Endothelin Receptors in Light-Induced Retinal Degeneration

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Excessive light exposure leads to retinal degeneration in albino animals and exacerbates the rate of photoreceptor apoptosis in several retinal diseases. In previous studies we have described the presence of endothelin-1 (ET-1) and its receptors (ET-A and ET-B) in different sites of the mouse retina, including the retinal pigment epithelium, the outer plexiform layer (OPL), astrocytes, the ganglion cell layer (GCL), and vascular endothelia. After light-induced degeneration of photoreceptors, endothelinergic structures disappear from the OPL, but ET-1 and ET-B immunoreactivities increase in astrocytes. Here, we present novel observations about the course of light-induced retinal degeneration in BALB- c mice exposed to 1500 lux during 4 days with or without treatment with tezosentan, a mixed endothelinergic antagonist. Retinal whole mounts were immunostained with anticleaved caspase-3 (CC-3) serum to identify apoptotic photoreceptor cells within the outer nuclear layer (ONL). Glial activation was measured as glial fibrillary acidic protein (GFAP) immunoreactivity in retinal whole mounts and in Western blots from retinal extracts. Tezosentan treatment significantly reduced both the number of CC3-immunoreactive cells and GFAP levels, suggesting that inhibition of endothelinergic receptors could play a role in photoreceptor survival. Using confocal double immunofluorescence, we have observed that ET-A seems to be localized in bipolar cell dendrites, whereas ET-B is localized in horizontal cells. Our observations suggest the existence of an endothelinergic mechanism modulating synaptic transmission in the OPL. This mechanism could perhaps explain the effects of tezosentan treatment on photoreceptor survival. Exp Biol Med 231:1095–1100, 2006

Key words: apoptosis; caspase 3; endothelin; glial activation; photoreceptor; retina

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Introduction

Excessive light exposure leads to retinal degeneration in albino animals and exacerbates the rate of photoreceptor apoptosis in several retinal diseases (1, 2). Different apoptotic pathways are involved in photoreceptor death (3, 4). Degeneration is accompanied by reactive gliosis (5), as evidenced by increased expression of filamentous proteins such as glial fibrillary acidic protein (GFAP) and vimentin. Retinal astrocytes normally express GFAP, whereas Müller glial cells only do so after photoreceptor degeneration (6, 7).

We have recently described the presence of endothelin-1 (ET-1) and its receptors (ET-A and ET-B) in different sites of the mouse retina, including the retinal pigment epithelium (RPE), the outer plexiform layer (OPL), astrocytes, the ganglion cell layer (GCL), and vascular endothelia (8). After light-induced degeneration of photoreceptors, endothelinergic structures disappear from the OPL, but increased ET-1 and ET-B immunoreactivities are present in astrocytes (8). Similar overexpression of ET-1 and ET-B is observed in a rat model of glaucoma (9). Acute light damage also leads to increased expression of ET-2 transcripts, presumably located in photoreceptors (10). On the other hand, ET-1 modulates anterograde fast axonal transport, which is essential for maintaining synaptic function and neuronal survival (11, 12). Therefore, the aim of this study was to analyze whether an endothelinergic antagonist would modify the course of light-induced degeneration.

Materials and Methods

Animals. Experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Male BALB/c mice were bred under a 12:12 hr light:dark cycle each with maximum illumination levels of 60 lux. Five animals 35–45 days of age were used for each experimental condition.

At the start of the experimental period (1200 hrs), mice were left in complete darkness. After 24 hrs, some animals were returned to basal illumination conditions, whereas others were transferred to transparent glass boxes placed under a light tube (OSRAM L58W/daylight; Osram, Munich, Germany). These were placed at the same distance from the light source and illumination at the level of the box floor was 1500 lux. Tezosentan (Actelion Pharmaceuticals,

