Estrogen Protects the Inner Retina from Apoptosis and Ischemia-Induced Loss of Vesl-1L/Homer 1c Immunoreactive Synaptic Connections

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PURPOSE. Protective effects of estrogen on nerve cells including retinal neurons have been described previously. However, subcellular effects on synaptic connectivity in mild ischemia more closely resembling ischemic conditions found in diabetic or sickle cell retinopathy and stenosis of the carotid artery have not been identified. The present study quantitatively analyzed effects of estrogen administration on synaptic connections of neurons in the ganglion cell layer (GCL) of the retina.

METHODS. Staining of Vesl-1L/Homer 1c (V-1L) immunoreactivity and TUNEL cytochemistry were used to quantify neuroprotective effects at the synaptic level in a model of mild retinal ischemia induced by temporary middle cerebral artery occlusion in the adult rat.

RESULTS. V-1L immunoreactivity was found in both synaptic layers, postsynaptic to glutamatergic ribbon synapses. Mild retinal ischemia led to a significantly higher percentage reduction in the number of V-1L-positive synapses in the inner plexiform layer (IPL) compared with the percentage of TUNEL-positive apoptotic neurons in the GCL. Estrogen prevented ischemia-induced loss of V-1L-immunoreactive synapses in the IPL and apoptosis of cells in the GCL.

CONCLUSIONS. Immunoreactivity for V-1L can be used as a synaptic marker for early changes before more severe neurodegenerative events. The present results suggest that estrogen protects neurons in the GCL including RGCs from both apoptosis and early changes in synaptic connections associated with ischemia and potentially preceding apoptosis. (Invest Ophthalmol Vis Sci. 2003;44:3155–3162) DOI:10.1167/iovs.02-1204

R etinal ganglion cells (RGCs), because of high sensitivity to cellular damage and neurotoxicity, offer a unique and effective model to study the mechanism of neurodegenerative disease progression.1–3 Pathologically, these neurons are the primary targets of processes leading to the loss of vision in neurodegenerative diseases such as glaucoma, but they are also directly affected in acute diseases characterized by retinal ischemia, such as diabetic retinopathy, sickle cell retinopathy, and stenosis of the carotid artery.4–7 These neurons are also known targets of steroid hormones.8–12 As such, RGCs serve as an ideal model to evaluate the neuroprotective effects of steroid hormones, and at the same time, can offer important insights for development of novel treatments for retinal degeneration and for other neurodegenerative diseases, as well.

Vesl/Homer proteins are cytosolic scaffold proteins that have been implicated in the clustering of neurotransmitter receptors and neuronal development and plasticity. The Vesl/Homer protein Vesl-1L/Homer 1c (V-1L) binds other proteins, such as metabotropic glutamate receptors and cation channels, and localizes them to glutamatergic synapses.13–19 V-1L was used as a marker for the assessment of changes in synaptic connectivity preceding apoptosis and during early stages of apoptosis of RGCs, because it has several unique properties. As a postsynaptic clustering molecule, it has the property of linking neurotransmitter receptors, plasma membrane ion channels, intracellular calcium channels, and the cytoskeleton.13–19,20,21 Therefore, V-1L serves as a unique marker to detect synaptic changes mediated or affected by extracellular signaling, intracellular signaling or cytoskeletal processes. V-1L is the ubiquitously expressed isoform of Vesl-1 with expression levels that are not affected by changing physiological conditions, as described for the conditionally expressed isoform of Vesl-1, Vesl-IS.15–16,22 Therefore, V-1L can be used as a synaptic marker that is sensitive to overall changes in synaptic connectivity—that is, neurotoxicity-induced loss of synapses, but with expression levels that are not influenced by changed physiological conditions.13–19,20,22 This specific function in cross-linking proteins at glutamatergic synapses in the central nervous system (CNS) was used in the present study to identify the distribution of ischemia-induced changes of postsynaptic elements of RGCs.

