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ORIGINAL ARTICLE

Bacterial lipopolysaccharide protects the retina from light-induced damage

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Abstract

Light-induced damage is a widely used model to study retinal degeneration. We examined whether bacterial lipopolysaccharide (LPS) protects the retina against light-induced injury. One day before intense light exposure for 24 h, rats were intravitreally injected with LPS in one eye and vehicle in the contralateral eye. At several time points after light exposure, rats were subjected to electroretinography and histological analysis. Bax, Bcl-xL, p-Akt, and p-Stat3 levels were assessed by Western blotting, and retinal thiobarbituric acid reactive substances levels were measured as an index of lipid peroxidation. One group of animals received injections of dexamethasone, aminoguanidine (an inducible NOS inhibitor), 5-hydroxydecanoic acid (a mitochondrial K^+/ATP channel blocker), or wortmannin [a phosphoinositide-3-kinase (PI3K) inhibitor] in order to analyze their effect on the protection induced by LPS. LPS afforded significant morphologic and functional protection in eyes exposed to intense light. Light damage induced an increase in mitochondrial Bax/cytoplasmic Bax ratio, and lipid peroxidation which were prevented by LPS. Dexamethasone and wortmannin (but not aminoguanidine or 5-hydroxydecanoic acid) prevented the effect of LPS. Moreover, wortmannin prevented the effect of LPS on p-Akt levels. These results indicate that LPS provides retinal protection against light-induced stress, probably through a PI3K/Akt-dependent mechanism.

Keywords: light damage, LPS, preconditioning, retina.

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Ischemia/reperfusion (I/R) injury, which plays an important role in the pathophysiology of several eye diseases, ultimately leads to retinal cell death. An option to increase the retina's resistance to such injury is the phenomenon of ischemic preconditioning (IPC), which consists in the application of brief episodes of I/R before a prolonged ischemic event. It has been demonstrated that IPC induces robust tolerance against retinal ischemic damage (Roth *et al.* 1998). Preconditioning differs from neuroprotection in several aspects. The prophylactic approaches to neuroprotection in essence represent acute or chronic pretreatments in which the drug is present when damage occurs, whereas in preconditioning, the singular or final treatment precedes the deleterious event by many hours or days, and the obligatory genomic reprogramming that largely defines the tolerant phenotype is promoted. Moreover, the

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Abbreviations used: Akt/PKB, protein kinase B; AMG, aminoguanidine; dexa, dexamethasone; ERG, electroretinogram; GCL, ganglion cell layer; 5HD, 5-hydroxydecanoic acid; INL, inner nuclear layer; IPC, ischemic preconditioning; IPL, inner plexiform layer; I/R, ischemia/reperfusion; Jak, Janus-associated kinase; LPS, bacterial lipopolysaccharide; mK^+/ATP channels, mitochondrial K^+/ATP channels; NOS, nitric oxide synthase; ONL, outer nuclear layer; PI3K, phosphatidylinositol 3-kinase; PS, photoreceptor segments; Stat3, signal transducers and activators of transcription 3; TBARS, thiobarbituric acid reactive substances; WT, wortmannin.

concept of preconditioning involves the use of subtoxic stress levels to provoke an endogenous protective response, which is not the case for neuroprotection (Gidday 2010). It has been postulated that differences in the intensity, duration, and/or frequency of a particular stress stimulus determine whether that stimulus is too weak to elicit a response, sufficient in magnitude to serve as a preconditioning trigger, or too robust and therefore harmful (Gidday 2006). In that context, it was demonstrated that while toll-like receptor 4 is activated in response to cerebral ischemia leading to substantial brain damage, mild activation of toll-like receptor 4 by preconditioning with low dose exposure to toll-like receptor ligands such as bacterial lipopolysaccharide (LPS) prior to cerebral ischemia, dramatically improves ischemia outcomes (Vartanian *et al.* 2011). Moreover, preconditioning with LPS reduces myocardial infarct size after I/R (Schober *et al.* 2008), and a single intravitreal injection of 1 μg of LPS provides protection against retinal I/R injury (Franco *et al.* 2008). The intracellular mechanisms related to the retinal effects of LPS involve different signals such as protein kinase C, protein-tyrosine kinases, mitogen-activated protein kinases, Janus-associated kinase (JAK), Signal transducers and activators of transcription (Stat), and NF-kB, among many others (Jang *et al.* 2007), but the mechanisms involved in the protection induced by low doses of LPS are not fully elucidated.

Ischemic preconditioning (IPC) and preconditioning with bright light protect the retina against light-induced damage (Liu *et al.* 1998; Casson *et al.* 2003). Retinal photoreceptor cells are uniquely adapted to function over a wide range of ambient light conditions. However, prolonged intense visible light exposure can lead to cell damage which can progress to cell death and loss of vision. Excessive light exposure leads to photoreceptor degeneration (Noell *et al.* 1966; Shahinfar *et al.* 1991) and can be a risk factor for onset and progression of age-related macular degeneration, and possibly some forms of retinitis pigmentosa (Cruickshanks *et al.* 1993). Notwithstanding, studies that support the hypothesis that sun exposure or excess light is a risk factor for age-related macular degeneration are not conclusive. In that context, there are several studies showing that sun exposure is not a risk factor (Darzins *et al.* 1997; Khan *et al.* 2006). Although ischemic damage and light injury may share some common mechanisms (such as calcium, NO, and oxidative stress), they seem to diverge in several aspects; for example, light damage depends on a functional visual cycle (reviewed by Wenzel *et al.* 2005), which seems not to be the case for I/R damage, suggesting a particular damaging pathway induced by light, but not by other deleterious stimuli. The exact mechanism of light-induced damage is still not fully understood; however, photoreceptor degeneration induced by light exposure has been widely used to study the capability of photoreceptor protection by different strategies (LaVail *et al.* 1992; Organisciak and Winkler 1994; Wen *et al.* 1996). Since preconditioning could be a strategy to protect the retina from light-induced degeneration, the aim of the present study was to examine whether an intravitreal injection of LPS induces functional and histological protection against light-induced retinal damage.

