

VEGF as a Survival Factor in Ex Vivo Models of Early Diabetic Retinopathy

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PURPOSE. Growing evidence indicates neuroprotection as a therapeutic target in diabetic retinopathy (DR). We tested the hypothesis that VEGF is released and acts as a survival factor in the retina in early DR.

METHODS. Ex vivo mouse retinal explants were exposed to stressors similar to those characterizing DR, that is, high glucose (HG), oxidative stress (OS), or advanced glycation end-products (AGE). Neuroprotection was provided using octreotide (OCT), a somatostatin analog, and pituitary adenylate cyclase activating peptide (PACAP), two well-documented neuroprotectants. Data were obtained with real-time RT-PCR, Western blot, ELISA, and immunohistochemistry.

RESULTS. Apoptosis was induced in the retinal explants by HG, OS, or AGE treatments. At the same time, explants also showed increased VEGF expression and release. The data revealed that VEGF is released shortly after exposure of the explants to stressors and before the level of cell death reaches its maximum. Retinal cell apoptosis was inhibited by OCT and PACAP. At the same time, OCT and PACAP also reduced VEGF expression and release. Vascular endothelial growth factor turned out to be a protective factor for the stressed retinal explants, because inhibiting VEGF with a VEGF trap further increased cell death.

CONCLUSIONS. These data show that protecting retinal neurons from diabetic stress also reduces VEGF expression and release, while inhibiting VEGF leads to exacerbation of apoptosis. These observations suggest that the retina in early DR releases VEGF as a prosurvival factor. Neuroprotective agents may decrease the need of VEGF production by the retina, therefore limiting the risk, in the long term, of pathologic angiogenesis.

Keywords: octreotide, somatostatin, PACAP, retina, mouse, apoptosis

Diabetic retinopathy (DR) is the most common complication of type 1 and type 2 diabetes. Most studies in DR focus on blood vessel pathology, and pharmacologic treatments mainly target VEGF. However, numerous reports have established that functional alterations and degeneration of retinal neurons precede and possibly activate microangiopathic processes and disruption of the blood-retinal barrier,¹⁻⁶ supporting the view that DR may be considered a neurodegenerative eye disease.³

We have recently reported that retinal neurons subjected to acute ischemic stress express and release VEGF.^{7,8} In this context, the significance of VEGF release is unlikely to be related to its proangiogenic effects. Rather, VEGF expression and release are probably related to a protective strategy of the retina, because VEGF may exert important neuroprotective actions in the presence of a variety of insults, as observed in neuronal cell lines,⁹⁻¹¹ in the spinal cord,¹² in the brain,^{13,14} and in the retina.¹⁴⁻²⁰ Our hypothesis is that, in early DR, VEGF is expressed and released to protect retinal neurons. In this phase, VEGF would not act as a proangiogenic but as a prosurvival factor. If the stress remains for a long time, the persistent high levels of VEGF may lead to negative

consequences, such as the formation of new, aberrant retinal vessels.

If neurodegeneration plays a primary role in initiating the neovascular response typical of DR, then a neuroprotective strategy may prove useful in early stages of DR to prevent the development of both functional and vascular pathologies. Many different substances have been tested for their neuroprotective and/or antiangiogenic properties. A consistent body of experimental evidence indicates neuropeptides or neuropeptide analogs as effective neuroprotective agents in models of retinal disease. In particular, the somatostatin analog octreotide (OCT) and the peptide pituitary adenylate cyclase activating peptide (PACAP) seem to be the most promising peptidergic substances in this context.^{7,8,21-23}

In this work, we used organotypic mouse retinal explants cultured in the presence of stressors that characterize the retinal environment in DR, namely high glucose (HG), oxidative stress (OS), or advanced glycation end-products (AGE) to: (1) verify that neuronal damage caused by diabetic stress is accompanied by expression and release of VEGF, (2) test the hypothesis that neuroprotection may limit the release of VEGF, (3) ascertain whether VEGF may act as a neuroprotective factor in the diabetic retina.



METHODS

Animals

The procedures were approved by the Ethics Committee in Animal Experiments of the University of Pisa (Pisa, Italy) and were in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, the Italian guidelines for animal care (DL 116/92), and the EU Directive (2010/63/EU). All efforts were made to reduce both animal suffering and the number of animals used. C57BL/6J mice (Charles River Laboratories, Inc., Wilmington, MA, USA) were used in these studies. They were kept in a regulated environment ($23 \pm 1^\circ\text{C}$, $50 \pm 5\%$ humidity) with a 12-hour light/dark cycle (lights on at 8 AM) with food and water ad libitum.

Ex Vivo Retinal Explants

Retinas from 4- to 5-week-old mice were dissected in Modified Eagle Medium (MEM; Sigma-Aldrich Corp., St. Louis, MO, USA) and cut into four fragments. Fragments were transferred onto Millicell-CM culture inserts (Merck Millipore, Darmstadt, Germany) with ganglion cells up. Inserts were placed in six-well tissue culture plates containing 1 mL of serum-free culture medium composed of 50% MEM/HEPES (Sigma-Aldrich Corp.) containing 6 mM D-glucose, 25% Hank's buffer salt solution (Sigma-Aldrich Corp.), 25% PBS, 25 U/mL penicillin, 25 mg/mL streptomycin, 1 $\mu\text{g}/\text{mL}$ amphotericin B, and 200 μM L-glutamine. The first day in culture was designated as incubation day 0. The explants were incubated up to day 10 at 37°C under a humidified 95%/5% (vol/vol) mixture of air and CO_2 . The medium was changed every other day. High glucose treatments were performed adding D-glucose to the culture medium to reach a concentration of 45 or 75 mM, according to previous studies. Indeed, although a HG treatment resembling a diabetes-induced glucose increase is generally set at 25 to 35 mM glucose, in many studies HG treatments have been performed using a wide variety of glucose concentrations. In particular, in rodent retinal explants maintained in serum-free medium, HG treatments were performed using 45 to 57 mM glucose,²⁴⁻²⁷ while 75 mM glucose has been used for hyperglycemic treatment of primary cultures of hippocampal neurons.²⁸ In our preliminary experiments, treatments with 75 mM glucose were necessary to induce significant increases of apoptotic markers in retinal explants maintained in serum-free medium up to 10 days. Oxidative stress treatments were performed adding H_2O_2 to reach a concentration of 10 μM or 100 μM ,²⁹ while AGE treatments were performed adding 10 $\mu\text{g}/\text{mL}$ or 100 $\mu\text{g}/\text{mL}$ AGE-BSA (BioVision, Milpitas, CA, USA) to culture medium.³⁰ Control experiments were performed incubating the explants in the culture medium with the addition of 69 mM mannitol (HG control) or of 100 $\mu\text{g}/\text{mL}$ BSA (AGE control). An analysis of apoptotic cell death using caspase-3 immunolabeling showed lack of significant differences between untreated and mannitol- or BSA-treated explants during a 10-day incubation period (Supplementary Fig. S1). These observations were in agreement with previous studies of rat retinal explants.²⁴⁻²⁶ On the basis of these findings, only one type of control (i.e., untreated explants) was used in the experiments with HG, OS, and AGE treatments.

