,⁴ had been considered a microvascular complication induced by hyperglycemia. However, several studies have shown that inflammation is

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Table 1 Sequences of Forward and Reverse Primer Sets Used in qPCR

Amplification	Forward primer	Reverse primer
GS	5'-GCTGCAAGACCCGTACCCT-3'	5'-TTCCACTCAGGTAAGTCTTCCACA-3'
GFAP	5'-GAGTACCACGATCTACTCAAC-3'	5'-CCACAGTCTTTACCACGATGT-3'
TNF- α	5'-ATGAGCACAGAAAGCATGA-3'	5'-AGTAGACAGAAGAGCGTGGT-3'
VEGF	5'-CACAGCAGATGTGAATGCAG-3'	5'-TTTACACGTCTGCGGATCTT-3'
TXNIP	5'-CGAGTCAAAGCCGTCAGGAT-3'	5'-TTCATAGCGCAAGTAGTCCAAAGT-3'
GAPDH	5'-CATGGCCTCCAAGGAGTAAGA-3'	5'-GAGGGAGATGCTCAGTGTGG-3'

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFAP, glial fibrillary acidic protein; GS, glutamine synthetase; qPCR, quantitative PCR; TNF- α , tumor necrosis factor alpha; TXNIP, thioredoxin-interacting protein; VEGF, vascular endothelial growth factor.

an important player in the progression of DR.^{5–7} Villarroel et al⁵ reported that retinal neurodegeneration was present before any microcirculatory abnormalities, which may be used to develop novel therapeutic targets in the early stages of type 2 DR. Barber et al⁷ also reported that neuronal function may be compromised prior to apoptosis, contributing to an early deterioration of vision in type 2 DR. These results suggest that blocking inflammation in the eyes in early T2D may decrease neuronal dysfunction and thus reduce vascular complications caused by type 2 DR.

Recent studies have shown that thioredoxin-interacting protein (TXNIP) is an important component in both T2D and type 2 DR. TXNIP binds to the redox-active cysteine residues of thioredoxin, inhibiting its oxidoreductase activity, linking this endoplasmic reticulum stress to inflammation, and resulting in target organ dysfunction.^{8–10} TXNIP is known to be highly expressed in the ganglion cell layer, internal plexiform layer, internal nuclear layer (INL), retinal endothelial cells, Müller cells, and pericyte cells in the retina, as well as human islet cells, under hyperglycemic states.^{11–14} TXNIP increases metabolic dysregulation, including hyperglycemia, oxidative and endoplasmic reticulum stress, and inflammation.^{9,10,15–18} In addition, TXNIP up-regulation leads to β -cell apoptosis in T2D,^{19–22} and angiogenesis in type 2 diabetic retinas by promoting vascular endothelial growth factor A (VEGFA), also known as vascular endothelial growth factor (VEGF), which is one of the most important players in the pathogenesis of proliferative DR.^{12,18,23}

Insulin, which is important for the treatment of T2D, suppresses TXNIP expression in β -cells by decreasing hyperglycemia and its induced oxidative stress.^{19,21,22,24,25} However, insulin has adverse effects such as weight gain and hypoglycemia, as well as increased vascular permeability causing an edematous state after the initiation of intensive insulin therapy.^{26–28} In a diabetic rat model, Bixler et al²⁹ concluded that several genes associated with inflammatory processes, microvascular integrity, and neuronal function showed altered expression patterns in a euglycemic state with insulin treatment. The Diabetes Control and Complications Trial (DCCT), a human clinical study, reported that vision can deteriorate with intensive insulin treatment in patients with long-standing poor glycemic control.³⁰

On the other hand, glucagon-like peptide-1 receptor agonist (GLP-1RA) has many beneficial effects in the treatment of T2D, including anti-inflammatory properties, glucose control, and β -cell protection, by both enhancing the secretion of insulin and down-regulating TXNIP expression.^{31–35} Additionally, through its anti-inflammatory properties, GLP-1RA has been shown to prevent microglial activation by toxins and is a good candidate for suppressing neuroinflammation and blocking the pathogenesis of Parkinson disease.^{36,37} In the field of ophthalmology, Hernandez et al³⁸ found that GLP-1R is concentrated in the retina, and that GLP-1RA had neuroprotective effects in the retinas of both humans and *db/db* mice, which are considered a typical animal model of T2D that exhibits hyperglycemia associated with obesity.

There have been several clinical reports analyzing the relationship between DR and macular thickness or retinal nerve fiber layer (NFL) thickness in T2D.^{30,39} However the timing of diabetes onset remains unclear, and these studies may not reflect very early changes in retinal layers. Furthermore, no study has explored the effect of GLP-1RAs on the inflammatory changes caused by TXNIP in the retina, or directly compared the early neuroprotective effects of GLP-1RAs on the retina with those of insulin.