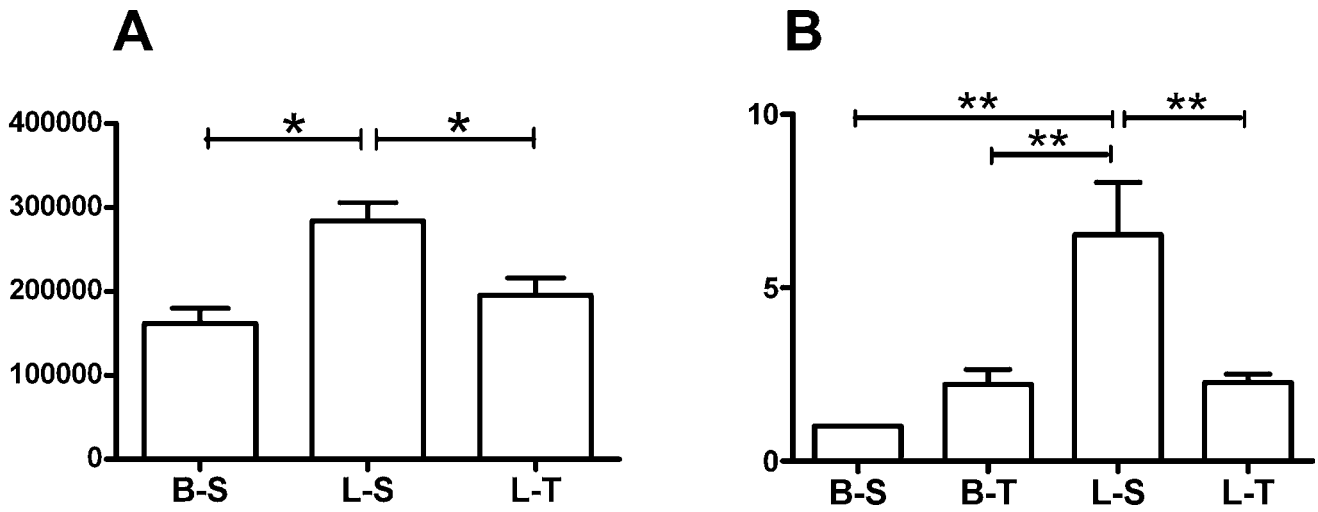


Figure 1. GFAP immunoreactivity under basal or high illumination conditions during 4 days. B-S, 60 lux, saline injections; B-T, 60 lux, tezosentan injections; L-S, 1500 lux, saline injections; L-T, 1500 lux, tezosentan injections. (A) Bars show GFAP immunoreactive areas (Px2) in samples obtained from the temporal hemisphere of retinal whole mounts. The area in L-S retinas is significantly higher than in the other two conditions ($P < 0.05$). (B) Quantitative analysis of GFAP by Western blotting. Bars represent the average of five tests in which optical density (O.D.) values of the different experimental points were normalized to the O.D. value of B-S retinas. O.D. values of L-S retinas were significantly higher than in the remaining conditions ($P < 0.01$).

Switzerland), a dual endothelin receptor antagonist (13, 14), was given daily by subcutaneous injection at noon (10 mg/kg/day) during 4 days. Control animals received the same number of saline injections. Four groups were compared: B-S, 60 lux, saline injections; B-T, 60 lux, tezosentan injections; L-S, 1500 lux, saline injections; and L-T, 1500 lux, tezosentan injections. A different experimental group was exposed to 1500 lux during 24 hrs without any pharmacologic treatment.

Immunohistochemical Procedures. Retinal whole mounts were obtained from mice perfused through the heart with a 4% paraformaldehyde solution in 0.1 *M* phosphate buffer (8). They were immunostained with a rabbit serum against cleaved caspase-3 (CC-3, Cell Signaling Technology Inc., Beverly, MA) to identify dying photoreceptor cells within the outer nuclear layer (ONL) (15). Other whole mounts were immunostained with a monoclonal anti-GFAP (BioGenex, San Ramon, CA) to evaluate gliosis at the level of the inner retina surface. For each experiment, whole mounts or sections from treated and nontreated retinas were processed simultaneously.

Cryosections were obtained from eyes that were immediately frozen after enucleation. These sections were briefly immersed (10 mins) in paraformaldehyde fixative before immunostaining with rabbit sera against ET-A or ET-B (Alomone Labs, Jerusalem, Israel) in combination with monoclonal antibodies against the synaptic protein SV2 (Developmental Studies Hybridoma Bank, Iowa City, IA) or a phosphorylated epitope of the 200-kDa neurofilament (clone RT97, Chemicon, Temecula, CA).

Whole mounts and sections labeled with fluorescein-5-isothiocyanate–conjugated goat antirabbit IgG or goat lissamine rhodamine-conjugated anti-mouse IgG (Jackson Immunoresearch Laboratories, West Grove, PA) were observed either with a Nikon E800 fluorescent microscope (Nikon, Tokyo, Japan) with a Nikon DN100 digital camera or with the Laser Scanning System Radiance 2000 (Bio-Rad, Hemel Hempstead, UK) using the 488 line of the argon laser followed by the 543 line of a helium-neon laser and the emission filters HQ515/30 and HQ590/70, respectively. Optical sections separated by 1 μm were performed in the z axis and processed using Confocal Assistant Software).

