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Autonomous Regulation of Retinal Insulin Biosynthesis in Diabetes

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Abstract

Diabetic retinopathy (DR) is a neurodegenerative disease that results as a complication of dysregulated glucose metabolism, or diabetes. The signaling of insulin is lost or dampened in diabetes, but this hormone has also been shown to be an important neurotrophic factor which supports neurons of the brain. The role of local insulin synthesis and secretion in the retina, however, is unclear. We have investigated whether changes in local insulin synthesis occur in the diabetic retina and in response to stressors known to initiate retinal neurodegenerative processes. The expression of insulin and its cleavage product, c-peptide, were examined in retinas of a Type I diabetes animal model and human postmortem donors with DR. We detected mRNAs for insulin I (Ins1), insulin II (Ins2) and human insulin (Ins) by quantitative real-time polymerase chain reaction (qRT-PCR) and in situ hybridization. Using an *ex-vivo* system, isolated neuroretinas and retinal pigmented epithelium (RPE) layers were exposed to glycemic, oxidative and inflammatory environments to measure insulin gene transcripts produced *de novo* in the retina under disease-relevant conditions. The expression of insulin in the retina was altered with the progression of diabetes in STZ mice and donors with DR. Transcription factors for insulin, were simultaneously expressed in a pattern matching insulin genes. Furthermore, *de novo* insulin mRNA in isolated

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retinas was induced by acute stress. RPE explants displayed the most pronounced changes in Ins1 and Ins2. This data reveals that the retina, like the brain, is an organ capable of producing local insulin and this synthesis is altered in diabetes.

Introduction

Retinal neurodegeneration is a major cause of blindness worldwide. A better understanding of the molecular pathways involved in retinal neuroprotection are important for the development of new therapies to halt vision loss due to ischemic retinopathies such as diabetic retinopathy (DR). Several molecular mechanisms have been implicated in the pathogenesis of DR including: increased glycemic, oxidative and nitrative stress and along with subclinical levels of inflammation (Lamoke et al. 2015; Arden and Sivaprasad 2011; Hammes et al. 1999). Retinal tissue injury could also result from loss of neuro- and vasculo-protective factors involved in maintenance of retinal neurovascular homeostasis.

Recent studies have emphasized the neuroprotective role of insulin beyond its ability to modulate glucose uptake from insulin-dependent tissues (Hui et al. 2005; Voll and Auer 1991). Most importantly, the discovery of insulin production in the central nervous system (CNS) has opened many possibilities for determining novel functions of insulin in extra-pancreatic tissues (Devaskar et al. 1994; Havrankova et al. 1978; Kovacs and Hajnal 2009). For example, its neurotrophic function has been shown to support for neuronal development and survival following ischemic insults in the brain (Voll and Auer 1991; Valenciano et al. 2006; Hui et al. 2005). The finding that immunoreaction of insulin was present in fetal, newborn and adult brains still did not differentiate between insulin of pancreatic origin that crossed the blood brain barrier versus synthesis within the central and peripheral nervous system. Not until the use of polymerase chain reaction was the presence of preproinsulin and insulin mRNA able to clearly differentiate that processing of the insulin protein occurred locally in these tissues, not at distal sites where the secreted protein traversed the blood-brain barrier (BBB) and relocated to the brain (Devaskar et al. 1994; Murakami-Kawaguchi et al. 2014; Deltour et al. 1993). Although insulin is involved in glucose metabolism, the brain was considered an insulin insensitive organ. It is now argued that insulin facilitates critical brain functions like metabolism, cognition and motivated behaviors in the CNS, along with being a potent mitogenic and neurotrophic factor (Ferrario and Reagan 2018; Mielke and Wang 2005; Abbott, Wells, and Fallon 1999; Wu et al. 2004; Naia et al. 2016). Anatomically and developmentally, the retina is known as an extension of the CNS; it consists of retinal ganglion cells, the axons of which form the optic nerve, whose fibers are, in effect, CNS axons (London, Benhar, and Schwartz 2013). The retina may then also depend on local insulin signaling to modulate pathways such as the insulin receptor substrate (IRS1) and PI3K-Akt signaling pathway to promote a neuroprotective environment, such as in the brain (Liu et al. 2018). In fact, insulin receptor (IR) autophosphorylation followed by the activation of downstream kinases, is downregulated in the diabetic retina (Reiter et al. 2006; Barber et al. 2001; Kondo and Kahn 2004). The origin of insulin that is upstream of this IR signaling cascade, however, is unclear. Here we have analyzed whether the retina contains insulin mRNA transcripts. We utilized the techniques others have used to examine secreted hormones, such as somatostatin, to detect the presence of intracellular

insulin mRNA required for de novo biosynthesis (Yamaguchi et al. 1990). Furthermore, we conducted experiments to determine whether changes in glycemia, redox stress, or inflammation has an effect on the temporal expression patterns of insulin in the retina.

Materials and Methods

Animals.

No randomization was performed to allocate subjects in the study. Normal euglycemic C57BL/6J mice purchased from the Jackson Laboratory. Mice at 6 weeks of age were made diabetic by injecting 75 mg/kg of streptozotocin (STZ) for 3 consecutive days. STZ was dissolved in a 0.01M sodium-citrate buffer (pH 4.5). Control animals were injected with the latter buffer only for the same duration of time. Mice were considered diabetic when fasting blood glucose levels were ≥250mg/dL. Mice that became severely diabetic (blood glucose levels ≥700mg/dL) and determined to be malnourished by veterinarian were sacrificed and not included in study (6 sacrificed and excluded). Experimental groups of diabetic mice were examined after being diabetic for 8 weeks and 23 weeks. *Ins2* ^{Akita} mice were used as a negative control for secreted insulin protein. *Ins2* ^{Akita} mice were monitored for blood glucose levels and because spontaneously diabetic at 4 weeks of age. *Ins2* ^{Akita} were examined after a duration of 8 weeks of hyperglycemia. For breeding, *Ins2* $^{Akita/+}$ female mice were bred to wild-type male mice, in accordance with institutional policy for appropriate care and use of animals. Genotyping of *Ins2* $^{Akita/+}$ mice was performed per the protocol recommended by The Jackson Laboratory. Only male mice were used for all experiments. Histologic processing of mouse eyes was performed following our previously published protocol (Martin et al. 2004). Mice were fasting for 24 hours prior to euthanizing the next morning between 9-10am. The care and use of all animals adhered to institutional guidelines for the humane treatment of animals and to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and Augusta University (Animal Use Approval #:2009-0214). Animals were housed in the vivarium of the Augusta University Laboratory Animal Services, which complies with federal laws governing the human care and treatment of laboratory animals. The facility and program is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC), a Registered Research Facility with the United States Department of Agriculture (USDA No. 57-R- 0002), and has an Office of Laboratory Animal Welfare OLAW Assurance Statement (A3307-01; dated February 26, 2009 on file). The institution abides by criteria established by the animal welfare act (PL99-198) and guidelines established by the Public Health Services in reviewing and approving protocols as well as in maintaining the facility. Mice were euthanized by CO_2 followed by immediate cervical dislocation. This method of euthanasia is consistent with the recommendations of the panel on euthanasia of the American Veterinary Medical Association. Animals were monitored closely for any signs of distress or discomfort, pain or injury during study following the injection of STZ. Animals displaying hunched posture, not mobilizing or eating properly, inflamed eyes, etc. were sacrificed immediately to minimize discomfort, distress, and pain.