Therefore, this study analyzed and compared the pathologic and associated morphologic changes caused by several inflammatory markers, including TXNIP on early diabetic retinas in an animal model of T2D. This investigation also compared the anti-inflammatory and/or neuroprotective effect in the retina and the optic nerve head of GLP-1RA treatment with insulin-treated, diabetic untreated (*db/db*) and nondiabetic mice (*db/dm*).

Materials and Methods

Animal Experiments

Five-week-old male *db/db* mice (type 2 diabetic) and *db/dm* mice (nondiabetic) were obtained from Charles River Japan (Kanagawa, Japan). Animals received an i.p. glucose tolerance test (baseline), which has been described previously.⁴⁰ The *db/db* mice were then divided into three groups: the lixisenatide group (LIX) ($n = 7$, GLP-1RA; Sanofi, Paris, France), the insulin group (INS) with a

Table 2 Body Weights and Serum Glucose Levels by Group

Parameter	<i>db/dm</i>	D-CON	INS	LIX
Body weight, g	27.3 ± 1.6	48.9 ± 7.0	54.2 ± 1.5	44.6 ± 8.4
<i>P</i> value*	<0.001			
Post hoc analysis†	D-CON, INS, LIX > <i>db/dm</i>			
Serum glucose, mg/dL				
0 min	63.6 ± 16.1	366.9 ± 44.9	278.6 ± 121.5	320.0 ± 125.5
30 min	89.2 ± 39.6	554.6 ± 92.9	554.3 ± 66.8	498.3 ± 36.7
60 min	87.2 ± 45.4	747.1 ± 138.0	639.1 ± 86.0	578.0 ± 123.1
90 min	79.2 ± 38.2	757.4 ± 60.0	693.7 ± 135.2	616.5 ± 100.3
120 min	71.2 ± 30.8	788.6 ± 132.5	698.7 ± 72.8	648.8 ± 102.9
<i>P</i> value (120 minutes)*	<0.001			
Post hoc analysis	D-CON > INS, LIX > <i>db/dm</i>			

Data are expressed as means ± SEM.

*Analysis of variance.

†Tukey honestly significant difference (HSD).

db/dm, nondiabetic mice; D-CON, untreated diabetic mice; INS, diabetic mice treated with insulin; LIX, diabetic mice treated with lixisenatide.

controlled glucose concentration based on the glucose concentration of lixisenatide ($n = 7$), and the diabetic control group (D-CON) (no treatment, $n = 7$). LIX mice were treated with i.p. injections of lixisenatide (500 µg/kg/day), and INS mice were treated with i.p. injections of Humulin R (human recombinant insulin, average 52 U/kg/day; Eli Lilly, Indianapolis, IN) for 8 weeks. Based on the previous studies,^{41–43} the authors decided the i.p. dose of lixisenatide as 500 µg/kg.

Nondiabetic mice were selected as the normal control group (*db/dm*; $n = 5$). Mice were housed under controlled conditions of temperature (20°C to 26°C) and humidity (50 ± 10%) with a 12-hour light-dark cycle. Mice received a gamma ray—sterilized diet (TD2018S; Harlan Laboratories, Indianapolis, IN).

Body weight, food and water consumption, and blood glucose concentrations were monitored daily. The dose of Humulin R was adjusted weekly based on the glucose concentration of lixisenatide. Blood glucose concentrations were measured by tail snipping (glucose assay kit ACCU-CHEK Performa; Roche, Basel, Switzerland).

At the end of the 8-week treatment, mice were anesthetized by an i.p. injection of a mixture containing ketamine (100 mg/kg) and xylazine (5 mg/kg) and were sacrificed by cervical dislocation, after which the eyes were enucleated. Whole-eye samples were taken to evaluate mRNA and protein levels, and for histologic examinations.

Ethics Statement

This study was approved by the institutional animal care and use committee and department of laboratory animals of the Catholic University of Korea, Songuei Campus, which was accredited as an Excellent Animal Laboratory Production Facility by the Korea Food and Drug Administration in 2017 and acquired Association for Assessment and Accreditation of Laboratory Animal Care International full accreditation in 2018. All animal research procedures were

conducted in accordance with the Laboratory Animals Welfare Act, NIH's *Guide for the Care and Use of Laboratory Animals*,⁴⁴ and the Guidelines and Policies for Rodent Experiments provided by the institutional animal care and use committee of the School of Medicine of the Catholic University of Korea (approval number: CUMS-2017-0249-04).