Materials and methods

Animals

Male *Wistar* rats (average weight, 250 ± 40 *g*) were housed under controlled conditions of humidity and temperature (21 ± 2°C), and under a 12 h light (200 lx) : 12 h dark lighting schedule (lights on at 07.00 hours), in a standard animal room with food and water *ad libitum*. Animal use procedures were in strict accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

LPS injections

Animals were anesthetized with ketamine hydrochloride (150 mg/kg) and xylazine hydrochloride (2 mg/kg) administered intraperitoneally. A drop of proparacaine (0.5%) was administered in each eye for local anesthesia. Rats were submitted to different treatments depicted in Fig. 1. With a Hamilton syringe and a 30-gauge needle, 4 μL of sterile pyrogen-free saline containing 1 μg LPS from *Salmonella typhimurium* (catalog # L-7261; Sigma Chemical Co., St.Louis, MO, USA) was injected into one eye of anesthetized rats, while an equal volume of vehicle (saline solution, 0.9% NaCl in sterile water, pH 7) was injected in the fellow eye. Injections were

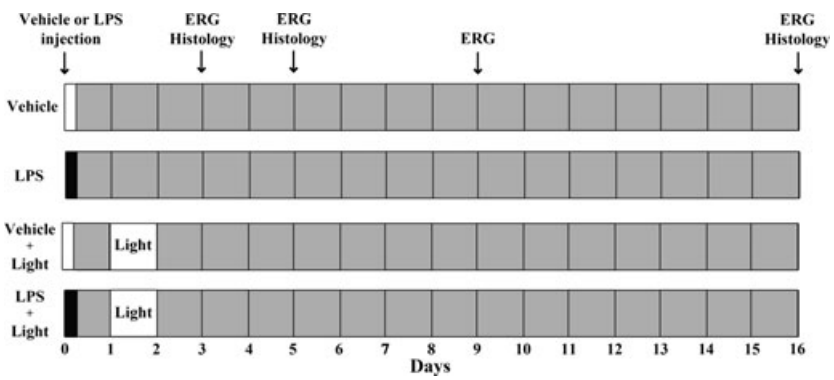

Fig. 1 Experimental groups and protocols for functional and histological studies. One day after intravitreal injection of vehicle or bacterial lipopolysaccharide (LPS), animals were exposed to intense light for 24 h. Electroretinograms were recorded at 1, 3, 7 and 14 days after light exposure. Retinal histology examination was performed at days 1, 3, and 14 after light treatment.

applied at 1 mm of the limbus and the needle was left in the eye for 60 s; this small volume prevented the increase in intraocular pressure and intravitreous volume loss.

Light damage

One day after intravitreal injections of vehicle or LPS, one group of rats were placed in an open acrylic box of 60 cm × 60 cm × 60 cm with 12 halogen lamps (12 V, 50 W each) located on top, with free access to food and water, as previously described (Piehl *et al.* 2007), whereas control animals were maintained under a 12 h light (200 lx) : 12 h dark lighting schedule. There were no areas of shadow in the cages and pupils were not dilated. Light exposure commenced consistently at 9.00 a.m., and was achieved using a light source positioned above the cages, at an intensity of 10 000 lx at the cage floor, as previously described (Piehl *et al.* 2007). Lighting level was determined using an analog Gossen illuminance meter. Temperature was monitored and the air-conditioned room was maintained at 24°C to avoid high temperature which may accelerate retinal damage (Organisciak *et al.* 1995). Light exposure was maintained over a period of 24 h, after which the animals were immediately returned to dim cyclic conditions for the post-exposure period. Animals were kept in dim light conditions following intense light exposure for a maximum period of 14 days. In the present study, age-matched vehicle-injected eyes served as the control group because in preliminary studies we found that in comparison with intact animals, the injection of vehicle did not affect retinal function and histology in non light-exposed eyes, or the effect of light on retinal function and histology (data not shown).

Preconditioning inhibitors

In order to test the involvement of immune responses in the effect of LPS, dexamethasone (3 mg/kg, catalog # D1756; Sigma Chemical Co.,) was injected 30 min before the injection of vehicle or LPS, as previously described (Schober *et al.* 2008). To assess the involvement of inducible nitric oxide synthase (iNOS) in the retinal protection afforded by LPS, aminoguanidine (AMG, catalog # 396494; Sigma Chemical Co.,) was intraperitoneally administered (100 mg/kg) 30 min before and 6 h after LPS. The timing and dose of AMG administration was selected on the basis of a previous report (Franco *et al.* 2008). In order to examine the involvement of mitochondrial K^+/ATP (mK^+/ATP) channels, the mK^+/ATP channel blocker 5-hydroxydecanoic acid (5HD, catalog # H-135; Sigma Chemical Co.,) was intraperitoneally injected (40 mg/kg) 15 min before LPS, as previously described (Roth *et al.* 2006). The involvement of phosphatidylinositol 3-kinase (PI3K) was examined by injecting 2 μl wortmannin (WT, catalog # W-1628; Sigma Chemical Co.,) (0.2 mM) which was intravitreally co-injected with LPS (2 μL, 0.5 μg/μL) or vehicle, as previously described (Kermer *et al.* 2000).

Electroretinography

Electroretinographic activity was assessed before, and at several time points after light exposure, as previously described (Franco *et al.* 2008; Fernandez *et al.* 2009). After 6 h of dark adaptation, rats were anesthetized under dim red illumination. Phenylephrine hydrochloride and tropicamide were used to dilate the pupils, and the cornea was intermittently irrigated with balanced salt solution to maintain the baseline recording and to prevent keratopathy. Rats were placed facing the stimulus at a distance of 20 cm. A reference electrode was placed through the ear, a grounding electrode was attached to the tail, and a gold electrode was placed in contact with the central cornea. A 15 W red light was used to enable accurate electrode placement. This maneuver did not induce detectable changes in dark adaptation and was switched off during the electrophysiological recordings. Electroretinograms were recorded from both eyes simultaneously and 10 responses to flashes of unattenuated white light (5 ms, 0.2 Hz) from a full-field/Ganzfeld stimulator (light-emitting diodes) set at maximum brightness (9 cd s/m^2 without filter) were amplified, filtered (1.5-Hz low-pass filter; 1000-Hz high-pass filter, notch-activated) and averaged (Akonic BIO-PC, Buenos Aires, Argentina). The a- and b-wave amplitude and latencies were measured as previously described (Franco *et al.* 2008; Fernandez *et al.* 2009). Electrophysiological responses were averaged for each run. Runs were repeated three times with 5-min intervals to confirm consistency and the mean of these three runs was used for subsequent analysis.

Retinal histology

One, 3 and 14 days after light exposure rats were killed and their eyes were immediately enucleated, immersed for 24 h in a fixative containing 4% formaldehyde in 0.1 M phosphate buffer (pH 7.2) and embedded in paraffin. The eyes were sectioned (5 μm) along the vertical meridian through the optic nerve head. Microscopic images were digitally captured (Eclipse E400 microscope with illumination: 6-V halogen lamp, 20 W, equipped with a stabilized light source; Nikon, Tokyo, Japan, via a Coolpix s10 camera; Nikon). The sections were stained with hematoxylin and eosin and analyzed by masked observers. The average thickness (in μm) of the retina and retinal layers for each eye was measured. The number of cells in the ganglion cell layer (GCL) was calculated by linear cell density (cells per 100 μm). No attempt was made to distinguish cell types in the GCL for enumeration of the cells. Measurements (× 400) were obtained at 1 mm dorsal and ventral of the optic disc. For each eye, results obtained from four separate sections were averaged, and the mean of five eyes was recorded as the representative value for each group. For histological studies of the effect of LPS *per se*, animals were sacrificed at 3, 5, and 16 days after injection of vehicle or LPS, as shown in Fig. 1.