Postmortem Retina Specimens.

Human retina samples from anonymous donors were obtained from the Georgia Eye Bank (Atlanta, GA). The following selection criteria, adapted from that of Barber et al were applied (Barber et al. 1998): adults younger than 75 years, with insulin-requiring diabetes or no diabetes (control), no life-support measures, and no chemotherapy. All eyes were enucleated 8 hours or less after time of death; retina samples were then obtained and frozen at −80°C.

Reverse transcription plus the polymerase chain reaction.

qPCR was used to quantify the steady-state levels of mRNA targets listed in Table 1. RNA was extracted from retinas of streptozotocin-induced diabetic mice after 8 weeks (2 months) and 23 weeks (~6 months) post-onset diabetes (n = 6 per group) using TRIzol. Age-matched, non-diabetic C57Bl/6J mice served as controls for each group (n = 6 per group). RNA (1 μg) was reverse transcribed using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Amplification of INS1 and INS2 mRNA was performed using Power SYBER green PCR master mix. The conditions used for the PCR were as follows: 95°C for 3 min (1 cycle) and 94°C for 20 sec, 55°C for 30 sec, and 72° C for 40 sec (40 cycles). The relative mRNA abundance was determined by normalizing to 18s ribosomal RNA (rRNA) using the 2Ct method (Ct refers to the threshold value). Multiple primer sets were tested. The sequences of the primers that span the exon-exon junction and were used in these reactions are shown in Table 1. A similar approach was used for amplification of INS in postmortem human retinal samples.

In situ hybridization.

To localize the mRNA transcript encoding Ins1 and Ins2 in intact mouse retina, retinal cryosections were prepared using our previously published method and used these sections for fluorescent in situ hybridization (FISH) analysis (Martin et al. 2009). Digoxigenin (DIG)-labeled riboprobes were generated using the described PCR primers for RT–PCR analysis (Table 1). Briefly, the 23 bp T7 promoter sequence (5′-TAA TAC GAC TCA CTA TAG GGA G-3′) was appended to the 5′ end of the antisense primer such that the promoter was incorporated into the PCR product. Subsequent amplification of the target DNA (cDNA prepared using RNA from mouse RPE/eyecup) yielded a PCR product that contained the T7 promoter sequence upstream of the mouse Ins1, Ins2 or human Ins sequence. To produce a template for transcription of the sense (control) strand of the amplified region, we designed an additional primer set with the T7 promoter sequence added to the sense-strand insulin primer. These templates were then used for the synthesis of riboprobes (antisense and sense) with T7 RNA polymerase. A DIG tag for antibody labeling was added to sense and antisense probes using a DIG RNA labeling kit (Roche). Mouse eyes were frozen immediately in Tissue-Tek Optimal Cutting Temperature compound (OCT; Sakura Finetek, Torrance, CA), and sections were made at 10-μm thickness and fixed in 4% paraformaldehyde for 30 min at room temperature. Formalin-fixed, paraffin embedded (FFPE) human sections were deparaffined by treatment with for approximately 10 minutes. Sections were then labeled with the each probe following an established protocol (Martin et al. 2009). Sections were

examined using a Zeiss Axioplan 2 fluorescent microscope equipped with an ApoTome, AxioVision 4.5 software, and an HRM camera (Carl Zeiss, Oberkochen, Germany).

C-peptide assay.

Retinal tissues from normal, euglycemic mice and streptozotocin-induced, diabetic mice were lysed and the resulting supernatants were collected after centrifugation. An Enzyme-Linked-Absorbatant-Assay (ELISA) assay was used to test for insulin and C-peptide in the tissues. The diluent (95 μL) was added to wells with 5 μL of sample and incubated for 1 hr at room temperature. The wells were emptied then washed 6 times using wash buffer. 10 μL of conjugate solution was added to each wells left for 1 hr at room temperature. The wells were washed 6 times again with wash buffer and incubated for 30 minutes with 100 μL of substrate solution. 100 μL of stop solution was added to the wells and the absorbance was read at 450/630 nm.

Ex vivo Retinal explant cultures tissue culture preparation.

Food was removed from mice, 24 hours prior to sacrifice. Mice were anesthetized by using CO_2 and then sacrificed by cervical dislocation. Retina and RPE-choroid cup were quickly separated and placed on the culture membrane (Millicell-CM, PICM030-50; Millipore, Billerica, MA) in a 35 mm Petri dish with 1.0 ml of Kreb's Ringer buffer containing penicillin/streptomycin cocktail and kept at a temperature of 37 °C. Retinal explants were incubated with 20mg/ml of actinomycin D (Sigma, #A1410) for 30 minutes or media only as a control. Explants were then treated exposed to 25mM D-glucose (high glucose, HG), H_2O_2 (.1M), or lipopolysaccharide from *Salmonella enterica* (LPS, 2.5μg/kg) for 1 hour.

Statistical Analysis.

Statistical analysis was performed using Prism 8 v.8.1.1. Select experiments with two experimental groups were analyzed using Student's t-test. One-way ANOVA was utilized to assess data with 4 or more groups. Tukey Kramer and Bonferroni tests were used for multiple comparisons. Outliers were not removed. Graphs are shown as standard box plots or dot plots. The ends of the box indicate the upper and lower quartiles, the line across the box indicates the median (50^{th} percentile), and the whiskers from the two ends of the box extend to the minimum and maximum data points. The level of statistical significance was set at $p<0.05$ for all tests.