Tissue Processing

After the eyes were enucleated, the neurosensory retina from one of the eyes was immediately separated. The other eye was dipped in formol acetic alcohol (mixture of 95% ethyl alcohol, 10% neutral formalin, and glacial acetic acid, 17:2:1) for 1 minute and fixed in 0.1 mol/L phosphate buffer (pH 7.4) containing 4% paraformaldehyde for 2 hours at 4°C. The anterior segment of the eye was dissected away, the vitreous was cleared, and the eyecup (posterior segment) was left for fixation overnight. Eyecups were embedded in paraffin and sectioned. Serial sections 7-µm thick near the optic nerve head were prepared for retinal immunofluorescence staining, including terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) and morphology.

RNA Extraction and qPCR

Total RNA was extracted from neurosensory retinas using the RNeasy Mini Kit (Qiagen, Valencia, CA). The RNA was reverse transcribed using a cDNA synthesis kit (PrimeScript RT Reagent Kit; Takara Bio, Kusatsu, Japan) according to the manufacturer's instructions. Quantitative PCR (qPCR) was performed using TOPreal qPCR 2× PreMIX (Enzynomics, Daejeon, Korea) on a Roche Diagnostics LightCycler 2.0 Real-Time PCR System (Roche, Mannheim, Germany). Reactions for each sample were run in triplicate, cycle thresholds were normalized to GAPDH expression, and comparative

Figure 1 Relative mRNA expression levels. **A:** Glutamine synthetase (GS) ($P = 0.007$). **B:** Tumor necrosis factor alpha (TNF- α). **C:** Vascular endothelial growth factor (VEGF) based on the average expression levels of the nondiabetic mice group (*db/dm*) (set as 1.0). Data are expressed as means $\pm$ SEM. $n = 5$ *db/dm*; $n = 7$ D-CON, INS, and LIX. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. *db/dm*, nondiabetic mice; D-CON, untreated diabetic mice; INS, diabetic mice treated with insulin; LIX, diabetic mice treated with lixisenatide.

quantitation was performed using LightCycler 167 software version 4.1 (Roche).

qPCR was performed using primers for glutamate-ammonia ligase (*GLUL*), also known as glutamine

synthetase (GS), and glial fibrillary acidic protein (*GFAP*), known glial cell activation markers^{45,46}; tumor necrosis factor (*TNF*), also known as tumor necrosis factor alpha (TNF- α) which induces expression of other inflammatory markers^{47,48}; *TXNIP*, which links oxidative stress to inflammation and apoptosis in T2D^{12,13,49,50}; and VEGF, an angiogenic factor induced in retinal ischemia and in inflammatory processes in T2D.^{12,18,23,51,52} Primer sequences are listed in Table 1.

Western Blot Analysis

The total protein from each neurosensory retina was extracted in 100 μ L of radioimmunoprecipitation assay buffer containing protease and phosphatase inhibitor (Sigma-Aldrich, St. Louis, MO) using a handheld homogenizer. Protein quantification was performed using the BCA protein assay kit (Pierce; Thermo Fisher Scientific, Waltham, MA). Then, 10 μ g of protein was loaded on 12% polyacrylamide gels and transferred to polyvinylidene difluoride membranes. Polyvinylidene difluoride membranes were blocked with 5% bovine serum albumin in Tween20/TBS at room temperature for 60 minutes and incubated at 4°C overnight with primary antibodies (GFAP 1:500; Abcam, Cambridge, UK; *TXNIP* 1:250; Abcam; and β -actin 1:500; Cell Signaling Technology, Beverly, MA). The blots were then incubated with species-specific horseradish peroxidase-conjugated secondary antibody for 60 minutes at room temperature. Immunoreactive bands were visualized using an ECL Advance Western Blotting Detection Reagent (GE Healthcare, Little Chalfont, UK) and determined using a luminescent image analyzer (LAS-4000 mini; Fujifilm, Tokyo, Japan). Densitometric data for the immunoreactive bands were analyzed using ImageJ software version 1.52 (NIH, Bethesda, MD; <http://imagej.nih.gov/ij>). Data were normalized to the specified loading controls.