Negative controls were made replacing the primary antibody by its diluent. Immunoreactivity of ET-A and ET-B antisera were tested by preabsorption with the appropriate antigenic peptide.

Image Analysis. Scoring procedures were carried out by a blinded operator and were limited to the temporal hemisphere, a region which is known to be especially sensitive to light damage (16, 17). For estimation of CC-3 positive cells, digital images (×40, $n = 10$) were collected from five retinas from different animals and positive cells were scored by direct observation of each image. Results are given as mean ± SEM. Values were compared using a one-tailed Mann-Whitney test.

Changes in GFAP immunofluorescence at the level of the astrocytic layer were measured in images (×40, $n = 20$) collected from the same region. A KS400 (Kontron System, Zeiss Vision, Oberkochen, Germany) was used to segment immunoreactive areas. Data were compared using analysis of variance followed by the Newman-Keuls multiple comparison test.

Western Blot Analysis. Retinas were homogenized in an extraction buffer containing 10 m*M* Tris-HCl with 2 m*M* EDTA, 150 m*M* NaCl, 1% Triton X-100, and protease inhibitor cocktail (Sigma-Aldrich, St Louis, MO). Extracts

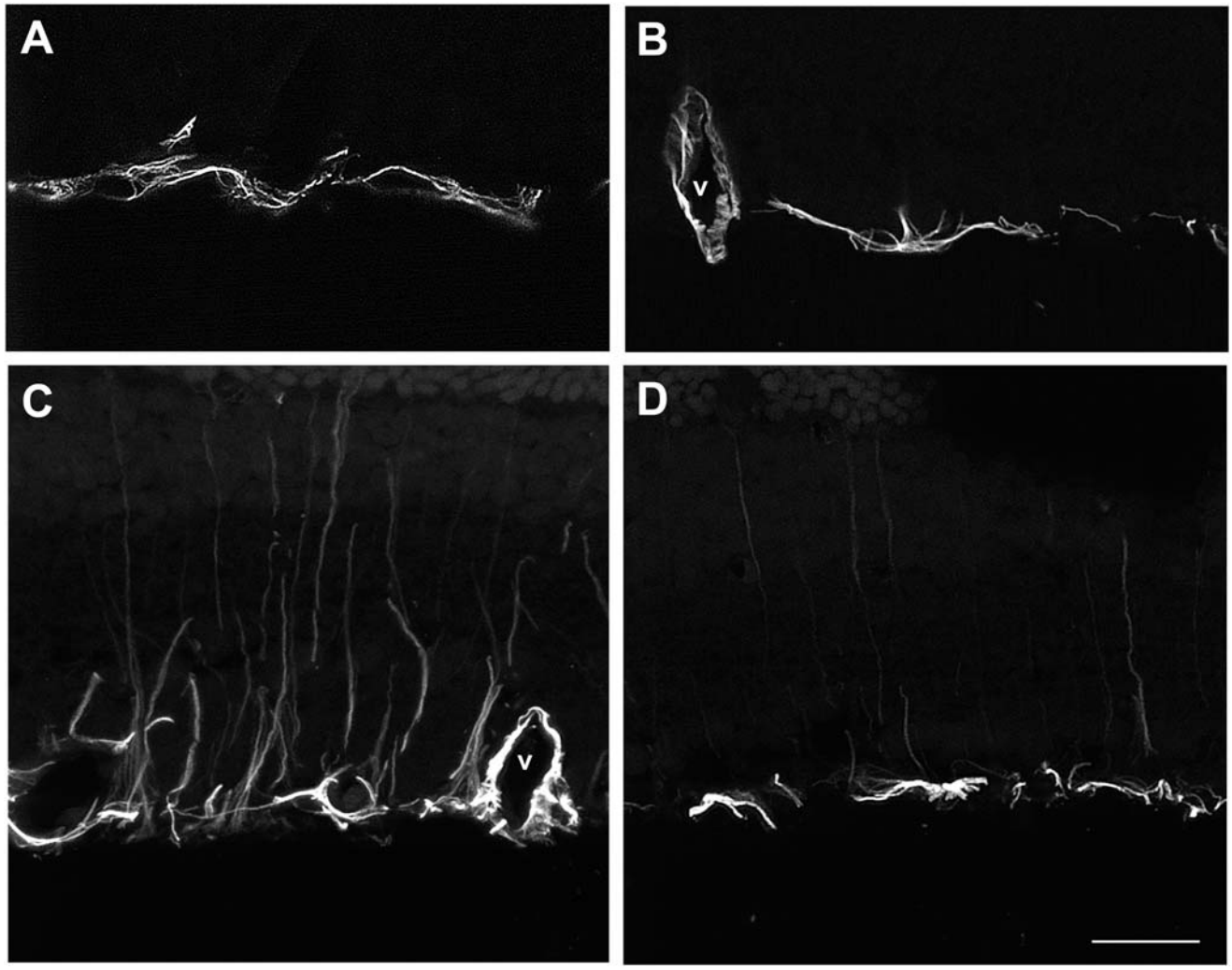


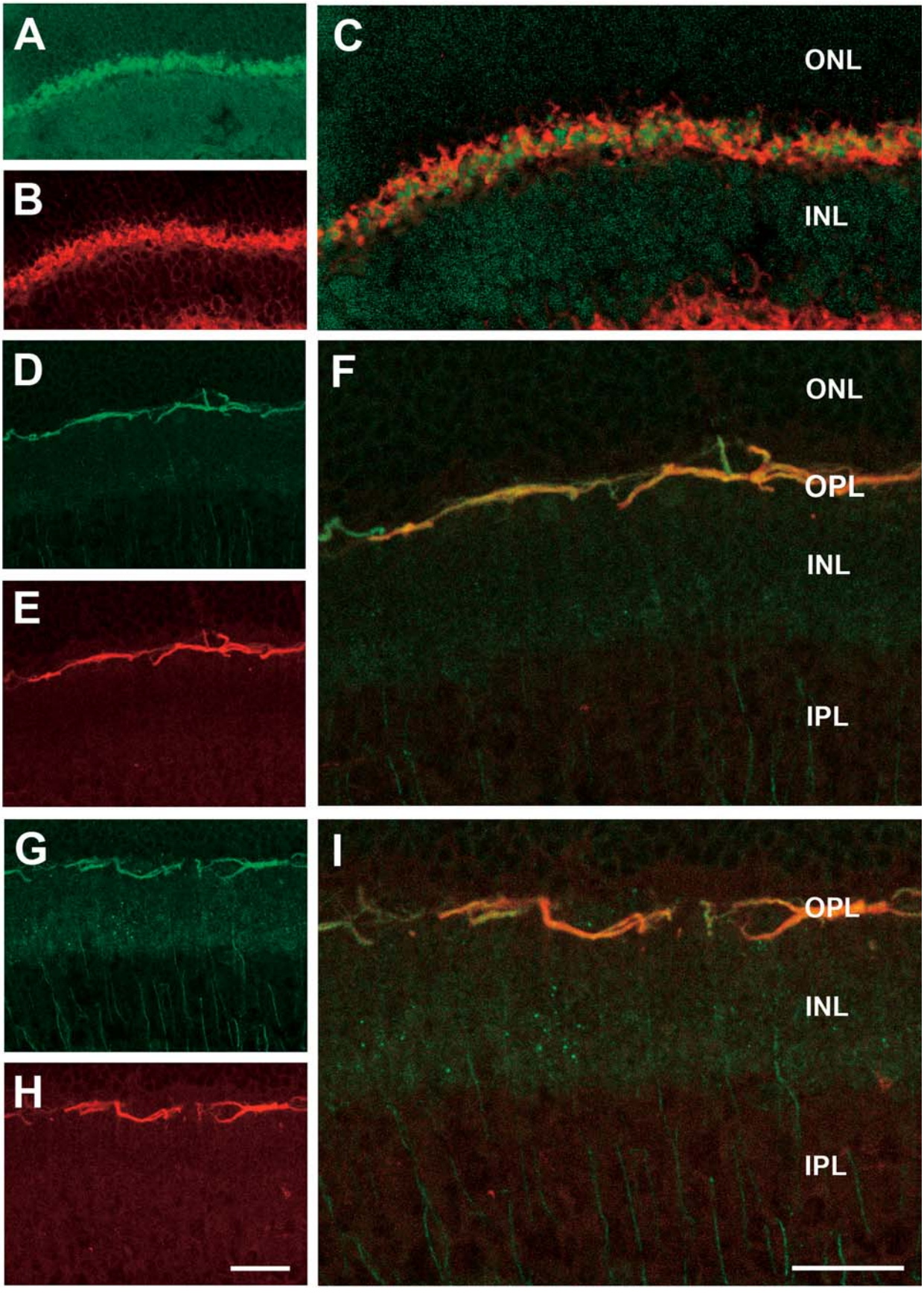
Figure 2. GFAP immunostaining in cryosections of the temporal hemisphere from different experimental groups. (A) In B-S retinas, immunoreactivity is selectively localized in astrocytes. (B) B-T retinas show similar distribution, but there is an enhancement of perivascular processes. v, vessel. (C) L-S retinas exhibit strong immunoreactivity in the astrocyte layer and perivascular processes. Müller cell radial processes are also positive. (D) In L-T retinas, there is still strong astrocyte GFAP labeling, but Müller cell immunoreactivity has almost disappeared. Bar = 25 μm.