Results

Reverse transcription-polymerase chain reaction analysis of preproinsulin, Ins1 and Ins2 transcripts in adult neuroretina

To determine whether mRNA transcripts encoding insulin were present in mouse retina, we used RNA isolated from neuroretinas and visualized the products using RT-qPCR. Preproinsulin I (PPI1) and preproinsulin II (PPI2) mRNA was observed in retinal tissues and corresponded with the expected amplification product (Fig. 1A). Ins1 and Ins2, the genes which encode mouse insulin were present in 3-month old mouse retinas (Fig. 1B). We then determined by *in situ* hybridization, that Ins1 and Ins2 mRNA was detected in layers of the neuroretina. In Figure 1C, positive Ins1 and Ins2 mRNA signals were detected in glia

or neurons in the ganglion cell layer (GCL), interneurons of the inner nuclear layer (INL), photoreceptors of the outer nuclear layer (ONL), and in the retinal pigment epithelium (RPE).

Ins1/Ins2 gene expression in murine retinas is modulated with diabetes

We compared the expression of insulin in retinal, brain and pancreatic tissue using semi-quantitative RT-PCR. Although the insulin expression level was significantly higher in the pancreas than in the brain and retina, retinal transcripts were present (Fig. 2A). After validating that retinal transcripts for insulin production were identical to that of other tissues known to express insulin, we observed whether changes in retinal insulin were observed in a mouse model of Type 1 diabetes. In this model pancreatic insulin is drastically decreased due to the destruction of beta cells of the pancreas using streptozotocin (STZ). C57BL6/J mice were rendered diabetic via intraperitoneal injection of STZ (75mg/kg) for 3 consecutive days. RNA from retinas of mice that were diabetic for 8 and 23 weeks were compared by RT-qPCR to age-matched controls for the expression of both Ins1 and Ins2. Interestingly, following an 8-week duration of diabetes, Ins1 and Ins2 mRNA transcripts (Fig. 2B and 2D, respectively) were found to be significantly upregulated (Ins1, $P<.0008$; Ins2, $P<.002$). This pattern was opposite to that of pancreatic Ins1/Ins2 transcripts, which showed a marked decrease at 8 weeks post-STZ injection (Fig. 2F and 2G). Expression of Ins1 and In2 at 23 wks in the pancreas was negligible, which is expected with STZ-injected mice at this duration of diabetes (data not shown). After 23 weeks of STZ-induced pancreatic β-cells destruction, retinal Ins1 downregulation was found to occur (Fig. 2C, $P<.01$). Furthermore, Ins2 expression after 23 weeks of hyperglycemia was not upregulated as seen at earlier time points (Fig. 2E). The fact that retinal insulin gene expression patterns did not mirror that of the pancreas, suggests that regulation of insulin production occurs locally in the retina and is decreased only after prolonged diabetic conditions.

C-peptide is found locally in retinas and is modulated with pancreatic insulin depletion (Type 1 diabetes model) with diabetes progression

RNA expression may or may not result in the production of the precursor protein, proinsulin, which is then cleaved into its active form. While the active form of insulin has a short half-life (5 minutes) and is difficult to measure, the secretion of c-peptide remains stable in the tissue and has a longer half-life (30 minutes). Therefore, the level of c-peptide in neuroretinas was examined by enzyme-linked immunosorbent assay (sandwich enzyme-linked immunosorbent assay; ELISA) in comparison to serum c-peptide levels. Mice were sacrificed in a fasting state to eliminate any post-prandial increases in insulin as a result of any residual β-cell- or hepatic- derived insulin that may remain in STZ-injected mice. Retinal c-peptide levels were significantly higher after only 8 weeks of diabetes ($.225\pm.01$ vs 1.22 ± 1, $P<.01$, $n=6$) (Fig. 3A). However, c-peptide levels significantly decreased after 23 weeks of hyperglycemia in comparison to controls ($.197\pm.01$ vs $.138\pm.03$, $P<.01$, $n=6$) (Fig. 3B).

C-peptide undetected in retinas of diabetic Ins2−/− Akita mice

As a comparison, we examined insulin genes and c-peptide concentration in a genetically modified diabetic model, or the Akita mouse. These mice have a mutation in the *Ins2* gene

that results in the incorrect folding of the insulin protein. The misfolded protein is retained in the ER, producing toxicity in pancreatic ß cells, reduced ß cell mass and reduced insulin secretion. Retinal complications include increased vascular permeability, alterations in the morphology of astrocytes and microglia, increased apoptosis and thinning of the inner layers of the retina (Barber AJ, et al., 2005). The expression of active insulin gene copies in null mutants for Ins2 transcripts were first analyzed by qRT-PCR. As shown in Figure 3C, Ins1 was detected and showed an upward trend in expression (possibly compensating for the absence of Ins2), but showed no significant difference in Wt versus *Ins2* $^{Akita/+}$ mice. In contrast, Ins2 was undetectable. The level of functional/active insulin was then examined at a protein level by measuring c-peptide. Figure 3D shows that c-peptide was not detected in retinas from *Ins2* $^{Akita/+}$ mice.

Changes in transcription factors that stimulate insulin gene transcription in retina

We then analyzed the expression of transcription factors essential for insulin expression such as pancreatic and duodenal homeobox (Pdx1), neurogenic differentiation (NeuroD), and v-maf musculoaponeurotic fibrosarcoma oncogene homolog (MafA). The expression patterns were analyzed by quantitative RT-PCR. There was a significantly higher expression (~2-2.5 fold changes) in all 3 genes at the 8-week mark and a downregulation of these genes at 23 weeks, which mirrored the pattern of c-peptide found in the retina (Fig. 4).