Colorimetric Analysis for Apoptosis Assessment

Apoptosis was evaluated using the Dead End Colorimetric TUNEL system (Promega Corporation, Madison, WI). Briefly, sections were deparaffinized, equilibrated in phosphate-buffered saline (PBS), permeabilized with proteinase K, post-fixed in 4% paraformaldehyde, and incubated in terminal deoxynucleotidyl transferase reaction mix for 1 hour at 37°C in the dark. The reactions were terminated by immersing the slides in 2 $\times$ saline-sodium citrate for 15 minutes at room temperature. After washing the slides three times in PBS for 5 minutes at room temperature, the slides were immersed in 0.3% H₂O₂ in PBS for 3 to 5 minutes at room temperature, and then washed three times in PBS. Subsequently, horseradish peroxidase-labeled streptavidin in the Dead End colorimetric TUNEL system was added to each slide, and the slides were incubated for 30 minutes at room temperature. Slides were washed three

Figure 2 **A** and **B**: Relative mRNA expression levels of thioredoxin-interacting protein (*TXNIP*) (**A**), and glial fibrillary acidic protein (*GFAP*) (**B**) based on the average expression levels of the nondiabetic mice group (*db/dm*) (set as 1.0); $P = 0.047$. **C**: *TXNIP* and *GFAP* assessment by Western blotting (**top panel**), statistical analysis of relative optic density of *TXNIP* (**bottom left panel**), and *GFAP* (**bottom right panel**) to β -actin among the groups based on the Western blot immunoreactive bands in the neurosensory retina. Data are expressed as means $\pm$ SEM. $n = 5$ *db/dm*; $n = 7$ D-CON, INS, and LIX. $*P < 0.05$, $***P < 0.001$. *db/dm*, nondiabetic mice; D-CON, untreated diabetic mice; INS, diabetic mice treated with insulin; LIX, diabetic mice treated with lixisenatide; qPCR, quantitative PCR.

times in PBS, and chromogen solution was added to each slide and developed until a light brown background was observed. Slides were washed four times in deionized water, and the stained cells were mounted onto microscope slides in Faramount aqueous mounting medium (Dako, Santa Clara, CA). Five microscopic images per group were captured using the Olympus cellSens Dimension software version 1.14 (IX83; Olympus Corporation, Tokyo, Japan).

Immunohistochemical Analysis for Glial Activation and Neuroinflammation

Glial activation was evaluated by fluorescence microscopy using specific antibodies against GFAP. Oxidative stress was evaluated using the same method with specific antibodies against *TXNIP*. Paraffin-embedded sections (3 μ m) were subjected to immunohistochemical examination. After deparaffinization, sections were placed in citrate-buffered

solution (pH 6.0) and heated for antigen retrieval. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide treatment, and nonspecific binding was blocked with normal nonimmune serum. Sections were then incubated with mouse anti-GFAP (1:50 dilution prepared in blocking solution; Abcam) and rabbit anti-*TXNIP* (1:100 dilution prepared in blocking solution; Abcam) overnight at 4°C in a humid atmosphere. After three washes in PBS for 5 minutes each, the sections were incubated with Alexa Fluor 488 goat anti-mouse and Alexa Fluor 549 goat anti-rabbit secondary antibodies (1:100 dilution prepared in blocking solution; Life Technologies, Madrid, Spain). The sections were washed three times in PBS, mounted with VectaStain mounting medium (VECTASHIELD with DAPI; Vector Laboratories, Burlingame, CA), and images were captured using inverted fluorescence microscopy (IX83; Olympus Corporation) using the Olympus cellSens Dimension program (Olympus Corporation). All captured neurosensory

Figure 3 Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL)-positive cells in the ganglion cell layer (GCL). **A:** *db/dm*. **B:** D-CON. **C:** INS. **D:** LIX. **Arrows** indicate TUNEL-positive cells. **E:** The counts of TUNEL-positive cells. **F:** The ratio of TUNEL-positive cells to normal cells in the GCL. Data are expressed as means $\pm$ SEM. $n = 5$ *db/dm*; $n = 7$ D-CON, INS, and LIX. * $P < 0.05$, ** $P < 0.01$. Scale bars: 25 μ m (**A–D**). *db/dm*, nondiabetic mice; D-CON, untreated diabetic mice; INS, diabetic mice treated with insulin; LIX, diabetic mice treated with lixisenatide.

retinal images were exported from the system into ImageJ software for analysis. To quantify immunofluorescence, each image was converted from 8-bit to three channels (red, green, and blue). Red channel values represented TXNIP staining (red fluorescence), and green values represented GFAP staining (green fluorescence). Sets of obtained values of TXNIP and GFAP were compared among the groups. Densitometric image analysis using ImageJ has been described previously,⁵³ and this method was applied for our analytical purposes. Results are presented as densitometry arbitrary units.