The neuroprotective effects of estrogens have been extensively assessed in animal models of cerebral ischemia. After the initial report of neuroprotection with estrogens in a model of transient cerebral ischemia,25 different estrogen treatments have been demonstrated to protect the brain from different forms of ischemic damage.21–36 Collectively, the ability of estrogens to preserve neuronal function in various experimental models of neurodegeneration suggests that they exert equally efficacious neuroprotective effects in retinal degeneration. The absence of estrogens, such as occurs after menopause, can have pathologic effects on vision. Age-related risks for development of ocular diseases can be reduced with estrogen treatment.37 Furthermore, just as postmenopausal women...
have been documented as having a higher prevalence of and risk of developing Alzheimer’s disease; they are also at a higher risk for development of macular degeneration—a risk that seems to be reduced with estrogen replacement.\textsuperscript{35,36} Shorter duration of estrogen exposure—that is, a shorter duration between menarche and the onset of the menopause, also increases a woman’s risk of age-related macular degeneration,\textsuperscript{41} further supporting the hypothesis that estrogen treatment may help stave off ocular tissue degeneration. Direct investigation of estrogen’s protective effects against ischemia-reperfusion-induced retinal damage has demonstrated that 17β-estradiol reduces leukocyte accumulation and consequent retinal damage, particularly in the inner retina.\textsuperscript{42} Mechanistically, excitotoxic cell death in the retina (such as that which occurs after ischemia) may be, at least in part, a consequence of changes in GABAergic signaling.\textsuperscript{43,44} Alternatively, estrogen may also exert its protective effects by influencing the vasculature or blood flow to the eye. To this end, the beneficial effect of estrogens in reducing the risk of developing age-related eye diseases, such as glaucoma and macular degeneration, has been linked to its positive effects on vascular hemodynamics.\textsuperscript{45}

The present study tested the hypothesis that estrogen (17β-estradiol) exerts protective effects on early changes in the synaptic connections between cone bipolar cells and RGCs associated with mild retinal ischemia and subsequent apoptotic events. Because of the profound and widespread effects that strong neurotoxic insults have on the retina, it is often difficult to differentiate between causal and secondary factors of neurotoxicity and necrotic or apoptotic events in neurons. Therefore, in the present study, we used an animal model, characterized by mild retinal ischemia, that is suitable for use in detecting early causal events leading to neurodegeneration. The use of model systems of mild ischemia rather than strong neurotoxic insults allowed us to evaluate early changes potently relevant to the prevention of neurodegeneration and acute retinal diseases.

**METHODS**

All experiments were approved by the University of North Texas Health Science Center (UNTHSC) Institutional Animal Care and Use Committee and were performed in compliance with the guidelines for the welfare of experimental animals issued by the NIH and in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**In Vivo Model of Mild Retinal Ischemia**

Retinal ischemia was induced by transient occlusion of the middle cerebral artery (MCA) in adult female Sprague-Dawley rats, as described previously.\textsuperscript{23,26,27} One-hour endovascular MCA occlusion caused a partial infarction of the ipsilateral ophthalmic artery and induced mild retinal ischemia affecting approximately 4% ± 1% of neurons in the ganglion cell layer (GCL), but not other retinal neurons, excluding secondary effects induced by presynaptic neurons of RGCs (see Fig. 5). After the 1-hour MCA occlusion, animals were reperfused for 24 hours. Blood samples were obtained at the time of MCA occlusion and after death, to determine plasma concentrations of estrogen. Rats were ovariectomized (OVX) two weeks before temporary MCA occlusion surgery. The OVX rats were divided into two groups that received either placebo replacement or estrogen replacement. All animals received placebo or hormone replacement by a single subcutaneous injection 2 hours before MCA occlusion surgery, as described previously.\textsuperscript{23,26,27,46} Estrogen was dissolved in inert corn oil (also used for placebo controls) and a dose of 100 μg/kg body weight was chosen to produce high physiological and pharmacological estrogen concentrations (50–500 pg/ml serum, with a peak concentration at 2 hours after injection).\textsuperscript{46}

**Tissue Preparation**

After ischemia treatment and 24 hours of reperfusion, rats were anesthetized deeply with halothane and decapitated. A detailed description of the preparation of the retinal tissue for light and electron microscopic immunocytochemistry is given in Brandstätter et al.\textsuperscript{47} Briefly, the eyes were opened and immersion fixed in 4% (wt/vol) paraformaldehyde (PA) in phosphate buffer (PB; 0.1 M, pH 7.4) for 30 minutes. After fixation, the retinas were dissected from the eyecup, cryoprotected in a graded series of sucrose in PB (10%, 20%, 30%) and sectioned vertically at 12-μm thickness on a cryostat. For electron microscopy, the fixation was in 0.05% glutaraldehyde and 4% (wt/vol) PA in PB for 10 minutes, followed by incubation in 4% (wt/vol) PA in PB for 40 minutes. After fixation for electron microscopy, the retinas were washed in PB and cryoprotected as described for light microscopy.