Response of mouse explant cultures to insults

The above *in-vivo* analysis of c-peptide in the retina cannot completely differentiate between systemic, extra-retinal sources of insulin production that may have remained in retinal vascular networks. Therefore, we utilized an *ex vivo* method to determine whether we can isolate acute changes insulin transcription in response to an insult or local metabolic imbalance (see flow-chart). Retinal explants were treated with and without an RNA synthesis inhibitor, actinomycin D, to determine whether synthesis of insulin gene expression was in fact *de novo* RNA synthesis that can be blocked in the presence of an RNA inhibitor. Figure 5 shows the results obtained by culturing RPE and neuroretina from normal, 3-month old mice exposed to 25mM D-glucose (high glucose, HG), H_2O_2 (.1M), or lipopolysaccharide from *Salmonella enterica* (LPS, 2.5μg/kg) for 1 hour (Fig. 5A-C). Neuroretinas and RPE from age-matched mice were used as controls. Mice were fasted for 24 hours to reduce the influence of pancreatic-derived insulin remaining in the retina. Interestingly, RPE explants induced a significant upregulation of Ins1 in response to H_2O_2 (P=.007) and LPS (Fig. 6A). No change was found in response to HG (Fig. 5A). Unlike RPE explants, neuroretina cultures expressed a 10-fold increase in Ins1 expression in response to HG and H_2O_2, but not LPS (Fig. 5C). Ins2 in the neuroretina explants, however, had a low expression and the changes were unquantifiable. These results indicate that the RPE is the major contributor to insulin gene expression in the mouse retina in response to acute redox and inflammatory stress.

Human INS transcripts decrease in RPE of diabetic post-mortem donors

Insulin processing in humans is not a two gene system, and instead consists of one ancestral gene containing two introns (Sures et al. 1980). We analyzed retinal sections from human donors using in situ hybridization and detected insulin mRNA-positive cells. Insulin-positive

signals were observed in the GCL, INL, and inner segments (IS) of the layer where photoreceptors reside (ONL) (Fig. 6A). Interestingly, the inner segment contains many ribosomes and is where most of the metabolism and regulation of membrane potential takes place in photoreceptors. In particular, the RPE was noted to have the strongest signal, suggesting that this is where the majority of insulin processing is occurring in the human retina. This was confirmed in the quantitative PCR that showed in separated neuroretina and RPE tissue, the relative expression on insulin in RPE is 4-7 times that of the neuroretina (Fig.6B). RPE tissue from diabetic donors showed a marked decrease in insulin transcripts in comparison to age-matched, normoglycemic donors ($P<.0001$) (Fig.6D). No difference was observed in neuroretinas of control, non-diabetic and diabetic human donors (Fig. 6C).

Discussion

Insulin deficiency is known to culminate in diabetic complications such as diabetic peripheral neuropathy affecting motor, sensory, and autonomic neurons (Wada et al. 2005). However, less attention has been paid to the direct role of *de novo* insulin loss in diabetic complications, including DR (Wada et al. 2005). This study demonstrates that insulin genes and the transcription factors essential for its expression, are found in the adult neuroretina and are modulated during the course of STZ-induced diabetes. The expression of insulin has been reported in extra-pancreatic tissues which include the neuroretina during embryogenesis (Valenciano et al. 2006; Rosenzweig et al. 1980; Reiter and Gardner 2003), but this is the first report of the presence and effects of metabolic alterations on insulin transcripts in the adult retina.

We aimed to distinguish whether gene expression versus the peptide form of insulin were altered. The presence of preproinsulin and insulin transcripts in the neuroretina suggests that the factor is produced locally, versus from a peripheral source that reaches the retina systemically. By analyzing the protein levels of its cleavage product, c-peptide, we determined that though in less abundance than pancreatic c-peptide, a low level of c-peptide is present in the murine neuroretina (Fig. 2F-G and Fig. 3A-B. Furthermore, the destruction of pancreatic beta cells, the main source of glucose-dependent insulin in the body, did not result in the attenuation of neuroretinal insulin genes as it did pancreatic insulin genes after 8 weeks of diabetes. By 23-weeks of uncontrolled hyperglycemia insulin genes and c-peptide levels were reduced under these conditions (Fig.2B-E and Fig. 3A-B). This was reflective of the pattern in which we found insulin mRNA to be along with the transcription factors, NeuroD1, Pdx1, and MafA (Figure 4). This demonstrates that temporal spikes in insulin release may occur in the retina in response to energy demands, stress, or insult. With *chronic* insult, such as in diabetes, this response is lost over time.

Insulin signals through its transmembrane receptor located neuronal cells in a manner similar to those expressed in liver and skeletal muscle. Insulin receptors (IRs) in the retina are highly specialized, with the most abundant expression levels within neuronal cell bodies, including photoreceptors, and in the plexiform layers, comprised of dendrites, and Müller cells (Reiter et al. 2003). The rate of insulin receptor activity in the *in vivo* retina is maintained in a tonic state, similar to the brain. This steady state of insulin receptor activation has been suggested to occur due to the stabilization of the circulating insulin

delivery by the blood retinal barrier (Reiter and Gardner 2003). As this study and previous studies in the brain suggest, this tonic level could also be due to the concurrent local insulin secretion by cells within these tissues. We cannot confirm whether Muller cells, that also have abundant IR expression also synthesize insulin, but others have shown positive immunoreactivity in Muller glia (Das et al. 1984). Our findings of insulin gene transcripts at multiple layers of the retina suggest that insulin may have both autocrine and paracrine functions in multiple cell types.

When exploring the molecular mechanism of how insulin resistance can lead to retinopathy, it has been hypothesized that dysfunctional insulin receptor signaling in the retina contributes to cellular dysfunction and death. It is widely accepted that insulin is a survival factor that acts through PI3K, Akt and the 70 kDa ribosomal protein S6 kinase, p70S6K (Wada 2009; Wada et al. 2005; White 2003). Activation of IRs in the retina protect and/or rescue the retina from apoptosis or light-induces damage (Wu et al. 2004). Retinal cell survival in chick embryos depend on insulin produced by the retina, and the inhibition of insulin action using antibodies caused a significant increase in apoptotic neural cells (Tesoriere et al. 1994). Despite an overwhelming amount of studies on neuroprotective roles of insulin receptor (IR) activity in the retina, the source of insulin has never been described in detail. Our results show that c-peptide levels are detected in early diabetic states, but chronic hyperglycemia leads to a decrease in local c-peptide detection in the retina. This suggests that the system that induces *de novo* insulin as a neuroprotective response becomes lost with the advanced stages of diabetes. This is supported by the observation in our model showing a decrease in transcription factors involved in insulin expression after ~6 months of diabetes.

Recent evidence shows that loss of IR-mediated signaling specifically in the RPE worsened rod photoreceptor abnormalities in diabetic mice (Tarchick et al. 2019). Interestingly, our results show higher expression of insulin genes in human RPE sections (Figure 6) and *ex vivo* experiments show that the RPE responds acutely to redox and inflammatory stress by upregulating insulin genes (Figure 5), suggesting that RPE is a more prominent source of local insulin production in the retina. This reveal that a mechanism of neuroprotection may be through the support of RPE by insulin secretion to the photoreceptors that overlay the RPE.