Tissue homogenization and subcellular fractionation

One day after 24-h light exposure rats were killed by decapitation, eyes were enucleated and retinas were dissected from the retinal pigment epithelium. For immunodetection of Bax and Bcl- xL proteins, tissues were gently homogenized in 150 μL of homogenization buffer (10 mM Tris-HCl, 1 mM $MgCl_2$, 10% glycerol, 0.5% Triton, 10 μg/mL leupeptin, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μM pepstatin, 2 μg/mL aprotinin, pH 7.4). The subcellular fractionation was preformed as described by Wang *et al.* (2008) with minor modifications. Briefly, samples were centrifuged at 800 *g* for 10 min at 4°C to remove unbroken cells and nuclear fraction. Supernatant A containing cytosolic and non-synaptosomal mitochondrial proteins was further centrifuged at 13 400 *g* for 30 min at 4°C to separate supernatant B from pellet B. Supernatant B was used as the cytosolic fraction, and pellet B was used as the non-synaptosomal enriched mitochondrial fraction, after resuspension in 40 μL homogenization buffer. For immunodetection of phosphorylated proteins (p-Akt/Thr308 and p-Stat3/Tyr705), the

homogenization buffer also contained a phosphatase inhibitor cocktail (2 mM imidazole, 1 mM sodium orthovanadate, 12 mM sodium fluoride, 1mM sodium molybdate, 4 mM sodium tartrate, dihydrate). Isolated fractions were stored at −80°C for further analysis.

Western Blot analysis

Retinal homogenates (supernatant A) or enriched mitochondrial and cytosolic fractions (30 μg protein/well) were denatured in sample buffer (50 mM Tris-HCl, 10% glycerol, 2% SDS, 0.5% bromophenol blue, and 10% β-mercaptoethanol, pH 6.8) at 95°C for 5 min and then separated by 12% Tris-glycine SDS-PAGE. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes and blocked overnight at 4°C with 5% bovine serum albumin (BSA), or with 5% non-fat dry milk Tris-buffered saline (TBS – 0.5% Tween) instead. Antibodies against Bax (1 : 1000, catalog # SC-493; Santa Cruz Biotechnology), Blc-xL (1 : 2000, catalog # 610747; BD Transduction Laboratories, Franklin Lakes, NJ, USA) or phospho-Akt/Thr308 (1 : 500, catalog # 9275; Cell Signaling Technology, Beverly, MA, USA) and mouse anti-phospho-Stat3/Thy705 (1 : 800, catalog # SC-8059; Santa Cruz Biotechnology, Santa Cruz, CA, USA) were incubated overnight at 4°C. Membranes were washed and incubated for 1 h at room temperature with a horseradish peroxidase-conjugated goat anti-rabbit (1 : 2000, catalog # 170-5046; Bio-Rad Laboratories, Hercules, CA, USA) or anti-mouse IgG antibody (1 : 10000, catalog # 115-035-146; Jackson ImmunoResearch Laboratories, West Grove, PA, USA), visualized by enzymatic chemiluminiscence (ECL; Western Blotting Analysis System, Amersham Biosciences, Buenos Aires, Argentina), and detected with Image Quant 350. Developed membranes were scanned and the intensity of bands was determined by using the ImageJ program (National Institutes of Health, Bethesda, MD, USA). Values were expressed as arbitrary units of Bax, Bcl-xL p-Akt, and p-Stat3 relative to β-actin. Cytosolic–mitochondrial Bax translocation was determined as mitochondrial Bax/cytosolic Bax ratio (normalized to β -actin).

Measurement of thiobarbituric acid reactive substances (TBARS) levels

Retinas were homogenized in 850 μL of 15 mM potassium buffer plus 60 mM KCl, pH 7.2 and TBARS levels were analyzed as previously described (Belforte *et al.* 2010). The reaction mixture contained: retinal homogenate (200 μL), 50 μL 10% sodium dodecyl sulfate (SDS), and 1.4 mL 0.8% thiobarbituric acid dissolved in 10% acetic acid (pH 3.5), and this solution was heated to 100°C for 60 min. After cooling, the precipitate was removed by centrifugation at 3200 *g* for 10 min. Then, 1.0 ml water and 5.0 mL of n-butanol-pyridine mixture (15 : 1, vol/vol) were added and the mixture was vigorously shaken and centrifuged at 2000 *g* for 15 min. The absorbance of the organic layer was measured at an emission wavelength of 553 nm by using an excitation wavelength of 515 nm with a Jasco FP 770 fluorescence spectrophotometer (Japan Spectroscopic Co. Ltd., Tokyo, Japan). The range of the standard curves of malondialdehyde bis-dimethyl acetal (MDA) was 10–2000 pmol. Results were expressed as nanomol MDA/mg of protein.

Protein level assessment

Protein content was determined by the method of Lowry *et al.* (1951) using bovine serum albumin (BSA) as the standard.

Statistical analysis

Statistical analysis of results was made by a two-way ANOVA followed by Tukey's test.