Tissues from three individual animals for each condition were used in the study, and sections with the same eccentricity (0.5 to 2 mm lateral to the optic nerve as a reference point) were compared. Orientation of the eye and the retina tissue during processing was maintained by labeling the eye with a permanent marker and by making asymmetric incision into the retina.

**Antiserum against V-1L**

An affinity-purified polyclonal antiserum against V-1L raised in rabbit against a GST-V-1L fusion protein was used in the present study. The specificity of this antiserum has been characterized.\textsuperscript{22}

**Light Microscopic Immunocytochemistry**

Immunocytochemical labeling was performed by the indirect fluorescence method, as described previously.\textsuperscript{48} The V-1L antiserum was used at a dilution of 1:1,000, and sections were incubated in the primary antiserum overnight at 4°C. The binding sites of the primary antiserum were revealed by a secondary antiserum, fluorescence-conjugated goat anti-rabbit IgG (Alexa 594; Molecular Probes, Eugene, OR) diluted 1:500. In control experiments, either the primary or secondary antiserum was omitted, or the primary antiserum was incubated with the antigen (GST-V-1L fusion protein) against which the antiserum had been raised (10-fold excess of the antigen, wt/wt) for 1 hour before application to the sections resulting in only background label in each case.

**Fluorescence TUNEL Cytochemistry**

Cells that had entered advanced stages of apoptosis were detected with a TUNEL assay performed with a fluorometric TUNEL system kit (DeadEnd; Promega, Madison, WI; fluorescein label, green fluorescence) according to the manufacturer’s instructions. The fluorescently labeled sections were counterstained with 4',6'-diamino-2-phenylindole (DAPI, blue fluorescence; Molecular Probes) to visualize the total number of cells.

**Light Microscopic Analysis**

Fluorescent images were collected digitally on a microscope set up for epifluorescence (Microphot FXA; Nikon, Melville, NY). Excitation wavelengths were selected by a computer-controlled filter wheel (Ludl Electronic Products Ltd., Hawthorne, NY). A single-emission filter was used that allowed the passage of DAPI (nuclear staining), fluorescent emission wavelengths (TUNEL staining, Alexa 488; Molecular Probes) and (immunocytochemistry, Alexa 594; Molecular Probes), depending on the excitation filter selected, which allowed multiple labels to be captured and the images overlaid without any spatial shifting of the image data. Images were captured with a cooled charge-coupled de-
vicer (CCD) camera (Photometrics SenSys; Roper Scientific, Tucson, AZ) as 10-bit, 1024 × 1024-pixel, gray-scale images. The camera and microscope automation were computer controlled (IP Laboratory Spectrum; Scanalytics, Fairfax, VA). Images were deconvolved (Microtome plugin or IP Laboratory Spectrum; Vaytek, Fairfield, IA). Deconvolved images were merged in the computer system to determine relative label distribution, count individual V-1L immunoreactive synapses in defined volumes of retina tissue, and visualize colocalization of labels (TUNEL staining). Images were reconstructed from 12 deconvolved optical sections through a 12-μm-thick vertical cryostat section and were merged in the system software. Tissues from three individual animals for each condition were used in the study, and sections with the same eccentricity (0.5–2 mm lateral to the optic nerve as a reference point) were compared. Orientation of the eye and the retina tissue during processing was maintained by labeling the sclera with a permanent marker and by making asymmetric incision into the retina. Sections from each animal (both the ipsilateral experimental tissue that had been subjected to ischemia and the contralateral control tissue were used from each animal) were analyzed after deconvolution and three-dimensional reconstruction (as just described); six Z-stacked images for each condition were quantified. Immunoreactive profiles were counted by using automated image acquisition and intensity analysis software (SimplePCI; Compix Inc., Cranberry Township, PA). Software parameters that were used to identify immunoreactive profiles (fluorescence intensity, area, volume) were kept constant for all analyses of different experimental conditions. Results are expressed in number of immunoreactive profiles per unit volume. Statistical analysis of immunoreactive labels and of the TUNEL assays was performed on computer by paired t-test (SPSS; SPSS Science Inc., Chicago, IL).