Pharmacological Treatments

The somatostatin analogue OCT (a long-lasting somatostatin subtype receptor 2 agonist; Sigma-Aldrich Corp.), was used at 1 μM , while PACAP-38 (Sigma-Aldrich Corp.) was used at 0.1 μM .⁸ A VEGF trap (R&D Systems, Minneapolis, MN, USA),

which is a recombinant soluble Flt-1/Fc chimera with homodimer structure binding all forms of VEGF with high affinity, was applied at 1 μM .⁷ Control experiments were performed using a VEGF trap that was preincubated overnight at 4°C in culture medium with double molar concentration of recombinant VEGF.

Immunofluorescence

Retinal fragments were fixed in 4% paraformaldehyde in 0.1M phosphate buffer for 1 hour. They were stored overnight in 25% sucrose in 0.1 M phosphate buffer and subsequently embedded in cryo-gel and frozen using liquid nitrogen. Immunofluorescence was performed using a rabbit antibody directed to active caspase-3 (1:500; Sigma-Aldrich Corp.) and appropriate secondary antibodies conjugated with Alexa Fluor 546 (Molecular Probes, Eugene, OR, USA). Retinal sections were counterstained with DAPI. Digital images were acquired at $\times 20$ or $\times 40$ magnification. To perform quantitative analysis, at least five representative images were selected for each retinal fragment and four fragments, each originating from a different retina, for experimental condition were analyzed. Each image was converted to grayscale and normalized to background using an image editing software (Adobe Photoshop CS3; Adobe Systems, Mountain View, CA, USA). Mean gray levels were then measured in the area encompassing the ganglion cell layer (GCL), the inner plexiform layer (IPL), and the inner nuclear layer (INL).

Western Blot

Four retinal fragments per condition, each originating from a different retina, were pooled and sonicated in 70 μL of 10 mM Tris/HCl, pH 7.6, containing 5 mM EDTA, 3 mM EGTA, 250 mM sucrose, and protease and phosphatase inhibitor cocktails. Homogenates were centrifuged at 22,000 g for 15 minutes at 4°C . The supernatants, containing cytosolic proteins, were used to detect cytochrome c. The supernatants were also used to measure VEGF content with ELISA (see below). Protein concentration was determined using a fluorometer (Qubit; Invitrogen, Carlsbad, CA, USA). Aliquots of each sample (30 μg protein) were subjected to SDS-PAGE. The gels were transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Membranes were blocked in 3% skim milk for 1 hour and then incubated overnight at 4°C with mouse monoclonal antibodies directed to cytochrome c (BD Biosciences, San Diego, CA, USA) diluted 1:500. The membranes were reblotted with a mouse monoclonal antibody directed to β -actin (1:2500; Sigma-Aldrich Corp.) used as the loading control. Finally, PVDF membranes were incubated for 1 hour with anti-mouse (1:12,500) horseradish peroxidase-labeled secondary antibodies (Santa Cruz Biotechnologies, Dallas, TX, USA) and developed with the enhanced chemiluminescence reagent. Images were acquired using Chemidoc XRS+ (Bio-Rad). Band optical density was evaluated with Image Lab 3.0 software (Bio-Rad). The data were normalized to β -actin. All experiments were run in duplicate. After statistics, data were averaged and plotted in the same graph.

Isolation of RNA and cDNA Preparation

Four retinal fragments per condition were pooled and total RNA was extracted using a RNA isolation kit (RNeasy Mini Kit; Qiagen, Hilden, Germany), purified, resuspended in RNase-free water and quantified using a fluorometer. First-strand cDNA was generated from 1 μg of total RNA using QuantiTect Reverse Transcription Kit (Qiagen).

TABLE. Stressor Doses and Incubation Times for Each Experimental Model

Model	Stressor	Low Dose	High Dose	Short Incubation	Long Incubation
HG	D-glucose	45 mM	75 mM	Day 2	Day 10
OS	H ₂ O ₂	10 μM	100 μM	Day 1	Day 5
AGE	AGE	10 μg/mL	100 μg/mL	Day 2	Day 7

Quantitative Real-Time RT-PCR

Quantitative real-time RT-PCR (qPCR) was performed as previously reported.³¹ To evaluate gene expression, qPCR experiments were performed using the SYBR Green PCR Kit (Qiagen). Forward and reverse primers were chosen to hybridize to unique regions of the appropriate gene sequence: Caspase-3 forward 5'-GCACTGGAATGTCATCTCGCTCTG-3'; Caspase-3 reverse 5'-GCCATGAATGTCTCTCTGAGGTTG-3'; VEGF forward 5'-GCACATAGGAGAGATGAGCTTCC-3'; VEGF reverse 5'-CTCCGCTCTGAACAAGGCT-3'; Rpl13a forward 5'-CACTCTGGAGGAGAAACGGAAGG-3'; Rpl13a reverse 5'-GCAGGCATGAGGCAAACAGTC-3'. Amplification efficiency was close to 100% for each primer pair. Each target gene was run concurrently with Rpl13a, a constitutively expressed gene encoding a ribosomal protein that is a component of the 60S subunit. To confirm that this transcript could be used as a suitable housekeeping/reference gene, it was validated in mouse retinal fragments both untreated and treated with the different stressors (Supplementary Table S1). Samples were compared using the relative threshold cycle (Ct Method).³² The increase or decrease (x-fold) was determined relative to a control after normalizing to Rpl13a. Three pools of fragments per condition, each made of four retinal fragments originating from different retinas, were analyzed and all reactions were run in triplicate. After statistical analysis, the data from the different experiments were plotted and averaged in the same graph.

Enzyme-Linked Immunosorbent Assay (ELISA)

Vascular endothelial growth factor levels were measured ($n = 3$ per condition) in culture mediums to evaluate VEGF release or in supernatants containing cytosolic proteins to evaluate VEGF content in the tissue, using a kit (R&D Systems). The ELISA plates were evaluated spectrophotometrically (Microplate Reader 680 XR; Bio-Rad). All experiments were run in duplicate. After statistical analysis, data from the different experiments were plotted and averaged in the same graph.