(30 μg of protein per lane) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred (100 v, 90 mins) onto a nitrocellulose filter using standard techniques. Equal sample loading for electrophoresis was confirmed by Bradford protein assay (Sigma-Aldrich) and equal transfer to the membrane was confirmed by Ponceau S staining. Immunoblotting was performed with the anti-GFAP monoclonal antibody. The membrane was further incubated with biotinylated anti-mouse IgG, extravidin-peroxidase (Sigma-Aldrich) and developed with an enhanced chemiluminescence detection kit (ECL, Amersham Pharmacia Biotech, Piscataway, NJ). Optical density was estimated using Scion Image Beta 4.02 Win software. Statistical comparison was made with analysis of variance for repeated measures followed by the Newman-Keuls multiple comparison test.

Results

CC-3. CC-3 immunoreactivity was not detected in retinas exposed to basal levels of illumination, notwithstanding their previous treatment with saline or tezosentan. By contrast, numerous CC-3–positive cells were found in light-exposed retinas. In saline-injected animals, the number of CC-3–positive cells (27.3 ± 3.0 cells/field) almost doubled those scored in tezosentan-treated animals (17.0 ± 2.7 cells/field). The difference was statistically significant ($P < 0.01$).

Expression of GFAP. Measurement of GFAP immunoreactivity in retinal whole mounts demonstrated a large increase of this immunoreactivity in animals exposed to 1500 lux receiving saline injections. By contrast, light-exposed retinas from tezosentan-treated mice were not different from those of animals under basal illumination conditions (Fig. 1A).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Similar differences were detected by Western blot analysis (Fig. 1B). The lowest GFAP levels were observed in animals under basal illumination conditions receiving saline, whereas the highest levels corresponded to animals submitted to 1500 lux and receiving saline. Tezosentan treatment significantly reduced the GFAP levels in light-exposed animals so they were not different from those in retinas under basal illumination conditions.

Cryosections (Fig. 2) demonstrated that, in animals kept under basal illumination conditions, GFAP immunoreactivity could only be detected in the astrocyte layer. In these animals, tezosentan administration during 4 days produced a minimal reaction in perivascular glial processes. Light-exposed retinas showed a large increase of astrocyte GFAP immunoreactivity. Moderate immunoreactivity also appeared in Müller radial glial processes, but only at the upper portion of the temporal hemisphere. Tezosentan treatment of light-exposed animals not only decreased astrocyte GFAP immunostaining, but nearly abolished GFAP expression in Müller glial radial cells.

Endothelinergic Receptors in the Outer Plexiform Layer. Effects of tezosentan might be explained by mechanisms operating directly or indirectly on photoreceptors. Therefore, we made further studies to understand the localization of endothelinergic receptors within the OPL, containing synaptic contacts among photoreceptor terminals, bipolar cell dendrites, and horizontal cell processes. In animals exposed to basal illumination levels, immunofluorescent labeling with ET-A antiserum demonstrated a layer of regularly distributed varicosities (Fig. 3A). ET-A immunofluorescence did not colocalize with SV2, a marker of photoreceptor terminals (Fig. 3B and C), or with RT-97, a marker for horizontal cells. Therefore, it follows that ET-A would be localized in bipolar cell dendrites. On the other hand, ET-B immunofluorescence was selectively colocalized with RT-97 immunofluorescence, indicating its presence in horizontal cells (Fig. 3D–F). Müller cells exhibited little or no ET-B immunofluorescence in animals exposed to basal levels of illumination (Fig. 3D).