Retinal Morphometry

After deparaffinization and rehydration, sections were stained with hematoxylin and eosin and images of these sections were acquired with a microscope (IX83; Olympus Corporation) using the Olympus cellSens Dimension program (Olympus Corporation). First, the central neurosensory retina was obtained and defined as the region within 300 μ m of the optic nerve head margin when the plane passed through the optic nerve. The peripheral neurosensory retina was then defined as a section acquired anywhere outside the 300- μ m section that had been described previously.⁵⁴ Measurements were taken at three different regions from both the central and peripheral neurosensory

retina, and the average was defined as the thickness of the corresponding layer. Layer measurements of sections were performed using the Olympus cellSens Dimension program (Olympus Corporation) to quantify the total neurosensory retinal, outer nuclear layer, INL, and NFL thicknesses.

Data Analysis

Quantitative variables were expressed as means $\pm$ SEM. Measured data were compared using analysis of variance. Statistical analyses were computerized using R software version 3.5.2 (R Foundation for Statistical Computing, Vienna, Austria). Statistical significance was set at $P < 0.05$.

Results

Body Weight and Blood Glucose Levels

The mean body weights and blood glucose concentrations of the experimental mice are shown in Table 2. At 8 weeks, the body weights of D-CON, INS, and LIX were higher than that of *db/dm*, and blood glucose concentrations at 120 minutes were significantly lower in *db/dm* than in the other groups. The blood glucose concentration of D-CON was

Figure 4 Comparisons of thioredoxin-interacting protein (TXNIP) immunofluorescence (red) among the groups. **A–D:** Central neurosensory retina. **E–H:** Peripheral neurosensory retina. **I:** Statistical analysis of TXNIP fluorescence intensity (A.U.) among the groups in the central neurosensory retina. **J:** Statistical analysis of TXNIP fluorescence intensity (A.U.) among the groups in the peripheral neurosensory retina. Data are expressed as means $\pm$ SEM. $n = 5$ *db/dm*; $n = 7$ D-CON, INS, and LIX. *** $P < 0.001$. Scale bars: 100 μ m (**A–H**). A.U., arbitrary units; *db/dm*, nondiabetic mice; D-CON, untreated diabetic mice; GCL, ganglion cell layer; GLP-1RA, glucagon-like protein-1 receptor agonist; INL, internal nuclear layer; INS, diabetic mice treated with insulin; IPL, internal plexiform layer; LIX, diabetic mice treated with lixisenatide; NFL, nerve fiber layer; ONL, outer nuclear layer; OPL, outer plexiform layer.

higher than those of INS and LIX. As mentioned in *Materials and Methods*, the blood glucose concentrations of INS and LIX did not differ significantly.

Quantitative RT-PCR

The mRNA levels of GS, TNF- α , VEGF (Figure 1), *TXNIP* and *GFAP* (Figure 2, A and B) were significantly higher in D-CON than in the *db/dm* mice (all $P < 0.05$). Both INS and LIX exhibited significantly lower mRNA levels of GS, TNF- α , VEGF (Figure 1), *TXNIP*, and *GFAP* (Figure 2, A and B) than D-CON (all $P < 0.001$). Compared with INS, LIX showed significantly lower levels of GS (Figure 1) ($P = 0.007$) and *GFAP* (Figure 2B) ($P = 0.047$), suggesting that lixisenatide treatment reduced the expression of glial activation markers in early type 2 DR.

Western Blot

D-CON significantly increased TXNIP expression compared with *db/dm* and LIX (all $P < 0.05$). The TXNIP concentration did not differ significantly between INS and D-CON (Figure 2C).

D-CON showed significantly increased GFAP expression compared with the other groups (all $P < 0.001$). LIX significantly decreased GFAP expression compared with INS ($P < 0.001$) (Figure 2C).

Apoptosis

The count and incidence of TUNEL positive cells to non-apoptotic cells in the ganglion cell layer was analyzed (Figure 3). D-CON showed a significantly higher apoptotic cell count ($P < 0.004$) and incidence ($P < 0.019$) than *db/dm*.

Figure 5 Comparisons of glial fibrillary acidic protein (GFAP) immunofluorescence (green) among the groups. **A–D:** Central neurosensory retina. **E–H:** Peripheral neurosensory retina. **I:** Statistical analysis of GFAP fluorescence intensity (A.U.) among the groups in the central neurosensory retina. **J:** Statistical analysis of GFAP fluorescence intensity (A.U.) among the groups in the peripheral neurosensory retina. Data are expressed as means $\pm$ SEM. $n = 5$ *db/dm*; $n = 7$ D-CON, INS, and LIX. *** $P < 0.001$. Scale bars: 100 μ m (**A–H**). A.U., arbitrary units; *db/dm*, nondiabetic mice; D-CON, untreated diabetic mice; GCL, ganglion cell layer; GLP-1RA, glucagon-like protein-1 receptor agonist; INL, internal nuclear layer; INS, diabetic mice treated with insulin; IPL, internal plexiform layer; LIX, diabetic mice treated with lixisenatide; NFL, nerve fiber layer; ONL, outer nuclear layer; OPL, outer plexiform layer.

dm. LIX showed significantly decreased apoptotic cell count ($P < 0.02$) and incidence ($P < 0.009$) compared with D-CON, whereas INS did not. However, the apoptotic cell count and incidence were not significantly different in both LIX and INS.