Statistics

Statistical significance was evaluated using ANOVA followed by Newman-Keuls Multiple Comparison post-test. The results are expressed as mean \pm SEM. Differences with P less than 0.05 were considered significant.

RESULTS

Analysis of Cell Death

For each experimental model, we used a low and a high dose of stressor and we determined a short and a long incubation time, as reported in the Table. The short incubation time was the day when caspase-3 immunolabeled cells were first detected using the high dose of stressor, while the long incubation time was the day when the maximum amount of caspase-3 immunolabeled cells was detected using the high dose of stressor. During the 10 days in culture, retinal explants maintained a normal morphology, with clear distinction of the

retinal layers and no apparent changes in layer thickness. Figure 1 shows the time-course of apoptosis in untreated control explants and in explants treated with high doses of stressors. In untreated explants (Fig. 1A), a few caspase-3 immunolabeled cells were detected in the INL and GCL from day 3. In HG- (Fig. 1B), OS- (Fig. 1C), and AGE- (Fig. 1D) treated explants, caspase-3 immunolabeled cells were first detected between day 1 and 2, while their highest numbers were observed between day 5 and 10. In particular, an analysis of immunofluorescence levels showed that apoptotic levels did not change significantly in untreated explants from day 3 to 10 (Fig. 1E), while maximum apoptotic levels were reached at day 10 in HG-treated explants (Fig. 1F), at day 5 in OS-treated explants (Fig. 1G), and at day 7 in AGE-treated explants (Fig. 1H). The apoptotic effects of each stressor were dose-dependent, as shown by Western blotting for cytochrome c, an apoptosis marker, in the presence of the low or the high dose of each stressor at the long incubation times (Fig. 2).

Protective Effects of OCT and PACAP

Incubation with OCT or PACAP protected the retinal explants from the stress induced by HG, OS, or AGE treatments with the high doses and for the long incubation periods, as assessed with caspase-3 immunofluorescence. Figure 3 shows representative images of HG-treated explants. Similar results were obtained with OS and with AGE-treated explants. Untreated explants (Fig. 3A), showed an amount of caspase-3 immunolabeled cells that was similar to that in the presence of OCT (Fig. 3B) or PACAP (Fig. 3C) alone. Caspase-3 immunoreactive cells were dramatically increased by HG treatment (Fig. 3D), but they were decreased back to untreated levels in HG-treated explants incubated in the presence of OCT (Fig. 3E) or PACAP (Fig. 3F). These results show that OCT and PACAP exert potent protective effects in stressed retinal explants. In addition, the incubation with OCT or PACAP alone did not affect caspase-3 immunolabeling and did not induce changes in the retinal layers or in retinal cells, as assessed with DAPI counterstain. The analysis of mean gray levels in immunofluorescence images (Fig. 3G) confirmed these observations.

Cell Death and VEGF Expression

We tested whether VEGF expression was correlated with the extent of neuronal damage and whether it may be reduced by protecting the neurons from the stress due to HG, OS, or AGE treatments with the high doses and for the long incubation periods. As shown in Figure 4A, the levels of caspase-3 mRNA, a measure of cell death, were significantly increased by HG, while they recovered to control values in explants treated with OCT or PACAP. In parallel, the levels of VEGF mRNA were almost doubled in HG-treated explants, while they were similar to those in control explants after treatment with OCT or PACAP (Fig. 4B). Similarly, OS treatment induced a 3-fold increase of caspase-3 mRNA expression, while OCT or PACAP inhibited this increase (Fig. 4C). Vascular endothelial growth factor mRNA levels were also increased by OS and they were reduced to control levels by OCT or below control levels by PACAP (Fig. 4D). In AGE-treated explants, caspase-3 mRNA