Pre-embedding Immunoelectron Microscopy

The labeling for pre-embedding immunoelectron microscopy was performed as described in detail previously. Briefly, after dissection and cryoprotection, retinas were frozen and thawed repeatedly to enhance tissue penetration by the antisera. Small pieces of retina were embedded in agar, and vertical sections (50 μm thick) were cut with a vibratome. The primary antisera was used at the same concentration and diluted in the same medium, but without Triton X-100, as used for light microscopy. Tissue sections were incubated in primary antisera for 4 days at 4°C. Binding sites of the primary antisera were visualized with biotinylated goat anti-rabbit IgG secondary antisera diluted 1:100 (Vector Laboratories, Burlingame, CA) and a peroxidase-based enzymatic detection system ( Vectastain Elite ABC kit; Vector Laboratories). The reaction product was silver intensified and gold-toned. Control experiments were performed as described for light microscopic immunocytochemistry. The analysis was performed with an electron microscope (EM910; Carl Zeiss Meditec, Thornwood, NY). Cell types were identified by using well-established anatomic criteria, such as the position of processes in defined sublayers of the inner plexiform layer, the size and morphology of neuronal processes, as well as the presence of electron-dense material and the presence and quantity of synaptic vesicles. Cone bipolar cell terminals were distinguished from rod bipolar cell terminals by their difference in size, the presence of multiple ribbon synapses and the absence of postsynaptic ganglion cell processes at rod bipolar cell terminals.

RESULTS

V-1L Expression in the Retina

V-1L immunoreactivity was present in both plexiform layers of the rat retina (Fig. 1). The immunoreactivity appeared predominantly as punctate staining indicative of clustering of V-1L at synapses. The presence of immunoreactive profiles of different sizes in the outer plexiform layer (OPL) indicates the expression of V-1L at both rod and cone photoreceptor terminals (Fig. 1), as has been shown previously for other synaptically localized proteins.

We further analyzed the distribution of the V-1L immunoreactivity to identify the subcellular localization at the ultrastructural level. To detect V-1L immunoreactivity at the subcellular level, a very sensitive immunocytochemical method combining peroxidase staining with silver intensification and gold toning of the label was used. Because of the diffusion of 3,3′-diaminobenzidine (DAB) reaction product, the spatial resolution was lower than when using gold-coupled secondary antibodies. However, the current method enabled us to localize low concentrations of antigen with an antisera that exhibits a high sensitivity to alterations in its antigen due to the fixation and embedding procedures.

We detected V-1L immunoreactivity specifically localized to the postsynaptic elements at bipolar cell dyads in the inner plexiform layer (IPL) of rat retina. Ganglion cells expressed V-1L at their contacts to cone bipolar cells both in the ON- and in the OFF-pathway (Figs. 2A, 2B, respectively). This specific immunoreactivity pattern is consistent with the clustering function of V-1L at synapses and with similar distribution patterns of V-1L-associated proteins, such as group I metabotropic glutamate receptors. Typically, both postsynaptic processes at the cone bipolar cell ribbon synapses were labeled for V-1L (Fig. 2). At cone bipolar cell...
Effect of MCA Occlusion on Neurons in the GCL