In agreement with the TUNEL results, D-CON showed significantly greater TXNIP and GFAP immunofluorescence staining than did *db/dm* in both the central and peripheral neurosensory retina (Figures 4, and 5, I and J). LIX showed significantly less TXNIP and GFAP immunohistochemical staining compared with D-CON in both the central and peripheral neurosensory retina, but INS showed significantly lower GFAP immunohistochemical staining compared with D-CON in the peripheral neurosensory retina only (Figures 4, and 5, I and J). LIX was associated with significantly reduced TXNIP and GFAP

immunohistochemical staining than INS in both the central and peripheral neurosensory retina (Figures 4, and 5, I and J).

In the optic nerve head, all groups showed similar TXNIP immunohistochemical staining (Figure 6). However, D-CON showed increased GFAP immunofluorescence staining compared with the other groups (Figure 7).

Retinal Morphometry

In the central neurosensory retina, there were no significant differences in total neurosensory retinal, outer nuclear layer, or INL thickness among all groups (Figures 8, A–D, and 9, A–C). The central NFL in D-CON was significantly thicker than that of *db/dm* ($P = 0.007$). The central NFL of LIX was significantly thinner than that of D-CON ($P = 0.001$),

Figure 6 Immunohistochemistry of thioredoxin-interacting protein (TXNIP) in the optic nerve head (ONH). **A–D**: All groups showed similar immunohistochemical staining. **E–H**: Merged immunohistochemical images among the groups. $n = 5$ *db/dm*; $n = 7$ D-CON, INS, and LIX. Scale bars: 100 μm (**A–H**). *db/dm*, nondiabetic mice; D-CON, untreated diabetic mice; INS, diabetic mice treated with insulin; LIX, diabetic mice treated with lixisenatide.

but there was no significant difference in thickness between INS and D-CON (Figure 9D).

In the peripheral neurosensory retina, total neurosensory retinal thickness was significantly greater in D-CON than in *db/dm* (Figures 8, E–H, and 10) ($P = 0.041$). There were no significant differences in thickness among D-CON, INS, and LIX.

The thickness of peripheral INL was significantly greater in D-CON than in *db/dm* (Figure 10) ($P = 0.049$). There was no difference in thickness between INS and D-CON, but the peripheral INL was significantly thicker in INS than in *db/dm* (Figure 10) ($P = 0.018$). The peripheral INL of LIX was significantly thinner than that of INS ($P = 0.021$), but did not differ significantly from that of *db/dm* (Figure 10).

The peripheral NFL in D-CON was significantly thicker than that of *db/dm* ($P < 0.001$), and those in INS and LIX were also significantly thinner than that of D-CON (all $P < 0.001$) (Figure 10). INS only showed significant differences in peripheral NFL thickness compared with D-CON, whereas LIX showed significant differences in the central and peripheral NFL (Figures 9D and 10D).

Discussion

This study showed that oxidative stress and inflammation increased in an animal model of early type 2 diabetic retina based on GS, TNF- α , VEGF, GFAP, and TXNIP mRNA levels, whereas insulin or lixisenatide treatment reversed these changes (Figures 1 and 2, A and B). However, there were no significant differences in TNF- α , VEGF, or TXNIP mRNA levels between INS and LIX at 8 weeks after treatment (Figures 1 and 2A). According to the Western blot analysis, D-CON showed significantly increased TXNIP and GFAP expression levels compared to *db/dm* in the neurosensory retina (Figure 2C). LIX showed significantly decreased TXNIP and GFAP expression compared with D-CON, whereas INS showed only decreased GFAP expression in the neurosensory retina (Figure 2C).