Results

The temporal course of the electroretinographic protection induced by 1 μg LPS against retinal light damage was studied. One day before light exposure, rats were intravitreally injected with vehicle in one eye and LPS in the contralateral eye, and ERGs were recorded at 1, 3, 7, and 14 days after light exposure (Fig. 2). The average ERG a- and b-wave amplitude in vehicle-injected and non-exposed eyes (control) or at several time points after light exposure, as well as representative scotopic ERG traces from rat eyes submitted to these treatments are shown in Fig. 2. Light treatment caused a marked attenuation of the scotopic ERG a- and b-waves which was already evident one day after light exposure, and persisted along the study, whereas their latencies remained unchanged (Table 1). LPS did not induce any significant protection on day 1 post-exposure, whereas at day 7 and 14, a significant prevention of the decrease in both parameters was observed. At 3 days after light exposure, the b-wave amplitude significantly increased in LPS-injected light-exposed eyes, and there was a trend for improvement the a-wave amplitude, but this trend was not significant. No significant differences in the ERG were observed between non-injected and vehicle-injected eyes, and between non-exposed eyes injected with vehicle and LPS (data not shown). In order to analyze whether the inhibition of immune responses precludes the effect of LPS, dexamethasone was intraperitoneally injected 30 min before the injection of vehicle or LPS, and retinal function was assessed at 7 days post-light exposure (Fig. 3). Dexamethasone, which showed no effect *per se* in non-exposed and light-exposed eyes, significantly prevented the protective effect of LPS on retinal function. Figure 4 shows a representative photomicrograph of a non-injected and non-exposed eye (Fig. 4a) a vehicle-injected and non-exposed eye (Fig. 4b) at 5 days after vehicle injection. LPS *per se* (in non-exposed eyes) did not affect the retinal structure at 3, 5, and 16 days after injection (Figs. 4c, 4d and 4e, Table 2). One day after 24-h light exposure, no major alterations in the retinal structure were observed (Fig. 4f), whereas clear pathological changes were evident in the retina from eyes injected with vehicle at day 3 and 14 after light exposure (Figs. 4g and 4h, respectively). At 3 days post-exposure, a significant decrease in photoreceptor outer and inner segment (PS) thickness was observed (Fig. 4g), whereas at 14 days post-exposure (Fig. 4h), a decrease in total retinal and ONL thickness and in ganglion cell layer (GCL) cell number, as well as a further decrease in PS thickness was evident in this group (Table 2). In LPS-injected and light-exposed eyes, the whole retinal structure was significantly preserved at 3 and 14 days post-exposure

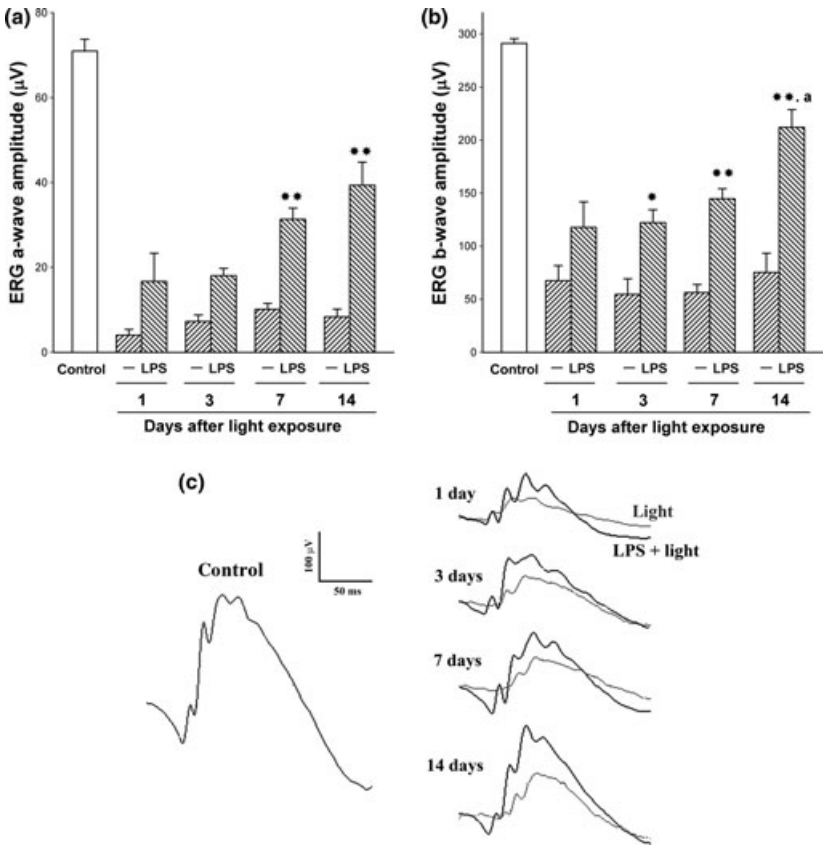

Fig. 2 Scotopic electroretinogram (ERG)s from rats injected with vehicle in one eye and LPS in the contralateral eye 24 h before intense light exposure. ERGs were recorded at 1, 3, 7 or 14 days post-exposure. Average amplitudes of scotopic ERG a-wave (a) and b-wave (b). A significant decrease in the amplitude (but not their latencies) of both ERG a- and b-wave amplitude was observed in light-exposed eyes injected with vehicle at all time points. In eyes injected with LPS a significant preservation of the ERG a- and b-wave amplitude was observed at 7 and 14 days after light exposure. At day 3 after light exposure, LPS afforded a significant protection on the b- wave (but not a-wave) amplitude. The ERG a- and b-wave amplitude from all the eyes exposed to intense light in the presence or absence of LPS significantly differ from that of non-exposed eyes ($p < 0.01$). Data are mean ± SE (n : 10 eyes/group). *$p < 0.05$, **$p < 0.01$ vs. light-exposed eyes injected with vehicle at the same time point; [a]$p < 0.05$ vs. LPS at 7 days after light exposure, by Tukey's test. (c) representative scotopic ERG traces from non-exposed eyes (control) and from an animal injected in one eye with vehicle and the contralateral eye with LPS whose ERGs were recorded at 1, 3, 7, and 14 days post-exposure.

(Fig. 4j and k, respectively, Table 2). The exposure to light in the absence or presence of LPS did not affect outer plexiform layer (OPL), inner plexiform layer (IPL), and inner nuclear layer (INL) thickness.

The effect of light exposure in vehicle- or LPS-injected eyes on the proapoptotic protein Bax and antiapoptotic protein Bcl-xL levels at 24 h after light exposure was assessed by Western blotting. Figure 5(a) shows representative Western blots of cytosolic and mitochondrial Bax levels in the retinas from eyes exposed to light in the absence or presence of LPS. Light exposure induced a significant increase in mitochondrial Bax/cytosolic Bax ratio, which was completely abrogated by an intravitreal injection of LPS. No differences in cytosolic Bcl-xL levels were observed among groups (Fig. 5b). LPS *per se* did not modify mitochondrial Bax/cytosolic Bax ratio or cytosolic Bcl-xL levels in non-exposed eyes.

Retinal lipid peroxidation was assessed at 1 or 3 days after light exposure, in the absence or presence of LPS. One day after light exposure, no differences in TBARS levels were observed among groups, whereas at 3 days after light exposure, a significant increase of this parameter was observed in vehicle-injected and light-exposed eyes, which was partly prevented by LPS injection (Fig. 6). LPS *per se* did not induce changes in TBARS levels at any of these time points in non-exposed rats.