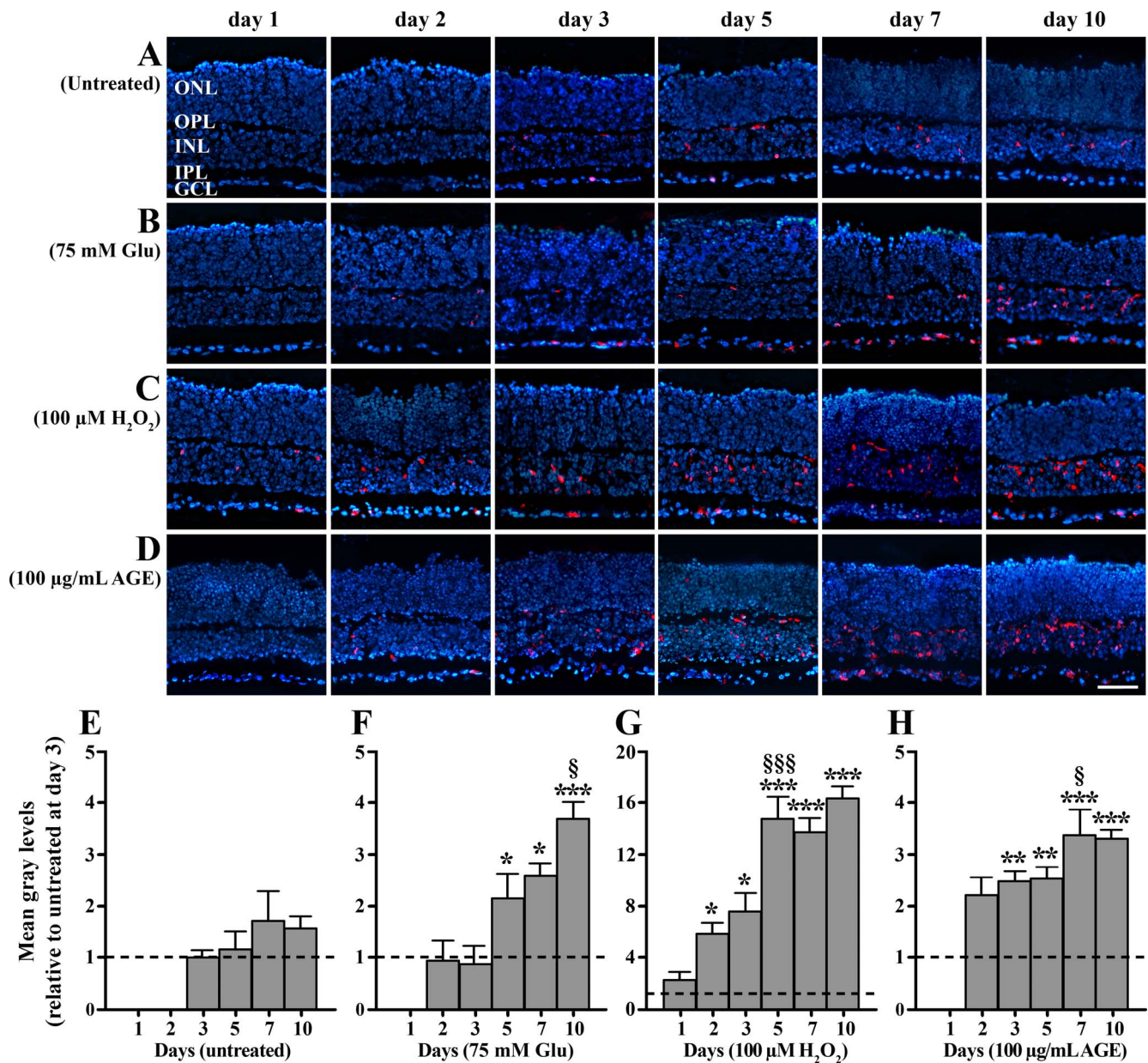


FIGURE 1. Representative immunofluorescence images showing active caspase-3 immunolabeled cells at different incubation times (days) in untreated explants (A) and in explants incubated with the high dose of HG (B), OS (C), or AGE (D) treatments, together with the quantitative analyses of immunofluorescence levels ([E] untreated; [F] HG-treated explants; [G] OS-treated explants; [H] AGE-treated explants). Retinal layers are visualized with DAPI counterstain. Scale bar: 50 μm. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 relative to untreated at day 3 (dashed lines); §*P* < 0.05, §§§*P* < 0.001 relative to previous incubation day. Glu, D-glucose; ONL, outer nuclear layer; OPL, outer plexiform layer.

expression was more than doubled with respect to untreated controls, while treatments with OCT or PACAP reduced caspase-3 mRNA expression to control levels (Fig. 4E). Vascular endothelial growth factor mRNA in AGE-treated explants was also markedly increased, but OCT or PACAP treatments completely prevented this effect (Fig. 4F).

VEGF Release

We evaluated VEGF release from stressed retinal explants and tested whether neuroprotection could limit this release. Vascular endothelial growth factor levels in the medium of untreated control explants did not change significantly between day 2 and 10, and they were approximately 0.6 pg/mL. Vascular endothelial growth factor was also measured in

the retinal explants and its value was approximately 25 pg/mg of protein. Vascular endothelial growth factor levels were measured in HG-, OS-, and AGE-treated explants with similar results. Representative examples are shown in Figure 5, which summarizes an analysis of VEGF levels in AGE-treated explants and in their incubation media at the short and the long incubation times in the presence of the low or the high dose of stressor. Vascular endothelial growth factor levels were significantly decreased in AGE-treated explants compared with untreated controls, independent of the incubation time and of the dose of stressor (Fig. 5A). In contrast, VEGF levels in the incubation medium increased both time- and dose-dependency (Fig. 5B). The values of the ratio between VEGF levels in the explant tissue and those in its incubation medium (Fig. 5C) were significantly reduced by AGE treatment at all

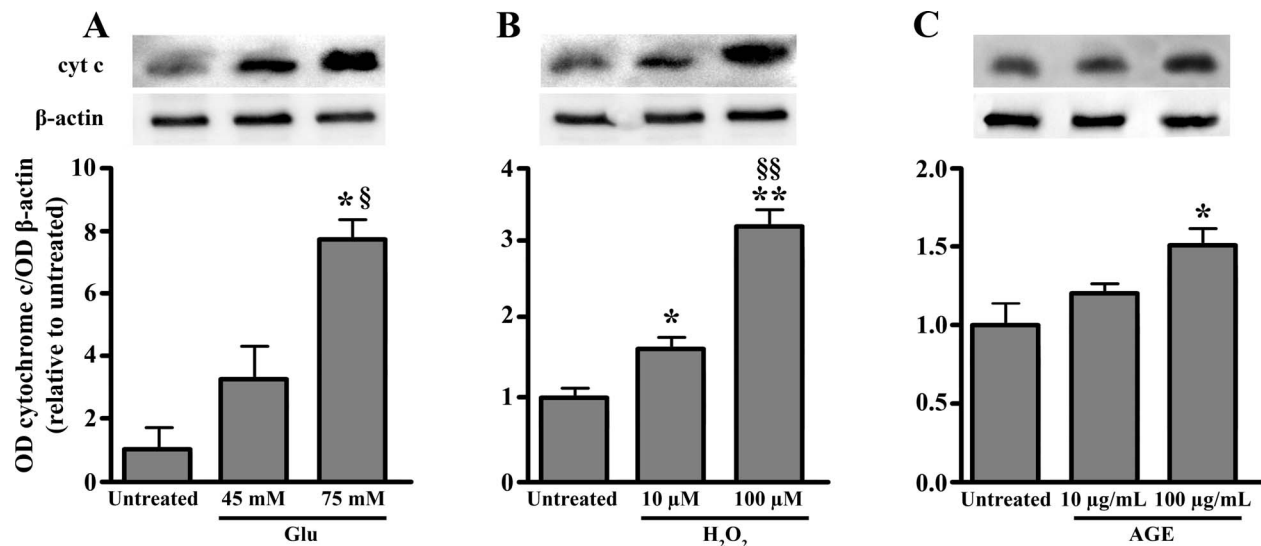


FIGURE 2. Western blotting for cytochrome c as a measure of apoptotic levels in retinal explants treated with the low and the high dose of each stressor in the HG (A), OS (B), and AGE (C) models at their long incubation times. * $P < 0.05$, ** $P < 0.01$ relative to untreated; §§ $P < 0.01$ relative to low dose.