In addition to being a neurotrophic effect, IR signaling in the retina may have a supportive role in glucose uptake in retinal cells. Although this may seem to go against the dogma that the retina is insulin insensitive, reports have challenged this concept by showing that GLUT4 is in fact expressed in retinal neural cells and translocation of GLUT4 is decreased in diabetic conditions (Sanchez-Chavez et al. 2012). The latter evidence, along with a study that shows pancreatic insulin does not reach the retina (James and Cotlier 1983), supports the idea that there is local secretion of insulin may impact glucose homeostasis in the retina. Results from our *ex vivo* experiments, in fact, found that the neuroretina responds to high glucose at least acutely, by upregulating Ins1, however Ins2 did not have reliable response (Figure 5).

In summary, our results show for the first time that insulin mRNA in an extrapancreatic tissue from adult human and mouse retinas is modulated over the progression of diabetes. The question of what directly impedes the expression of insulin- dependent transcription factors in diabetes still remains. This study suggests that the regulation of local insulin production may be an important process in the pathogenesis of diabetic retinopathy. Furthermore, anti-diabetogenic drugs that target insulin synthesis, may have a direct effect on the expression of insulin locally in retinal cells.

Acknowledgements/Conflict of interest

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Highlights

- Insulin gene transcripts Ins1 and Ins2 and found in the retina and are altered in a model of Type 1 Diabetes.
- The neuroretina and RPE are both sources of insulin that is intrinsic to the retina.
- Secreted insulin in the retina is regulated independently of pancreatic-derived insulin.

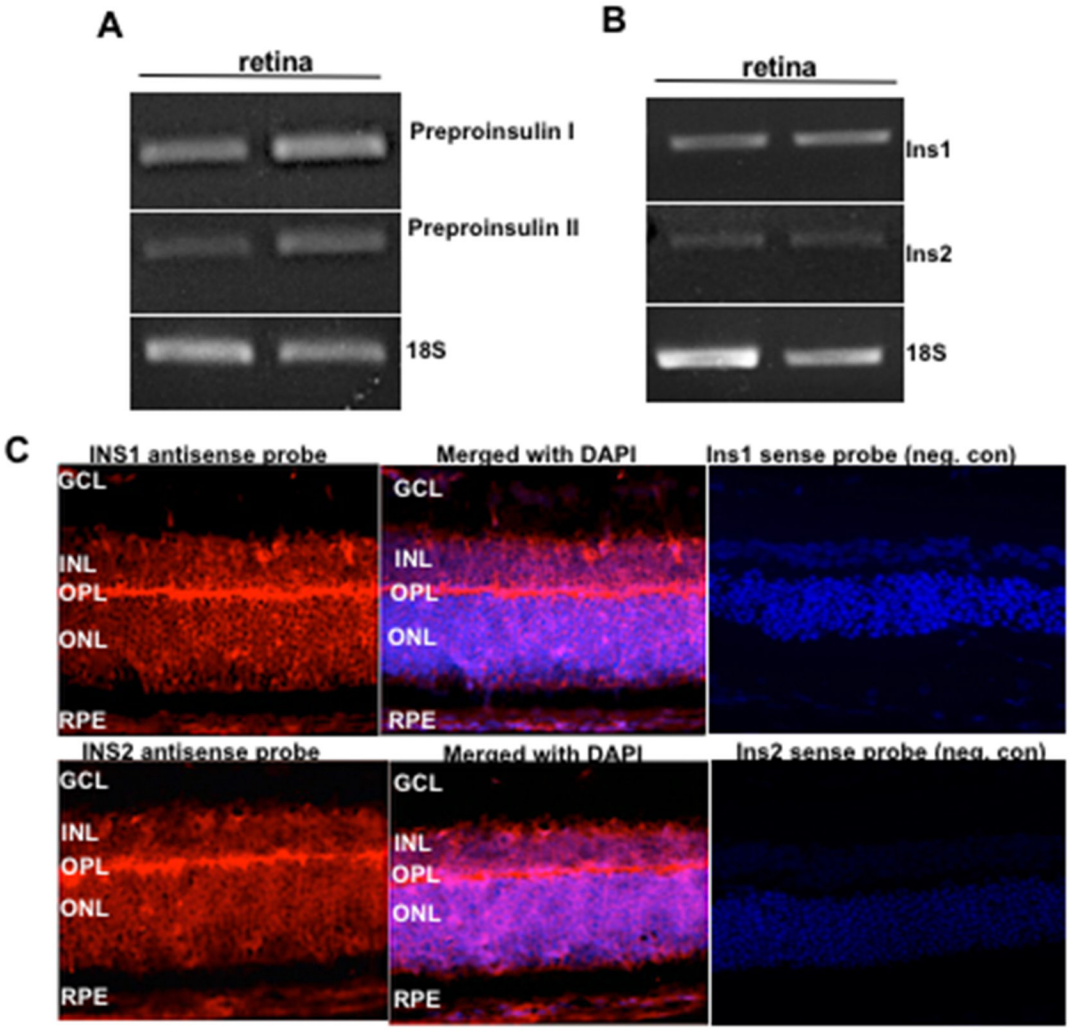

Figure 1.

Insulin gene expression in mouse retina. Total RNA collected from mouse retina was used for RT–PCR analysis of PPI1, PPI2 (**A**), Ins1, and Ins2 expression (**B**). 18S rRNA was used as an internal control. **C**) Localization of Ins1 and Ins2 mRNA transcripts was evaluated by fluorescent in situ hybridization (FISH). Hybridization of mouse retinal cryosections with anti-sense probes specific to Ins1 and Ins2. Red labeling is indicative of Ins1 and Ins2 transcripts present in the GCL, INL, ONL, and RPE. No positive signal was detected in sections hybridized with sense (negative control) probe. Abbreviations: ganglion cell layer (GCL); inner nuclear layer (INL); outer plexiform layer (OPL) outer nuclear layer (ONL); retinal pigmented epithelial layer (RPE).