The effect of AMG, 5HD, and WT on the retinal protection induced by LPS against light-induced damage was examined by electroretinography at 7 days after light exposure. The average ERG a- and b- wave amplitude of these groups are shown in the upper panel of Fig. 7, while representative scotopic ERG traces are shown in the lower panel of Fig. 7. Neither 5HD nor AMG had any significant effect on the

Table 1 Scotopic ERG a- and b-wave latencies from rats injected with vehicle in one eye and LPS in the contralateral eye 24 h before intense light exposure

		day 1		day 3		day 7		day 14	
Latency (ms)	Control	Light	LPS + light	Light	LPS + light	Light	LPS + light	Light	LPS + light
a wave	22.2 ± 0.3	22.2 ± 1.2	23.8 ± 0.7	25.8±1.5	24.5 ± 1.5	22.9 ± 0.8	22.1 ± 0.3	20.8 ± 1.7	22.4 ± 0.5
b wave	44.8 ± 0.8	46.5 ± 2.4	49.8 ± 1.8	48.9±4.5	45.9 ± 2.1	43.4 + 0.7	43.7 + 0.8	41.6 + 1.1	42.7 + 0.6

ERG a -and b-wave latencies (ms).
No differences in scotopic ERG a- and b-wave latencies were observed among groups. Data are mean ± SE (*n*: 10 eyes/group).

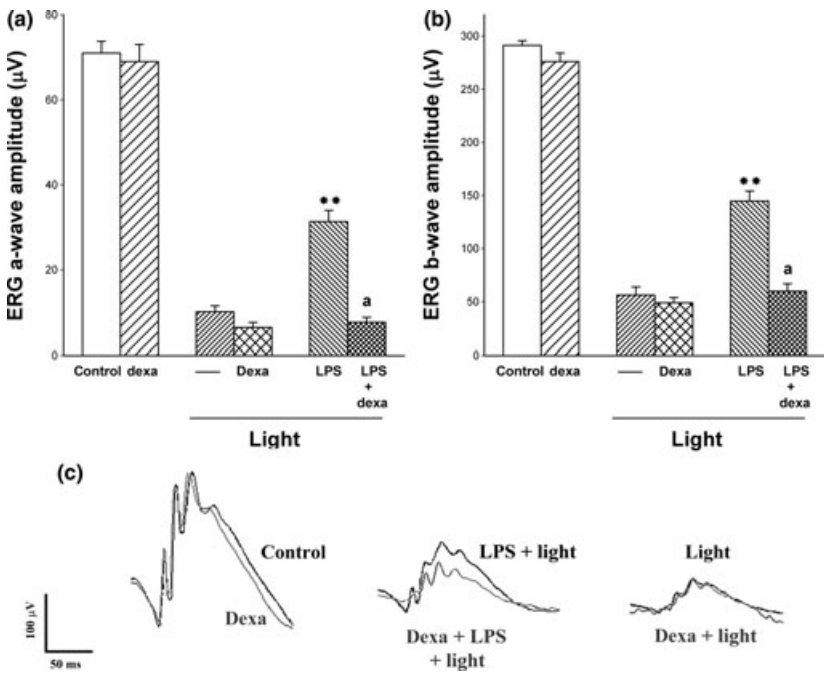

Fig. 3 Electroretinogram (ERG)s from control eyes and eyes injected with vehicle or LPS, 24 h before intense light exposure, in the presence or absence of dexamethasone. Dexamethasone was i.p injected 30 min before vehicle or bacterial lipopolysaccharide (LPS) injection, and ERGs were recorded at 7 days post-exposure. Average amplitudes of scotopic ERG a-wave (a) and b-wave (b). Dexamethasone did not affect the ERG a- and b-wave amplitude in non-exposed and light-exposed eyes, but it significantly decreased the protective effect of LPS on these parameters. Data are mean ± SE (n : 10 eyes/group) $^{**}p < 0.01$ vs. light-exposed eyes injected with vehicle. $^{a}p < 0.01$ vs. light-exposed eyes injected with LPS. (c) representative scotopic ERG traces from non-exposed eyes (control) and from an animal injected in one eye with vehicle and the contralateral eye with LPS in the presence of absence of dexamethasone (Dexa).

protection induced by LPS, whereas WT significantly prevented its effect on the retinal dysfunction induced by light exposure. The preventive effect of WT was also evident at histological level, as shown in Fig. 8. LPS induced a preservation of the retinal structure in light exposed eyes (Fig. 8e), whereas WT prevented this protection (Fig. 8f). Wortmannin *per se* did not affect the retinal structure in non-exposed (Fig. 8b) or light-exposed eyes (Fig. 8d).

In order to further analyze the mechanism involved in the protection induced by LPS, the levels p-Akt and p-Stat3 were assessed in retinas from control eyes and eyes exposed to intense light, in the absence or presence of LPS, at 3 days after LPS injection (Fig. 9). LPS induced a significant increase in p-Akt levels in non-exposed and light-exposed eyes, and wortmaninn prevented the increase in this parameter, as shown in Fig. 9(a). On the other hand, LPS increased p-Stat3 in non-exposed eyes, whereas it was ineffective in light-exposed eyes (Fig. 9b). Wortmannin did not modify the effect of LPS on p-Stat3 levels in non-exposed and light-exposed eyes.

Discussion

The present results indicate that an intravitreal injection of a low dose of LPS protected the retina from light-induced