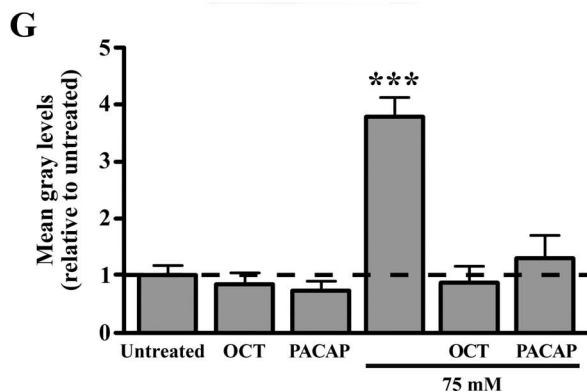
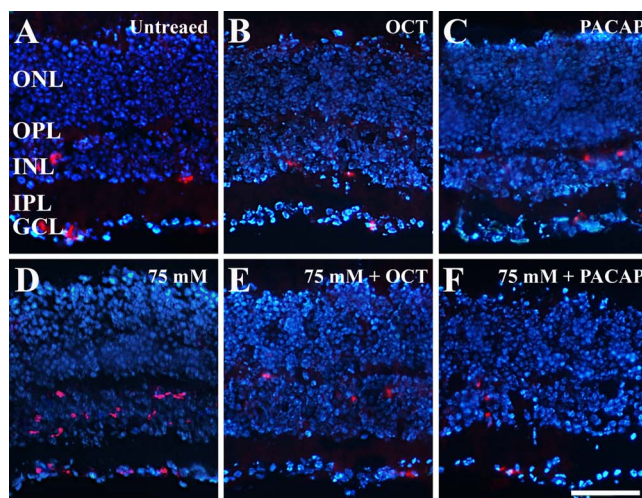


FIGURE 3. Representative immunofluorescence images showing active caspase-3 immunolabeled cells at day 10 in untreated explants (A), in explants incubated with OCT (B), or PACAP (C), and in explants treated with the high dose of HG alone (D) or in combination with OCT (E) or PACAP (F). Retinal layers are visualized with DAPI counterstain. (G) Quantitative analysis of immunofluorescence levels. Scale bar: 50 μm. *** $P < 0.001$ relative to untreated (dashed line).

incubation times and doses, indicating that a considerable part of the VEGF contained in the tissue was released upon exposure to AGE. The graph with the sum of VEGF in the explant tissue plus VEGF in the medium (Fig. 5D) shows a tendency of these values to increase with the incubation time and with the stressor dose, reaching statistical significance at day 7 with the dose of 100 μg/mL and indicating that the treatment determined an overall increase of the VEGF produced by the explants.

The effects of the treatment with OCT or with PACAP on the levels of VEGF release were evaluated at the short incubation time of each experimental model to ascertain whether VEGF release is initiated before substantial cell death is detected and whether neuroprotection may have an effect on VEGF release at this early phase. As shown in Figure 6, in all three experimental models an important increase of VEGF release was observed in the stressed explants, but it was completely abolished by OCT or PACAP. In particular, in HG-treated explants, both OCT and PACAP treatments significantly reduced VEGF release below control levels.

Neuroprotective Effects of VEGF

These experiments were conducted on HG explants treated with 45 mM glucose and with a VEGF trap, analyzed at day 10. The low dose of stressor was used to avoid massive cell death that could mask the expected effects of the VEGF trap. Active caspase-3 immunofluorescence revealed only a few labeled cells in untreated control explants (Fig. 7A) or in explants treated with the VEGF trap alone (Fig. 7B). A slight increase in active caspase-3 immunolabeling was observed in the presence of 45 mM glucose (Fig. 7C), but the immunolabeling dramatically increased in the presence of 45 mM glucose together with the VEGF trap (Fig. 7D). This increase was not seen when using a VEGF trap that had been preincubated with recombinant VEGF (Fig. 7E). The analysis of mean gray levels in immunofluorescence images showed that the increase of active caspase-3 immunolabeling caused by 45 mM glucose was very limited and not statistically significant, but the addition of the VEGF trap determined a 10-fold increase of the immunolabeling relative to untreated controls, while a VEGF trap preincubated with VEGF had no effects (Fig. 7F). These