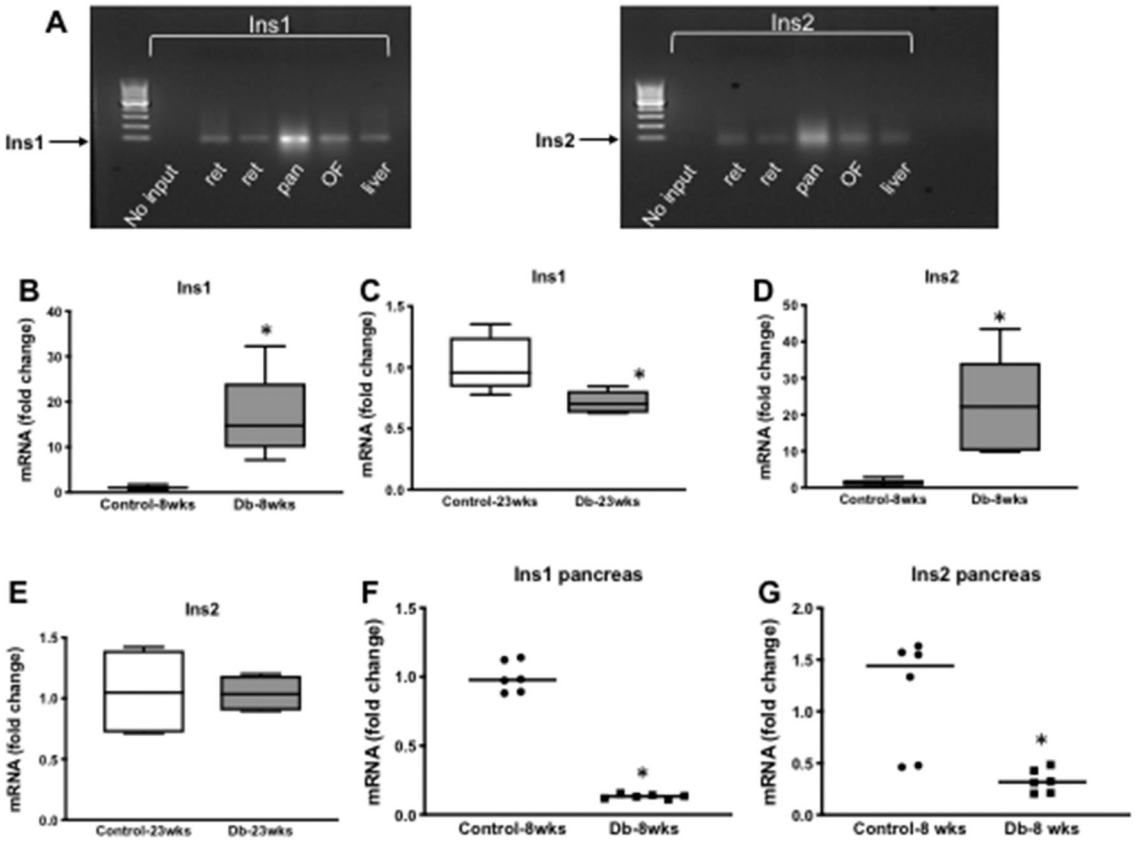

Figure 2.

Changes in Ins1 and Ins2 expression. **A**) Expression of Ins1 and Ins2 were compared in different murine tissues (retina, pancreas, olfactory bulb, liver). Real-time quantitative PCR (qPCR) analysis of Ins1 and Ins2 mRNA expression in retinas of normoglycemic (controls) and STZ-injected, diabetic (Db) groups (**B-E**). mRNA from Db retinas revealed an increase in insulin genes, (Ins1, p=0.0008, Ins2, p=0.0023) over time when measured at after 8 weeks (**B,D**). At 23 weeks (**C,E**) of diabetes there was a significant decrease in Ins1 (p=0.012) and no change in Ins2 (n=6 samples per group and amplified in 3 separate runs). *p<0.05 vs. control. **F-G**) Real-time quantitative PCR (qPCR) analysis of Ins1 and Ins2 mRNA expression in pancreatic tissue from normoglycemic (controls) and diabetic (Db) show decrease in expression of Ins1 and Ins2. n=6 samples and amplified in 3 separate runs. *p<0.05 vs. control.

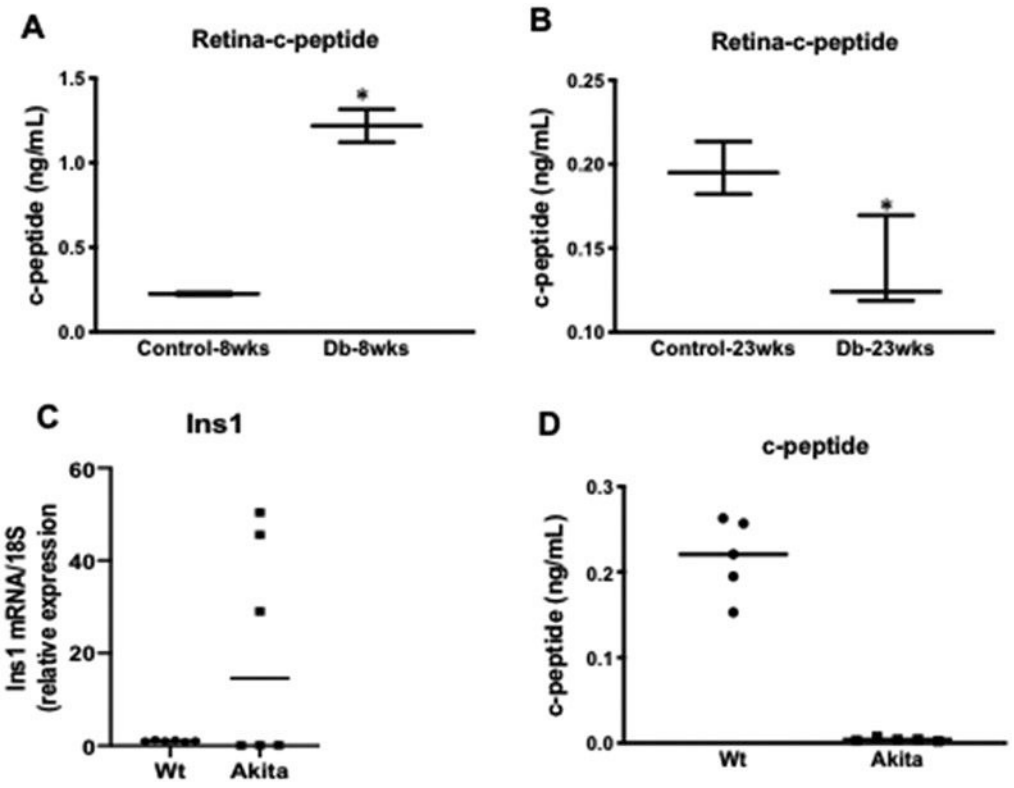

Figure 3.