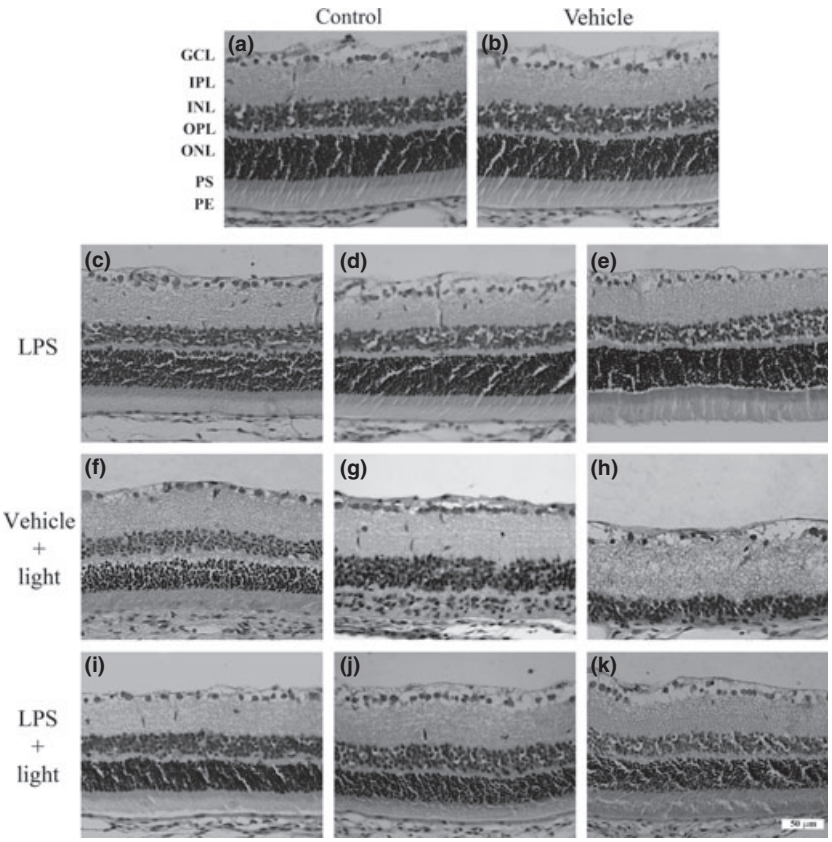

Fig. 4 Representative photomicrographs showing histological appearance of non-injected and non-exposed eyes (a), eyes injected with vehicle at 5 days after injection (b), eyes injected with LPS at 3, 5, and 16 days post-injection (c, d, and e, respectively), and eyes exposed to light for 24 h in the presence of vehicle (f, g, h) or LPS (i, j, k) at 3, 5, and 16 after injection (1, 3, or 14 days after exposure, respectively). Severe retinal damage is shown in the retina from eyes exposed to light in the presence of vehicle at 3 or 14 days after light exposure, whereas in LPS-injected eyes, the retinal structure was notably preserved. Scale bar = 50 μm. GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer, PS, photoreceptor outer and inner segment; PE, pigment epithelium.

Table 2 Histological analysis of retinas from eyes exposed to light in the absence or presence of LPS

	Total Thickness	PS	ONL	OPL	INL	IPL	cell number inGCL
Control	148.4 ± 1.4	31.8 ± 4.5	32.8 ± 2.7	8.1 ± 1.0	21.5 ± 3.7	33.4 ± 1.5	8.5 ± 0.4
Light							
Day 1	141.0 ± 4.0	24.1 ± 1.2	32.3 ± 1.5	9.5 ± 0.3	26.6 ± 0.9	35.9 ± 0.6	8.4 ± 0.5
Day 3	135.6 ± 0.8	13.1 ± 0.3**	19.0 ± 0.4	8.9 ± 0.1	29.2 ± 2.8	47.5 ± 0.2	9.0 ± 1.3
Day 14	101.4 ± 15.1*	2.7 ± 2.6**	4.6 ± 3.1**	9.1 ± 1.5	25.5 ± 6.2	43.0 ± 8.7	5.6 ± 0.5*
LPS + Light							
Day 1	142.2 ± 8.3	28.7 ± 2.6	35.3 ± 3.8	8.9 ± 0.7	29.4 ± 2.3	37.6 ± 1.3	8.8 ± 0.1
Day 3	141.4 ± 19.2	23.2 ± 4.4	36.8 ± 10.3	8.5 ± 0.4	28.8 ± 0.6	44.1 v 0.3	8.8 ± 0.4
Day 14	145.8 ± 9.9[a]	26.3 ± 3.l[b]	30.9 ± 4.6[b]	8.3 ± 0.8	22.0 ± 1.8	40.0 ± 2.0	8.3 ± 0.3[a]

Total retinal and retinal layer thicknesses (in μm) in control eyes, or eyes exposed to light in the absence or presence of LPS, at different time points after light exposure. At 3 days after light exposure, a significant decrease in PS thickness was observed in vehicle-injected eyes, whereas at 14 days after light exposure a further decrease in PS and a decrease in total and ONL thickness and the number of GCL cells /100 μm was observed. Data are mean ± SEM (n = 5 eyes/group). The presence of LPS significantly reduced the effect of light stress on these parameters. *$p < 0.05$, **$p < 0.01$ vs. non-exposed eyes, [a]$p < 0.05$, [b]$p < 0.01$ vs. light exposed eyes injected with vehicle at the same time interval post-exposure, by Tukey's test. PS, photoreceptor outer and inner segment; OPL, outer plexiform layer; ONL, outer nuclear layer; IPL, inner plexiform layer; INL, inner nuclear layer; GCL, ganglion cell layer.

damage. LPS preserved retinal function and histology, decreased Bax translocation from the cytoplasm to mitochondria, and oxidative stress in light-exposed eyes. In a previous report we showed that a single intravitreal injection of 1 μg LPS *per se* does not affect the electroretinographic activity or retinal histology at 1 or 7 days post-injection (Franco *et al.* 2008). It has been shown that LPS pretreatment induces myocardial and cerebral protection against I/R injury (Rosenzweig *et al.* 2004; Schober *et al.* 2008). Schober *et al.* (2008) have shown that dexamethasone

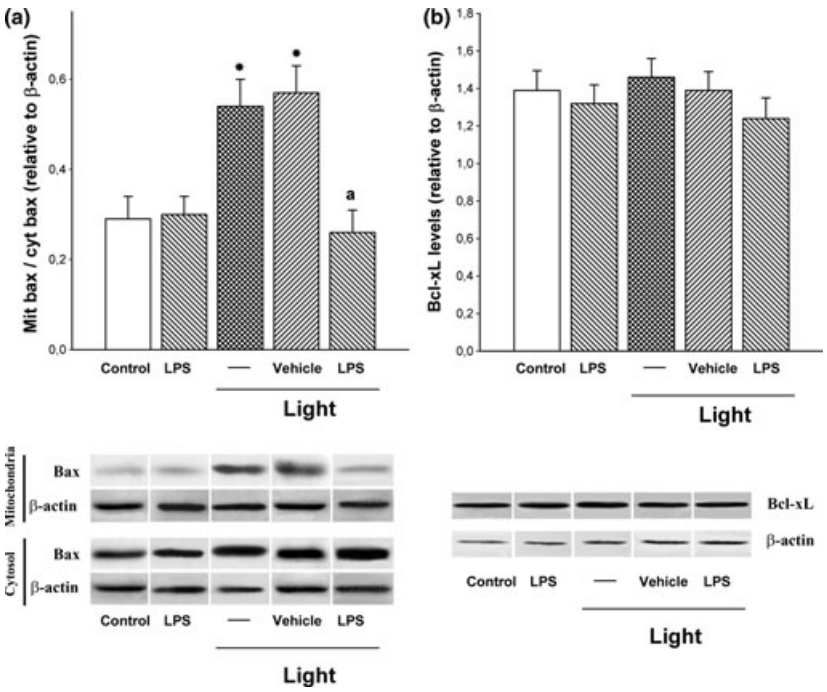

Fig. 5 Effect of LPS and light exposure on retinal mitochondrial and cytosolic levels of Bax and cytosolic levels of Bcl-xL. Upper Panel: one day after light exposure densitometric analysis of the samples revealed a significant increase in mitochondrial Bax/cytosolic Bax ratio (a) in the absence of LPS, whereas LPS which showed no effect *per se*, prevented the effect of light exposure. No differences in cytosolic Bcl-xL levels (b) were observed among groups. Lower panel: representative gel for cytosolic and mitochondrial Bax (left) and for cytosolic Bcl-xL (right) for all the experimental groups. Data are mean ± SEM ($n = 5$ eyes per group). $^{*}p < 0.05$ vs. non-exposed eyes; $^{a}p < 0.05$ vs. light-exposed eyes injected with vehicle, by Tukey's test.