In detail, TXNIP protein levels were significantly higher in the central and peripheral neurosensory retina in INS compared with *db/dm* and LIX in the early type 2 diabetic retina (Figure 4), suggesting that lixisenatide suppressed TXNIP in the early type 2 diabetic retina to a level similar to

Figure 7 Immunohistochemistry of glial fibrillary acidic protein (GFAP) in the optic nerve head (ONH). **A–D:** D-CON showed increased immunohistochemical staining. **E–H:** Merged immunohistochemical images among the groups. **Arrows (B and F)** indicate locations showing increased GFAP staining of D-CON. $n = 5$ *db/dm*; $n = 7$ D-CON, INS, and LIX. Scale bars: 100 μm (**A–H**). *db/dm*, nondiabetic mice; D-CON, untreated diabetic mice; INS, diabetic mice treated with insulin; LIX, diabetic mice treated with lixisenatide.

that in normal control mice, whereas insulin did not. To control for the effect of hyperglycemia, insulin doses were adjusted in the INS mice based on the glucose concentrations in the LIX mice; thus, these anti-inflammatory results

of TXNIP suppression were dependent on the effects of GLP-1RA after accounting for hyperglycemia. Additionally, insulin had little or no suppressive effect on TXNIP in the early type 2 diabetic retina in this study.

Central neurosensory retina

Peripheral neurosensory retina

Figure 8 Sections of neurosensory retinal images stained with hematoxylin and eosin. **A–D:** Central neurosensory retina. **E–H:** Peripheral neurosensory retina. Scale bars: 100 μm (**A–H**).

Figure 9 Comparison of central neurosensory retinal thickness among the groups. **A:** Central total neurosensory retina. **B:** Central outer nuclear layer. **C:** Central inner nuclear layer. **D:** Central nerve fiber layer. In the central nerve fiber layer, D-CON shows significantly greater thickness than *db/dm*. LIX shows significantly reduced thickness compared with D-CON, whereas INS did not. Data are expressed as means $\pm$ SEM. $n = 5$ *db/dm*; $n = 7$ D-CON, INS, and LIX. $^{***}P < 0.01$. *db/dm*, nondiabetic mice; D-CON, untreated diabetic mice; INS, diabetic mice treated with insulin; LIX, diabetic mice treated with lixisenatide.

The mechanism of the effects of insulin and GLP-1RA on TXNIP remains unclear, but shares the common *PI3K/Akt* signaling pathway in pancreatic β -cells.^{14,34,55} Both insulin and GLP-1RA reduce caspase-3 activation, which is induced by TXNIP up-regulation and leads to cell death in the ganglion cell layer,¹³ via the *PI3K/AKT* signaling pathway by directly phosphorylating caspase-9 in diabetic rat retinas.^{56,57} Insulin also suppresses TXNIP transcription by activating histone deacetylases via the *PI3K/AKT* signaling pathway.⁵⁵ On the other hand, exendin-4, a GLP-1RA, also uses the *PI3K/AKT* signaling pathway and mediates proteasome-induced degradation of TXNIP via the cyclic AMP signaling pathway.³⁴ Based on the study results and known mechanisms described above, the primary reason for the significant difference in TXNIP protein levels between INS and LIX is TXNIP proteasomal degradation in the early type 2 diabetic retina. However, further studies are required to support this hypothesis.

In the current study, lixisenatide reduced neuroinflammation in early type 2 diabetic retina more efficiently than insulin. LIX had lower mRNA levels of GS and GFAP, which are Müller cell- and glial cell-specific activation markers, than D-CON and INS (Figures 1 and 2B). A previous study reported different results regarding the mRNA expression of GS under diabetic versus nondiabetic conditions. Zhou et al⁵⁸ reported that TXNIP suppressed the mRNA expression of GS by inducing oxidative stress in retinal Müller cells. However, their study was based on *in vitro* Müller cell cultures, in contrast to the current *in vivo* study. Lieth et al⁵⁹ reported similar results to Zhou et al⁵⁸ using experimental rat models, but even they observed an increase in GS activity at 1 month after diabetes exhibition,

although it was not statistically significant. It may be that early type 2 DR differs in terms of gene expression profile compared with typical type 2 DR, although further study is needed to confirm this.

TUNEL staining demonstrated that LIX had significantly lower apoptotic cell count and incidence than D-CON, whereas LIX did not show significant differences compared with *db/dm* (Figure 3). At the protein level, LIX showed decreased immunohistochemical staining for both TXNIP and GFAP in the central and peripheral neurosensory retinas compared with levels in D-CON and INS (Figures 4 and 5). Similarly, the INL in the peripheral neurosensory retina and the NFL in the central and peripheral neurosensory retina in LIX were significantly thinner than those in D-CON and INS (Figures 8, 9, and 10). In addition, GFAP immunohistochemical staining in the optic nerve head was lower in LIX than in D-CON (Figure 7). These results indicate that neuroinflammation in the early type 2 diabetic retina was significantly decreased with lixisenatide treatment compared with insulin treatment when controlling for hyperglycemia. These results support previous reports on neuroprotection via GLP-1RA treatment in type 2 DR.^{38,60,61}