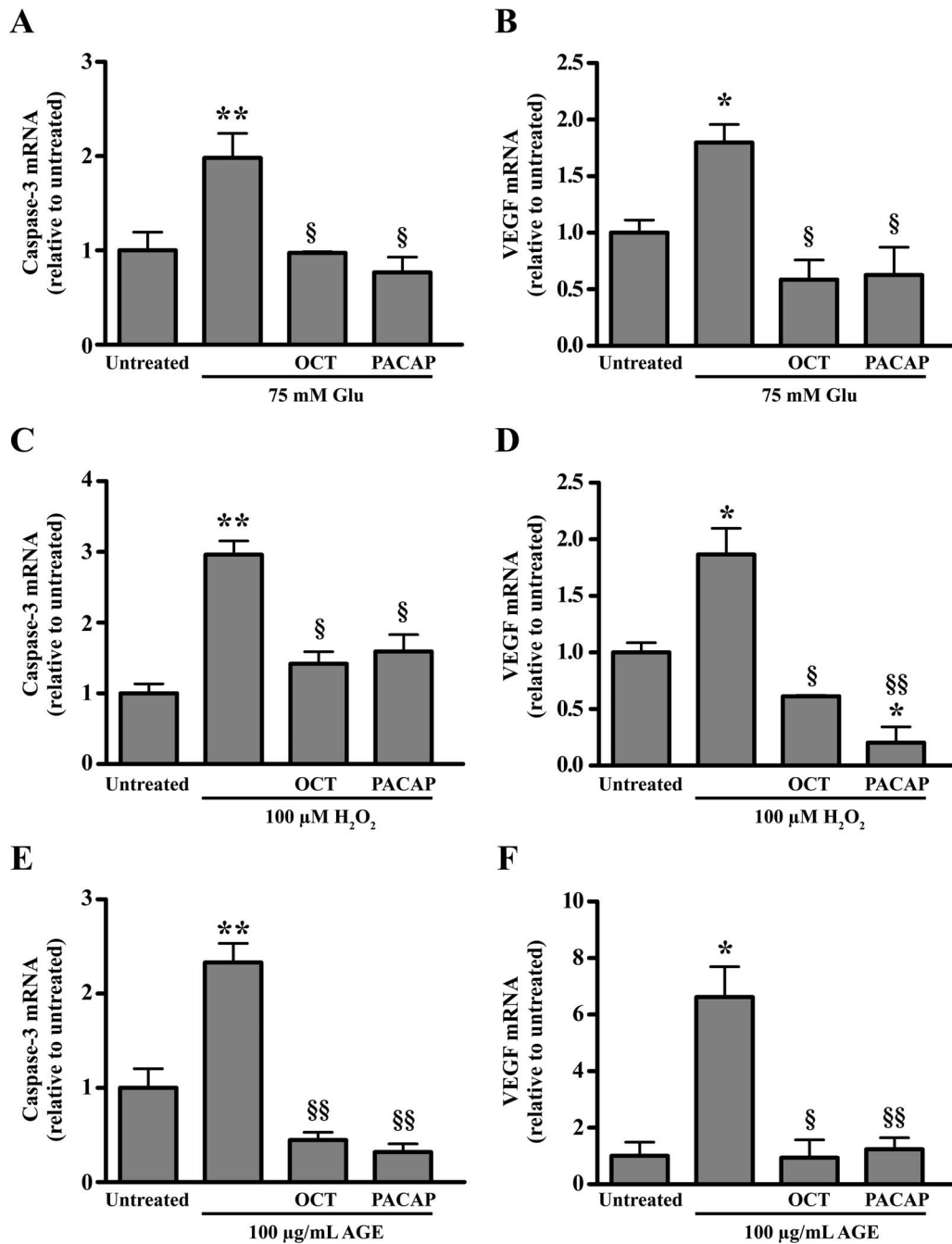


FIGURE 4. Caspase-3 and VEGF mRNA expressions, evaluated with qPCR, in HG- (A, B), OS- (C, D), and AGE- (E, F) treated explants incubated with the high stressor dose and for the long incubation time in the presence or absence of OCT or PACAP. **P* < 0.05, ***P* < 0.01 relative to untreated; §§*P* < 0.01 relative to treatment with stressor alone.

observations were confirmed with qPCR measures of caspase-3 mRNA expression. Indeed, as shown in Figure 7G, the pattern of caspase-3 expression in the different experimental conditions was the same as that observed with the gray level analysis of immunolabeled sections. The levels of caspase transcripts in HG explants treated with VEGF trap was 4- to 5-fold that in untreated controls or in VEGF trap-only-treated explants.

DISCUSSION

In early DR, high levels of glucose result in retinal damage because they activate a series of negative intracellular responses,

including (although not limited to) OS and AGE formation. Oxidative stress may be secondary to disrupted retinal glutamate homeostasis and excitotoxicity or to a variety of intracellular pathways activated by HG and may cause inflammation, reduction of naturally expressed neuroprotectants and cell death, while AGE formation is induced by HG and has been observed to result in neuronal death in rodent retinal explants.³³ Oxidative stress and AGE production are likely to be interrelated, because AGE can induce OS, while AGE synthesis is accelerated by OS.^{34,35} Therefore, the experimental models used in our studies, consisting of HG-, OS-, or AGE-treated retinal explants, are modeling the three main factors characterizing the condition of the retina in early DR

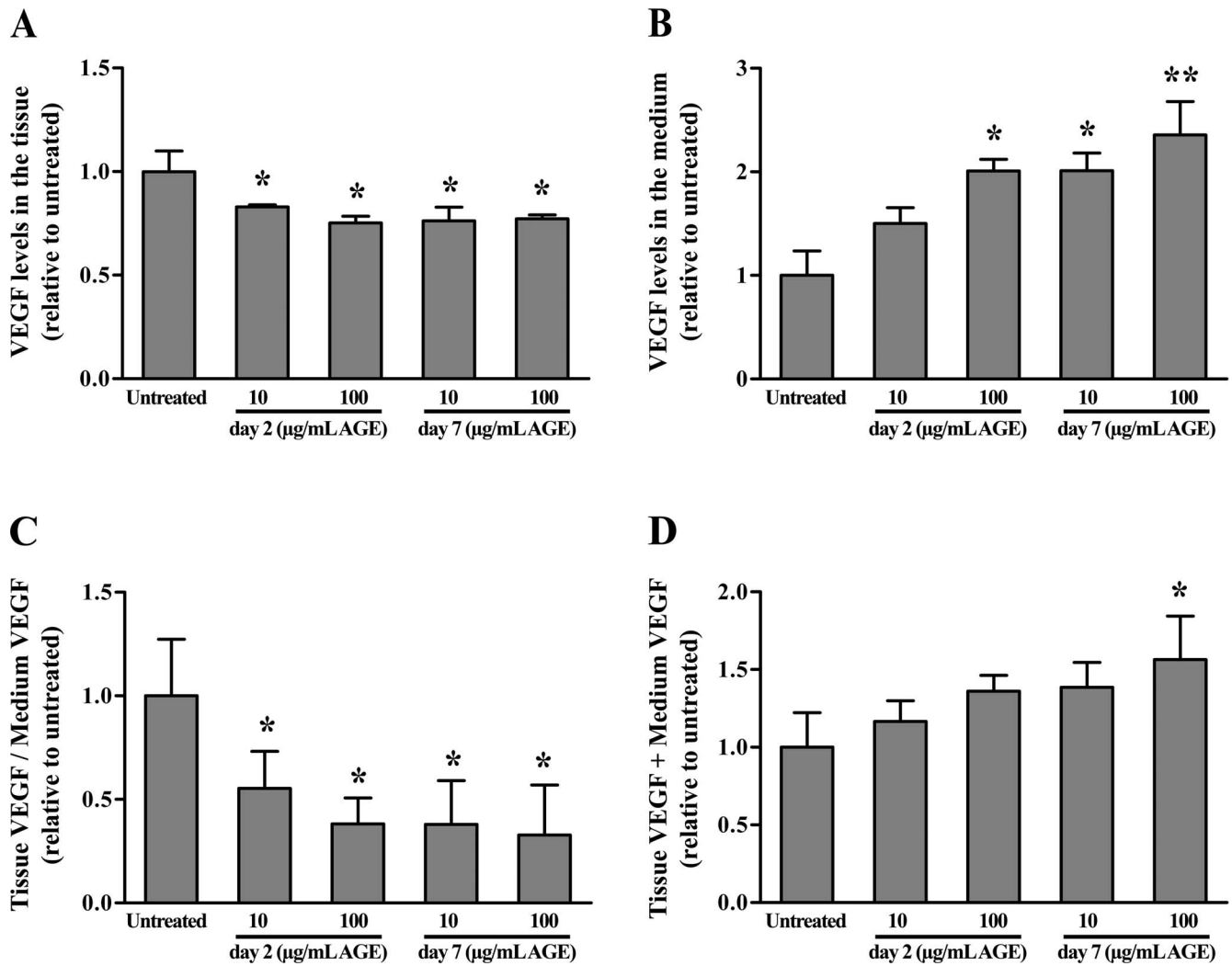


FIGURE 5. Vascular endothelial growth factor levels, evaluated with ELISA, in AGE-treated explants and in their incubation media at the short and the long incubation times in the presence of the low or the high dose of AGE. Vascular endothelial growth factor levels were measured in the retinal tissue (A) or in the incubation medium (B). The ratio VEGF in the tissue/VEGF in the medium (C) indicates whether VEGF is mainly released or retained in the cells; the sum VEGF in the tissue + VEGF in the medium (D) indicates possible variations in total VEGF **P* < 0.05, ***P* < 0.01 relative to untreated.