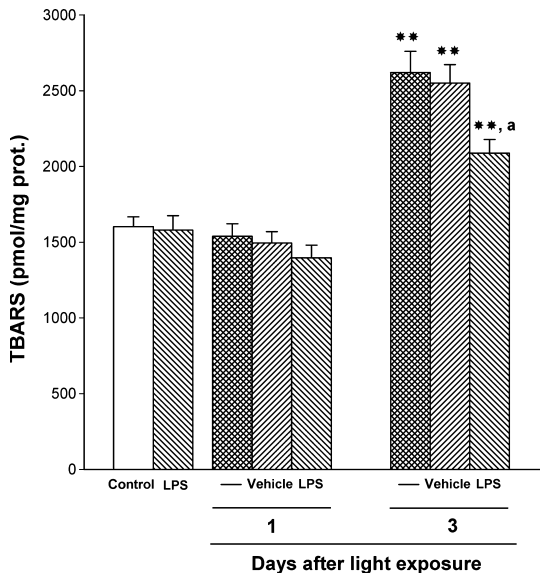

Fig. 6 Retinal TBARS levels in the retina from non-exposed eyes or eyes exposed to intense light in the presence of vehicle or LPS, at 1 or 3 days after light exposure. LPS per se did not affect TBARS levels at 4 days post-exposure. At 3 days after light exposure, this parameter was significantly higher in light-exposed eyes injected with vehicle than in those injected with LPS. At 1 day post-exposure, retinal lipid peroxidation did not differ among groups. Data are mean ± SEM ($n = 6$ retinas/group), $^{**}p < 0.01$, vs. non-exposed eyes, $^{a}p < 0.01$ vs. vehicle injected eyes at day 3 after light exposure, by Tukey's test.

inhibits the protective effect of LPS on acute myocardial infarction. In agreement, dexamethasone prevented the protective effect of LPS against light-induced damage, supporting that the underlying mechanism in the protection induced by LPS is dependent upon induction of an inflammatory response. Although Wenzel *et al.* (2001) have demonstrated that dexamethasone completely protects the retina against light damage, in our experimental setting, dexamethasone alone was ineffective. The use of higher doses of dexamethasone (20–52 mg/kg) as well as the experimental model used in this report (BALB/c mice) could account for this discrepancy.

Despite limitations of our method for ERG assessment due to using a single intensity recordings (lack of intensity response plot, risk of artifact and error), light exposure induced a significant decrease in the scotopic ERG a- and b-wave amplitude which persisted along the study, while preconditioning induced by LPS did not merely delay but prevented retinal alterations, since the recovery in the ERG was a sustained effect noted up to 14 days after light exposure.

Classically, it was assumed that prolonged light exposure exclusively damages retinal photoreceptors. However, more recent findings show that excessive light *in vivo* (Sang *et al.* 2011) and *in vitro* (Wood *et al.* 2007; Osborne *et al.* 2008) can negatively affect retinal ganglion cell survival. In this regard, it should be noted that most of the observations regarding intense light-induced photoreceptor degeneration have been made immediately or shortly after cessation of illumination, whereas long-term effects of light damage on the retina have seldom been investigated. As shown herein, a highly significant decrease in photoreceptor segment thickness was observed at 3 days, and with extended post-exposure survival times (i.e. 14 days), cell loss becomes significant in the GCL. In agreement with these results,

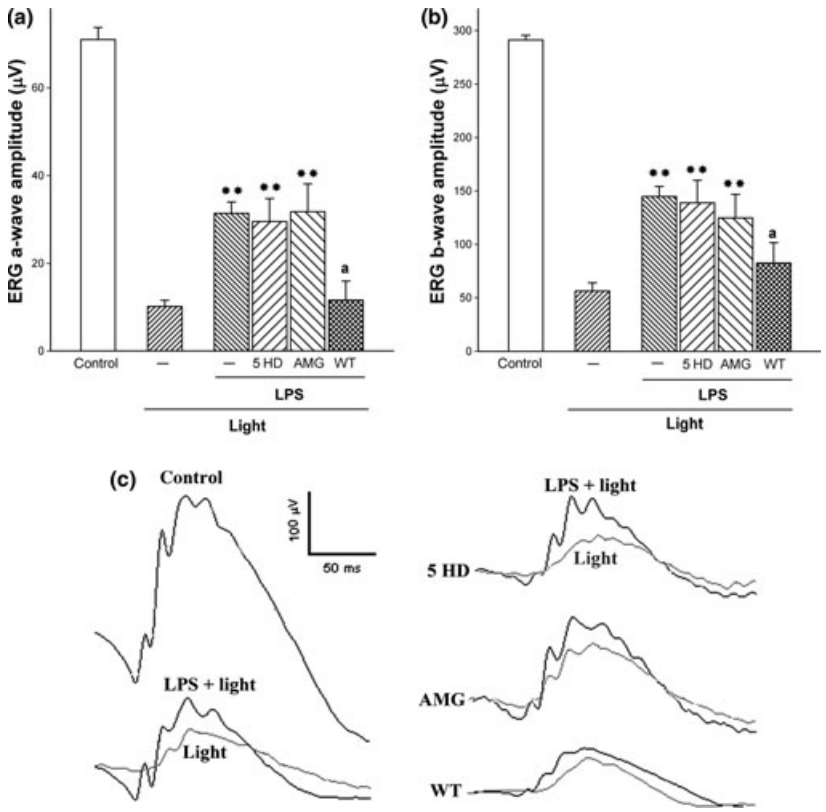

Fig. 7 Effect of 5-hydroxydecanoic acid (5HD), aminoguanidine (AMG), and wortmannin (WT) on the functional protection induced by LPS against light damage. Eyes were injected with 5HD, AMG or WT, as described in Material and methods. Average amplitudes of scotopic ERG a-wave (a) and b-wave (b) 7 days after light exposure. Wortmannin significantly decreased the protection induced by LPS on the retinal dysfunction induced by light exposure, whereas 5HD and AMG were ineffective. Data are mean ± SE (*n*: 5 eyes/group). $^{**}p < 0.01$ vs. light-exposed eyes injected with vehicle, $^{a}p < 0.01$ vs. light-exposed eyes injected with LPS, by Tukey's test. (c) Representative scotopic ERGs from all the experimental groups.

some groups have shown a delayed ganglion cell loss after different intervals post-exposure to intense light (Wasowicz *et al.* 2002; Marco-Gomariz *et al.* 2006). Our results indicate that the decrease in total retinal, PS, and ONL thickness, as well as in GCL cell number in eyes exposed to intense light was prevented by the intravitreal injection of LPS.