The neuroprotective mechanisms of GLP-1RA remain unclear, but may involve regulation of transcription factor *NF- κ B*, an important downstream target of the aforementioned *PI3K/AKT* signaling pathway that regulates inflammatory gene expression and mediates the proinflammatory response of microglial cells in Parkinson disease.^{62,63} GLP-1RA improved insulin sensitivity in an obese mouse model by directly inhibiting inflammatory pathways in adipocytes, enhancing glucose uptake, and decreasing hyperglycemia-induced inflammation.^{64–66} In the retina, some reports

Figure 10 Comparison of peripheral neurosensory retinal thickness among the groups. In the peripheral retina, D-CON showed significantly greater thickness in the total neurosensory retinal, inner nuclear layer, and compared with *db/dm*. **A:** Peripheral total neurosensory retina. **B:** Peripheral outer nuclear layer. **C:** In the peripheral inner nuclear layer, LIX shows significantly reduced thickness compared with D-CON, whereas INS does not. **D:** In the peripheral nerve fiber layer, LIX and INS show significantly decreased thickness compared with D-CON. Data are expressed as means $\pm$ SEM. $n = 5$ *db/dm*; $n = 7$ D-CON, INS, and LIX. * $P < 0.05$, *** $P < 0.001$. *db/dm*, nondiabetic mice; D-CON, untreated diabetic mice; INS, diabetic mice treated with insulin; LIX, diabetic mice treated with lixisenatide.

have shown that the *PI3K/AKT* signaling pathway is important for the survival of retinal neurons.^{56,67} In addition, Vuong et al⁶⁸ showed that the GLP-1RA liraglutide prevented retinal *p53* activation in T2D.

In this study, only lixisenatide induced a significant reduction in peripheral INL thickness compared to insulin treatment in early type 2 diabetic mice (Figure 10). This is noteworthy because physiological dysfunction of the INL in T2D patients was supported by electroretinogram, even without DR.^{69–71} The oscillatory potentials in the electroretinogram reflect inner retinal activity,^{72–74} particularly indicating ischemia in the DR,^{75–77} and predict the development of proliferative PDR.^{78,79} The somata of Müller cells in the retina are located in the middle of the INL⁸⁰ and are negatively affected by inner retina dysfunction in DR. In an experiment in type 2 diabetic mice, Hernandez et al³⁸ reported that GLP-1RA prevented the decreased a-wave, b-wave, and oscillatory potentials, as well as the increase in implicit time compared with type 2 diabetic mice. In addition, recent studies reported that peripheral lesions were predictive of an increased risk of DR progression.^{81,82} Silva et al⁸² argued that an increasing number of peripheral lesions substantially increased the risk of progression of DR and proliferative DR, independent of baseline DR severity and glycated hemoglobin levels. Therefore, the results from the current study suggest that GLP-1RAs are beneficial for the prognosis of the early type 2 DR by decreasing neuroinflammation in the peripheral INL.

The main difference between the current study and previous studies is that most other studies reported a decrease

in the thickness of retinal layers in type 2 DR.^{30,39,83–87}

However, the onset of T2D in patients was not clear in these studies, suggesting that their clinical course did not clearly reflect the early type 2 diabetic retina. The initial evaluation of patients may occur more than eight weeks after the onset of T2D. In contrast, this study initiated treatment as soon as T2D was confirmed because *db/db* mice are known to exhibit hyperglycemia associated with obesity as early as 4 to 8 weeks after birth,⁸⁸ and effects can be measured at 8 weeks after treatment. Therefore, the current study has advantages in its methodology.

A previous study reported different results under similar conditions to those in the current study. Bogdanov et al⁵⁴ reported that total retinal, outer nuclear layer, and INL thicknesses were significantly decreased in the central and INL in the peripheral retinas of diabetic mice at 8 weeks compared with nondiabetic controls, which is not in agreement with this study's results. These differences may be due to weight or glucose concentrations at 8 weeks, which was the time point of mice sacrifice in this study. However, the results from the current study are more reasonable because INS and LIX showed similar neurosensory retinal layer thicknesses as *db/dm*, whereas parts of the retinal layers of D-CON were significantly thicker than those of *db/dm*, and these increased thicknesses were the result of retinal edema caused by inflammation.

Conclusions

In conclusion, neuroinflammation was significantly reduced in lixisenatide-treated retinas compared with untreated or

even insulin-treated retinas of early type 2 diabetic mice, independent of hyperglycemia. The neuroprotective effects of lixisenatide treatment should be evaluated in the peripheral INL, and may improve the prognosis of the early type 2 DR.

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