No changes could be detected in ET-A or ET-B immunofluorescence at the OPL after exposure to 1500 lux during 24 hrs. However, the same exposure increased ET-B immunofluorescence in Müller cells (Fig. 3D–I).

Discussion

These results provide experimental evidence that stimulation of endothelinergic receptors may modulate photoreceptor survival and glial activation. Tezosentan decreased the number of CC-3 cells in the ONL, suggesting that blockade of endothelinergic receptors reduced the apoptotic response to light exposure. Besides, immunohistochemical and Western blot evidence indicates that tezosentan treatment also downregulated GFAP expression in astrocytes and Müller cells of light-exposed animals. Decrease of the glial response could probably reflect two different mechanisms: the reduction of photoreceptor death shown by CC3 labeling and the inhibition of ET-B receptors in astrocytes, which are upregulated in the degenerating retina (8).

A slight increase of perivascular GFAP immunoreactivity was observed in mice treated with tezosentan under basal illumination conditions. This probably reflects the increase in circulating ET-1 levels (13) that follows blockade of ET-B receptor scavenging functions (18). It also points to the importance of ET-1 in the maintenance of endothelial-astrocytic barriers (19).

Both ET-A and ET-B were selectively localized in different structures of the photoreceptor triad synapse, with ET-A probably present in bipolar dendrites and ET-B in horizontal cell processes. Although further studies are required, previous observations suggest that ET-1 immunoreactivity is present in photoreceptors (8). Moreover, by *in situ* hybridization, ET-2 transcripts localize to photoreceptors (10). Endothelins could play a role in photoreceptor synaptic transmission, and this would require a tight control of endothelin extracellular concentration. Scavenging by ET-B receptors in horizontal cells could be adequate under basal conditions, whereas under extreme illumination levels, additional ET-B receptors would be activated in Müller cells and astrocytes (8) to maintain proper extracellular endothelin levels. Modulation of synaptic transmission might affect photoreceptor survival, perhaps by regulating glutamate release (20).

A role of the RPE in endothelin-mediated photoreceptor survival cannot be excluded, because this retinal layer contains ET-1, prepro-ET1, and ET-A immunoreactivities (8, 21). Effects of tezosentan could also depend on endothelial mechanisms, because both ET-A and ET-B receptors are present in retinal vascular endothelium (8).

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Figure 3. Immunoreactivity of endothelinergic receptors in the OPL. (A–F) Basal illumination conditions; (G–I) 1500 lux during 24 hrs. (A) ET-A immunofluorescence appears in fine varicosities regularly distributed along the OPL. (B) Immunofluorescence of the synaptic protein SV2, a photoreceptor marker. (C) Merged image demonstrates that ET-A and SV2 labels occupy different sites, suggesting that ET-A would be localized in bipolar dendrites. (D) In the OPL, ET-B is exclusively present in thick processes that show the typical branching of horizontal cells. A few Müller cell processes are weakly labeled in the inner retina. (E) RT-97 immunofluorescence is restricted to the OPL and shows the same distribution pattern of ET-B. (F) Perfect colocalization of ET-B and RT-97 demonstrates that ET-B is present in horizontal cells. (G) After light exposure, ET-B immunofluorescence in the OPL is similar to that shown in (D), whereas ET-B–labeled Müller cell processes are more numerous than under basal illumination conditions. Notice that ET-B labeling is always stronger in horizontal than in Müller cells. (H) RT-97 labeling of horizontal cells. (I) The merged image confirms that horizontal cells exhibit ET-B labeling. Bar = 25 μm.

GFAP upregulation after light injury seems to be associated with a fast increase of glial ET-B receptors, both in astrocytes (8) and Müller cells (10). ET-1 overload induces astrocyte hypertrophy associated with uncoupling of the astrocytic gap junctional network (22). Infusion of an ET-B–selective antagonist attenuates the increase of astrocytes after injury of the brain cortex, indicating that induction of reactive astrocytes depends on the activation of ET-B receptors (23). Excess ET-1 released by injured glial cells can be compensated by scavenging ET-B receptors (18), and it has been suggested that blockade of these receptors after central nervous system injury might modulate glial scar formation, providing a more permissive substrate for neuronal survival and regeneration (24).

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