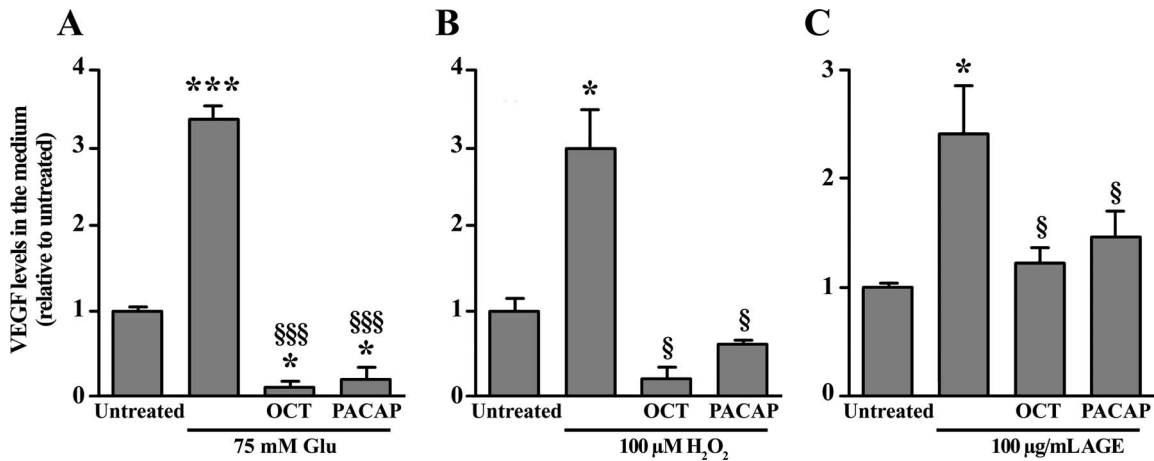


FIGURE 6. Vascular endothelial growth factor release, evaluated with ELISA, in HG- (A), OS- (B), and AGE- (C) treated explants incubated with the high stressor dose and for the short incubation time in the presence or absence of OCT or PACAP. **P* < 0.05, ****P* < 0.001 relative to untreated; §*P* < 0.05, §§§*P* < 0.001 relative to treatment with stressor alone.

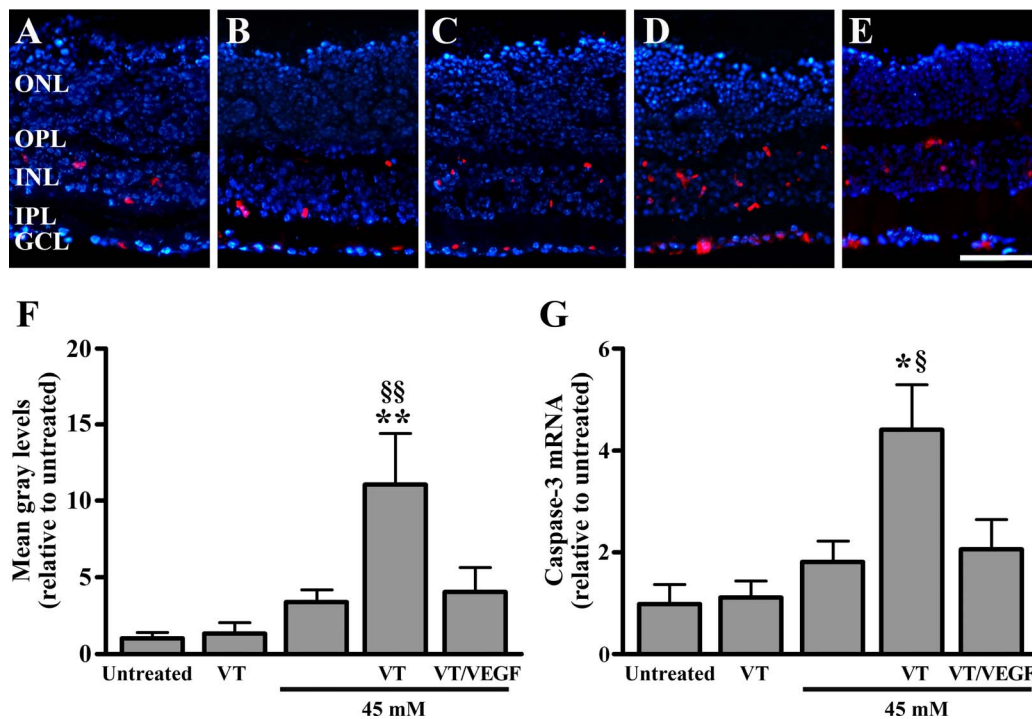


FIGURE 7. Immunofluorescence for active caspase-3 in untreated retinal explants (A), or in explants incubated with a VEGF trap (VT, [B]), with the low dose of the HG model (C), with low dose-HG and VT (D), or with low dose-HG and VT preincubated with recombinant VEGF (E) for the HG long incubation period. (F) Quantitative analysis of mean immunofluorescence levels. (G) Caspase-3 mRNA expression evaluated with qPCR. Retinal layers are visualized with DAPI counterstain. Scale bar: 50 μ m. * $P < 0.05$, ** $P < 0.01$ relative to untreated; § $P < 0.05$, §§ $P < 0.01$ relative to treatment with stressor alone.

In *in vivo* models of DR and in the retinas of patients with diabetes, cell death has been reported mainly in ganglion cells and in amacrine cells, while the data relative to photoreceptor death or damage in diabetic retinas are not conclusive, as recently reviewed.³⁶ Similarly, apoptotic cells in our retinal explants, visualized with caspase-3 immunoreactivity, were primarily localized to INL and GCL, thus reinforcing the similarities of these *ex vivo* models with the retina in early DR

VEGF as a Survival Factor in Early DR

Our observations show that VEGF expression and release are concomitant and proportional to retinal damage induced by HG, OS, or AGE. Possible interpretations of this finding are: (1) VEGF increase and retinal damage are two independent phenomena, (2) retinal damage induces VEGF increase, and (3) VEGF increase provokes retinal damage. Our data strongly suggest that inhibiting cell death also inhibits VEGF increase; however, inhibiting VEGF does not result in reduced retinal damage but rather provokes a significant increase of cell death. Therefore, it appears that VEGF increase and retinal damage are not independent phenomena. In addition, these observations suggest that VEGF levels in the early diabetic retina may directly depend on the level of retinal damage.