Detection of c-peptide levels in retinas of T1D; STZ-injected and diabetic Akita mice. ELISA values of average c-peptide protein concentration in the retinas of control versus 8-week diabetic, STZ-injected (**A**) and 23-week diabetic, STZ-injected (**B**) mice. n=6 samples per group *p<0.05 vs. control. **C**) Real-time quantitative PCR (qPCR) analysis of Ins1 and Ins2 mRNA expression in retinal tissue from normoglycemic (controls) and diabetic Akita (Akita). Student t-test showed that there was no significant change in Ins1 expression in diabetic Akita mice compared with Wt mice and Ins2 was undetected. n=6 samples and amplified in 3 separate runs. **D**) Retina c-peptide protein concentration was determined in samples obtained from WT mice and diabetic Akita mice. Negligible amounts of the protein were detected in Akita mice, which are unable to synthesize active insulin or its cleavage product, c-peptide. Values are means ± SE of group size ($n = 6$).

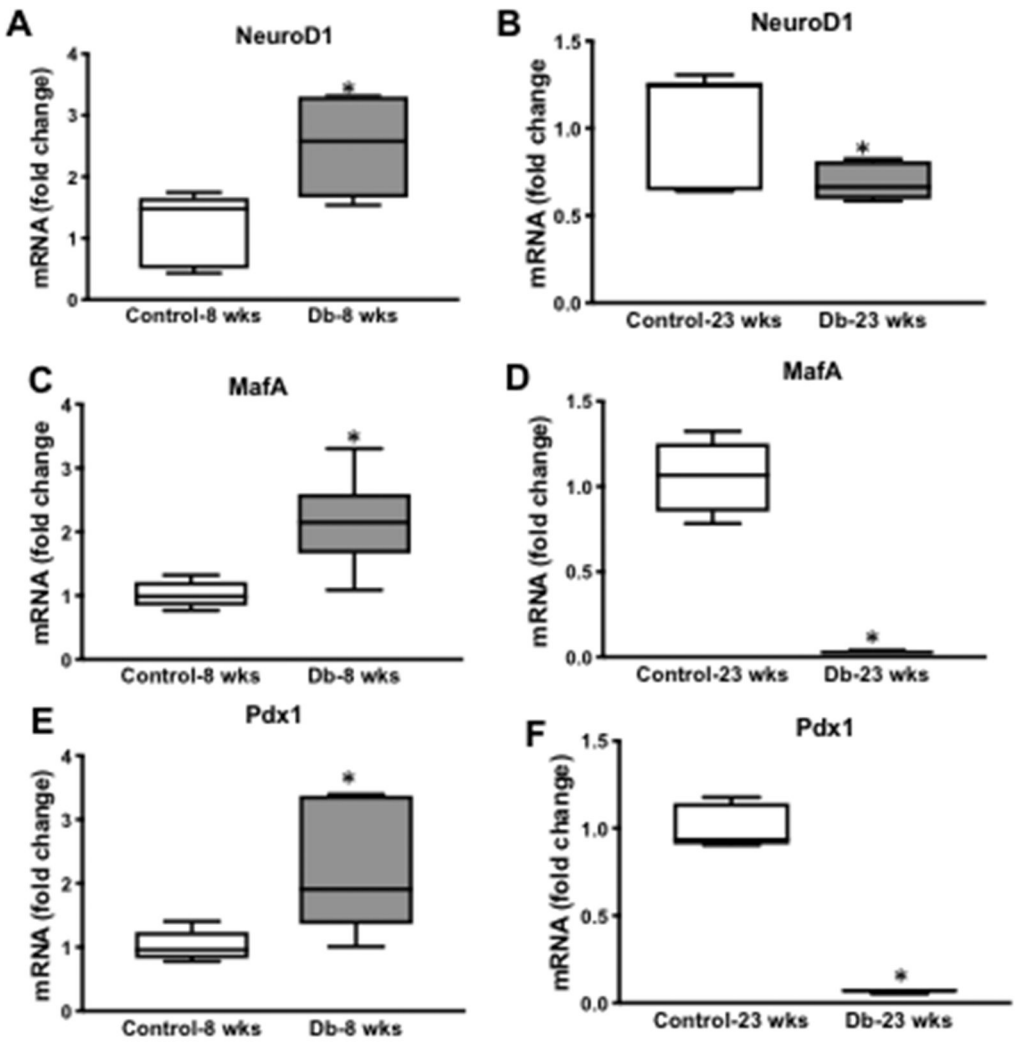

Figure 4.

Expression of insulin transcription factors. mRNA of NeuroD1 **(A-B)**, MafA (**C-D**), and Pdx1 (**E-F**) in retinal tissue from normoglycemic (controls) and STZ-injected diabetic (Db). mRNA expression of genes were normalized to 18s expression (n=6 samples per group and amplified in 3 separate runs). Data are presented as mean±SEM, *p<0.05 compared to age-matched, non-diabetic controls.