In our experiments, we used a model for cerebral ischemia, temporary endovascular MCA occlusion, which causes a partial infarction of the ipsilateral ophthalmic artery and leads to mild retinal ischemia. To further characterize the effect of the mild retinal ischemia model caused by temporary MCA occlusion we investigated the neurotoxic effects on retinal neurons using the TUNEL staining technique. Apoptotic cells were identified in vertical sections of control (eye contralateral to the ischemic insult), ischemic and estrogen-treated ischemic
FIGURE 4. Effects of mild retinal ischemia and estrogen treatment on the number of V-1L-immunoreactive synapses in the IPL of the rat retina. (A–C) Vertical cryostat sections through rat retinas show the distribution of V-1L immunoreactivity under different experimental conditions after induction of mild retinal ischemia. All four different experimental conditions are shown: retinas contralateral (A) or ipsilateral (B) to the ischemic lesion from OVX rats that had received placebo replacement therapy or retinas ipsilateral (C) or contralateral (D) to the ischemic lesion from OVX rats that had received estrogen replacement therapy. The strong punctate immunoreactivity throughout the IPL was reduced in the ischemic condition (B) when compared with control (A) or estrogen treated animals (C, D). The general distribution of immunoreactivity was not changed in any of the experimental conditions. No reduction in the number of either large or small immunoreactive profiles in the OPL (presumably cone and rod photoreceptor synapses, respectively) were observed. The images were reconstructed from 12 deconvolved optical sections through a 12-μm-thick vertical cryostat section and were merged in the imaging system software. These Z-stack images allowed visualization of V-1L immunoreactivity in a three-dimensional space of a defined volume of retina tissue. (E) The effects of mild retinal ischemia and estrogen treatment on the number of V-1L-immunoreactive synapses in the IPL of the rat retina are graphically summarized after quantification of six Z-stack images for each condition. Four different experimental conditions are shown: retinas contralateral (control) or ipsilateral (ischemia) to the ischemic lesion from OVX rats that had received placebo replacement therapy or retinas ipsilateral (ischemia + estrogen) or contralateral (control + estrogen) to the ischemic lesion from OVX rats that had received estrogen replacement therapy (dose, 100 μg/kg body weight). Data are presented as mean ± SEM for each condition and were compared with control data for statistical analysis by paired t test. A significant change in the number of V-1L-immunoreactive synapses in the IPL was observed only in ischemic retinas (P < 0.01). Estrogen replacement therapy prevented this ischemia-induced loss of V-1L-positive synapses. Scale bar, (A–D) 25 μm.

Neuroprotection in the Inner Retina

Effects of Mild Retinal Ischemia on the Number of Vesl-1L/Homer 1c Immunoreactive Synapses

The distribution of Homer was quantitatively analyzed in the IPLs of retinas of OVX rats that had experienced retinal ischemia and were treated with a prolonged regimen of 17β-estradiol. The number of synapses in a given volume of all sublayers of the IPL was compared between animals with retinal ischemia, with or without estrogen treatment and controls. The number of V-1L-positive synapses was significantly decreased by approximately 25% in animals with retinal ischemia (Fig. 4B) compared with control animals without ischemia (contralateral to the ischemic insult: Fig. 4A; contralateral to the ischemic insult after estrogen treatment: Fig. 4D) and this effect was reversed by estrogen treatment (Fig. 4C). Additional control experiments included animals that had undergone sham ischemia with and without estrogen treatment and the contralateral control eyes for all treatment conditions. The quantitative assessment of the effects of retinal ischemia with or without estrogen treatment on density of V-1L-positive synapse in defined volumes of rat retina IPL is summarized in Figure 4E. The loss of V-1L-immunoreactive synapses was uniform across the different sublaminas of the IPL, indicating

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that both ON- and OFF-pathways are affected similarly. Only under ischemic conditions was a significant difference between the morphologic and physiological changes, which was attributable to the fact that the model mimics a slow-onset neurodegenerative disease and acute neurotoxic insults to RGCs. This difference in the efficacy of estrogen may be attributable to two factors: Ligating the optic nerve can lead to permanent or transient damage of the optic nerve and/or the axons of RGCs, as well as to the impairment of axonal transport of RGCs, which has been shown to play a role in RGC function, viability, and survival. This severe damage, which has been found to be associated with irreversible morphologic changes, is potentially unresponsive to estrogen’s neuroprotective effects.

In the present study, cell death was solely induced by ischemia and the subsequent signaling events associated with decreased blood supply to the retina. Estrogen has been shown to be a potent neuroprotectant under these conditions of low oxygenation and supply of nutrients (e.g., leading to oxidative stress). A second factor may be related to the fact that approximately 4% of neurons in the GCL, approximately 23% of V-1L-positive synapses in the IPL, and presumably no interneurons were affected by the mild retinal ischemia, which may indicate that the model mimics a slow-onset neurodegenerative disease similar to glaucoma and retinal ischemia during diabetic retinopathy, sickle cell retinopathy, or stenosis of the carotid artery that is responsive to neuroprotection by estrogen.

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References