In summary, our data support the hypothesis that VEGF represents a sort of an emergency survival factor whose production is commensurate with the extent of retinal cell suffering in early DR. Consistent with this view, we have observed increased VEGF release by retinal explants in acute ischemia, which was reduced when the retinas were treated with neuroprotective peptides.^{7,8} In addition, glutamate excitotoxicity, one of the major causes of retinal neuronal death in DR, has been reported to upregulate VEGF production in diabetic retinas,³⁷ while inhibition of glutamate receptors

results in a decrease of vitreoretinal VEGF in diabetic rats.³⁸ Similarly, the antioxidant resveratrol has been found to block the early increase of retinal VEGF caused by streptozotocin-induced diabetes.³⁹ In general, observations of brain reaction to damage suggest that, in addition to death pathways, also protective mechanisms are activated in the injured brain and imply that the central nervous system possesses intrinsic cytoprotective mechanisms that are likely to be mediated by chemical signals derived from the injured brain itself.⁴⁰ Vascular endothelial growth factor may be one of such signals released by the retina in the early phases of DR in response to the damage caused by increased glucose levels. Interestingly, it has been reported that the optic nerve and retinal ganglion cells in a rat model of experimental glaucoma are protected from degeneration by short-term hyperglycemia.⁴¹ Recent findings indicate that, in DR, VEGF may exert neuroprotective effects mediated by Müller cells responding to VEGF with their VEGFR2 receptors.⁴²

In keeping with the proposed role of VEGF as a survival factor expressed and released in the retina in response to acute diabetic stress, our results provide direct evidence of neuroprotective effects of VEGF in our *ex vivo* models. Several studies have convincingly demonstrated the neuroprotective potential of VEGF in a variety of retinal pathologic conditions,¹⁵⁻¹⁸ although VEGF blockade does not seem to affect the healthy retina.^{43,44} Our data strongly suggest that VEGF is the main protective factor released by the retina to protect itself from the damage caused by DR. One may wonder why, in the course of evolution, VEGF has been preferred to other neuroprotective factors (for instance neurotrophins) to protect the retina in DR, in spite of its detrimental proangiogenic effects resulting, in the long run, in the proliferative phase of DR. It should be considered that under diabetic conditions, not only retinal neurons but also vascular endothelial cells and

pericytes undergo progressive degeneration.⁴⁵ Vascular endothelial growth factor is known to be an important growth factor for endothelial cells⁴⁶ and its upregulation in early DR may have the dual objective of protecting both retinal neurons and endothelial cells. This view is supported by recent findings documenting both neurodegeneration and vascular damage induced by repeated intravitreal injections of VEGF inhibitors in the eyes of Ins2(Akita) diabetic mice.¹⁸

Neuroprotective Effects of OCT and PACAP

In our experiments, neuroprotection was provided by OCT or PACAP. In principle, we could not exclude potential side effects of OCT or PACAP treatments, which may affect the structure of retinal explants or induce apoptosis in a caspase-independent manner.⁴⁷ The retinal structure, as seen in DAPI counterstained sections, was apparently similar in untreated explants and in explants treated with OCT or PACAP; therefore, we can reasonably conclude that no side effects are induced by OCT or PACAP treatments.

We assumed that neither OCT nor PACAP could significantly influence VEGF levels per se. In effect, if OCT has well-documented neuroprotective effects in the retina,^{7,8,22} it has also been demonstrated to efficiently inhibit hypoxia-induced VEGF expression both in the in vivo model of oxygen induced retinopathy (OIR)⁴⁸ and in ex vivo retinal explants cultured in hypoxia, where the intracellular mechanisms affected by OCT to inhibit VEGF expression have been identified.³¹ Although we cannot exclude, in principle, that such mechanisms are also activated by OCT in our ex vivo models of DR, it should be noted that our explants were cultured in normoxia and were never subjected to hypoxic conditions. Therefore, a direct action of OCT on hypoxia-induced VEGF expression, such as that described in the literature, is unlikely in our experimental settings. These questions will be better addressed with further studies to define the intracellular signaling mechanisms activated by OCT in our ex vivo models of DR.

Pituitary adenylate cyclase activating peptide has been reported to exert neuroprotective functions in a variety of retinal injuries²¹ and in the retina of streptozotocin-treated rats.^{23,49,50} Regarding possible direct effects on VEGF, most experimental evidence reports an inducing, and not inhibitory, effect of PACAP on VEGF. Indeed, PACAP treatment results in increased VEGF in rat retinal hypoperfusion,⁵¹ in lung cancer cells,⁵² and in pituitary folliculostellate cells.⁵³ In addition, PACAP does not appear to influence the hypoxia-induced neoangiogenic response characterizing the OIR model (G. Casini, R. Amato, and M. Dal Monte, unpublished observation, 2015). In a recent paper, intraocular PACAP administrations have been reported to decrease the expression of hypoxia-inducible factor 1 α , a positive VEGF regulator, in the retina of streptozotocin-treated rats after 3 weeks from diabetes induction, although data on actual VEGF expression were not reported.⁵⁴ In summary, we can reasonably assume that the actions of OCT and of PACAP in our studies were prevalently neuroprotective, with only little or no direct effects on VEGF expression. On the other hand, it should be also considered that if OCT and/or PACAP were direct inhibitors of VEGF in our models, their effects should have been similar to those of a VEGF trap, that is induction rather than limitation of cell death.

CONCLUSION

The present study not only confirms and expands the notion that neurodegeneration is likely to play an essential role in the pathogenesis of DR, but also indicates that VEGF is a

critical survival factor for retinal cells stressed by factors (HG, OS, and AGE) characterizing the early phases of DR. Therefore, if, on the one hand, it is reasonable to hypothesize that therapeutic strategies based on neuroprotection will be effective in preventing or arresting DR development, on the other it appears that an anti-VEGF approach would be detrimental if performed in early DR. Based on our and others' data, protecting retinal neurons reduces possible loss of function and at the same time limits VEGF expression and release, thus reducing the possibility of progression to the proliferative phase of DR. Naturally occurring substances such as peptides with neuroprotective action represent a stimulating perspective for treatment of early DR. In this respect, topical administration of somatostatin have been found to prevent retinal neurodegeneration in streptozotocin-induced diabetic rats.⁵⁵ With this basis, a multicentric, phase II-III, randomized controlled clinical trial (EUROCONDOR) to assess the efficacy of topically administered somatostatin to counteract retinal neurodegeneration in DR is ongoing.

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