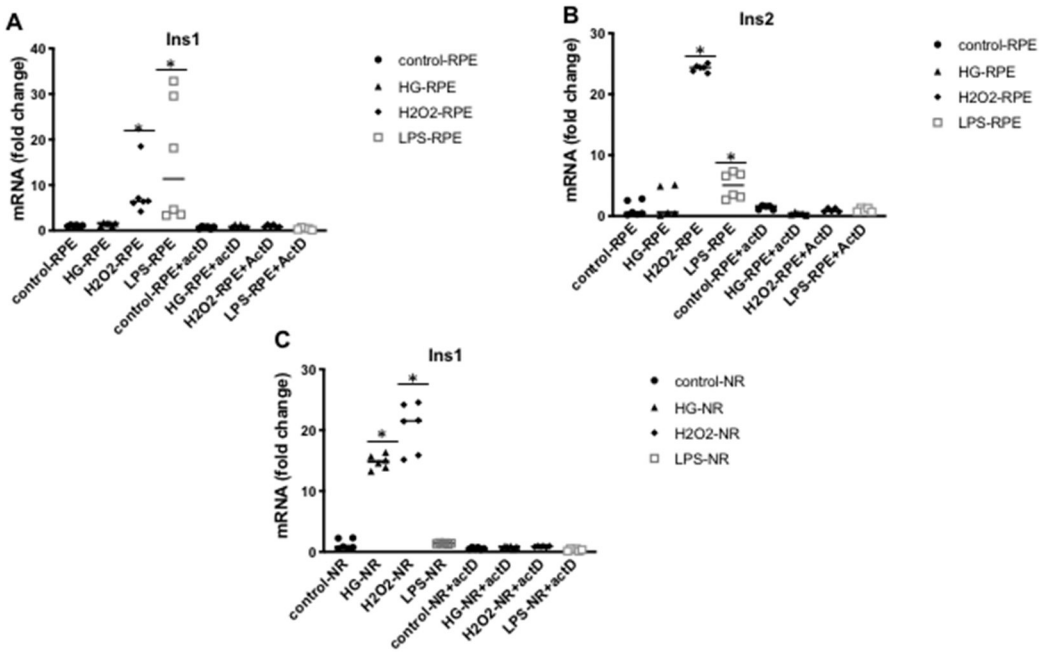

Figure 5.

Ex-vivo retinal cultures. RNA derived from RPE explants cultured in HG, H_2O_2 and LPS-conditioned media were utilized to measure the expression of Ins1(**A**) and Ins2(**B**) for 1 hour. The expression of Ins1 from mRNA derived from NR explants exposed to HG, H_2O_2 and LPS for 1 hour was detected in samples. Actinomycin D (actD) was utilized as an inhibitor of RNA synthesis. Data is expressed as mean±SEM, *$p<0.05$. n=6 samples per group and amplified in 2 separate runs.

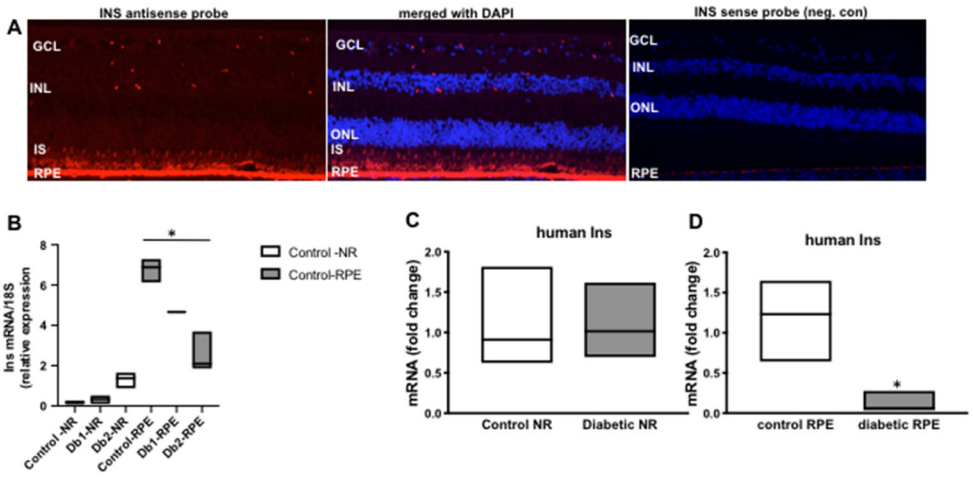

Figure 6.

Ins expression in human retinas. (**A**) In situ hybridization was used to determine the location of Ins in the retinas of control and diabetic donors. A weak signal detected in sections hybridized with sense (negative control) probe in RPE was determined to be non-specific in experiments which compared slides without any probe incubated with anti-DAB only (not shown) **B**) Expression profiling of *Ins* in neuroretinas and RPE isolated from control and diabetic donors. Relative expression of NR versus RPE samples are depicted in bar graphs (n=5 samples per group and amplified in 3 separate runs). **C-D**) qPCR analysis of mRNA from control and diabetic donors in NR and RPE tissue samples. Data is expressed as mean±SEM, *p<0.05, (n=5 samples per group and amplified in 3 separate runs).

Table 1.

Average weights and blood glucose levels of control and diabetic mice. Data are expressed as the mean ± SEM.*Significantly different from age-matched control mice (*p<0.05).

Experimental Groups	Body Weight (g)	Blood Glucose (mg/dL)	Duration of diabetes (weeks)	age
Control	25.01± .07	111± 8.78		14
Diabetic (by STZ injection)	21.45± .92*	328± 13.5*	8	14
Control	29.92± .84	111±8.02		29
Diabetic (by STZ injection)	19.61± 1.01*	352± 25.7*	23	29
Wt Control for Akita	18.47± 1.82	110± 9.2		12
Diabetic (Akita)	16.76± 1.29	325± 23.9*	8	12

Table 2.

Primer sequences.

Gene	Primer sequences
Ins1 (mouse)	5'- AATGGGCCAAACAGCAAAGTC -3' 5'- GAAACAATGACCTGCTTGCTGA -3'
Ins2 (mouse)	5'- GCAAGCAGGAAGGTTATTGTT -3' 5'- ACACACCAGGTAGAGAGCCT -3'
Preproinsulin 1 (mouse)	5'- CCCACCCCTCTGCAATGAATA -3' 5'- CCTGGTGTTTTATCACAAGCTTCA -3'
Preproinsulin 2 (mouse)	5'- AGCCACAATAGCCCCTAAAAGA -3' 5'- TCATGATCCTCCCGGACAAAC -3'
MafA (mouse)	5'- ATCATCACTCTGCCCACCAT -3' 5'- TGGAGCTGGCACTTCTCGCT -3'
NeuroD/Beta2 (mouse)	5'- CCTTTTAACAACAGGAAGTGG -3' 5'- TAGACAGTTTCTGGGTCTTG -3'
Pdx1 (mouse)	5'- CCTTCGGGCCTTAGCGTGTC -3' 5'- CGCCTGCTGGTCCGTATTG -3'
18S (mouse)	5'- CCAGAGCGAAAGCATTTGCCAAGA -3' 5'- AGCATGCCAGAGTCTCGTTCGTTA -3'
Ins (human)	5'- AGGCCATCAAGCAGATCACT -3' 5'- TTCCCCGCACACTAGGTAGA -3'
18S (human)	5'- CCCGTTGAACCCCATTCGT -3' 5'- GCCTCACTAAACCATCCAATCGGTA -3'