Exposure to intense light induces retinal apoptotic cell death (Noell *et al.* 1966; '; Remé *et al.* 1998). Bax is a major effector of apoptotic cell death in the retina after ischemia, excitotoxicity, axotomy, and retinal degeneration (Isenmann *et al.* 1999; Tezel and Wax 1999; Podestà *et al.* 2000). Although light exposure increases Bax and induces apoptosis in other cell types such as lens epithelial cells, melanocytes, and retinal pigment epithelial cells (Waster and Ollinger 2009; Kernt *et al.* 2010, 2011), to our knowledge, the present results are the first demonstration that light exposure triggered retinal Bax translocation from the cytoplasm to mitochondria which was prevented by LPS, supporting the role of Bax in the cell death pathway provoked by intense light, as well as in LPS-induced protection. In agreement, it was previously shown that deficiency of Bax and Bak protects the retina against light damage (Hahn *et al.* 2004), and that IPC prevents the increase in retinal Bax levels induced by I/R damage (Zhang *et al.* 2002). As previously shown by Grimm *et al.* (2000), no changes in retinal Bcl-xL levels were observed after light exposure.

Compelling evidence exists that intense light leads to retinal oxidative stress. The effectiveness of a wide variety of antioxidants indicates that reactive oxygen species are formed during intense light (Organisciak and Vaughan 2010). In agreement with Chen and Zhang (1994), light exposure induced a significant increase in retinal TBARS levels (an index of lipid peroxidation) at 3 (but not 1) days, and this effect was prevented by LPS. These results suggest that a low dose of LPS could behave as an antioxidant therapy in the paradigm of retinal light-induced injury.

Several signaling pathways and/or cellular survival proteins underlie the neuroprotective effects of retinal IPC, such as adenosine (Li and Roth 1999), mK^+/ATP channels (Roth *et al.* 2006), and NO (Sakamoto *et al.* 2006), among many others (reviewed by Gidday 2006). We examined some mechanisms whose involvement in the protective effect of IPC against I/R damage has been previously demonstrated. Retinal protection induced by LPS was not influenced by an mK^+/ATP channel blocker or by AMG, supporting that mK^+/ATP channels and iNOS are not involved in the protection against light-induced injury triggered by LPS. We have previously shown that AMG prevents the protective effect of LPS against I/R injury (Franco *et al.* 2008). Since AMG did not affect the protection induced by LPS against light damage, it seems that the mechanism involved in the protective effect of LPS could depend on the type of damage. The protective effect of LPS, both at functional and histological level was completely prevented

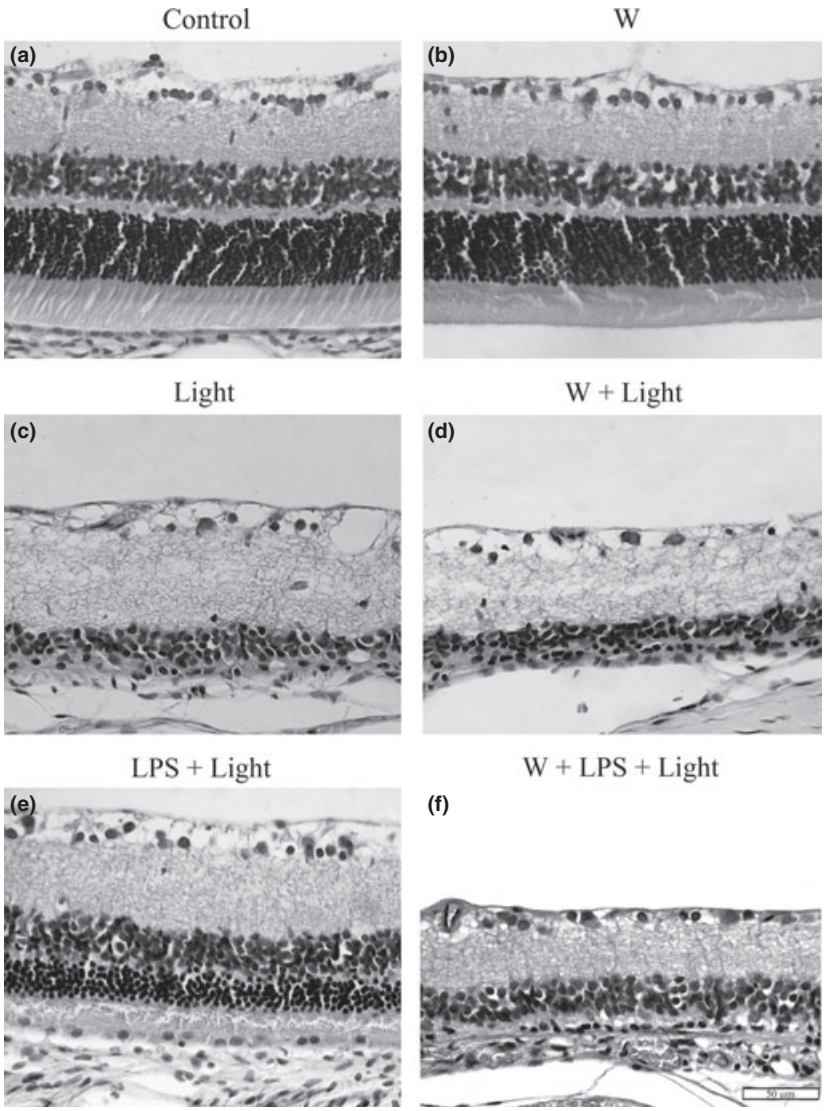

Fig. 8 Effect of wortmannin (WT) on the histological protection against light damage induced by bacterial lipopolysaccharide (LPS) at 7 days after light exposure. Shown are representative photomicrographs of histological appearance of non-injected and non-exposed eyes (a) and of light- exposed eyes injected with vehicle (c), WT (d), LPS (e), or LPS + WT (f). The preservation of the retinal structure induced by the injection of LPS was substantially decreased in the presence of WT. Wormannin *per se* did not affect the retinal structure in non-exposed eyes (b) or in light-exposed eyes (d). Scale bar = 50 μm.

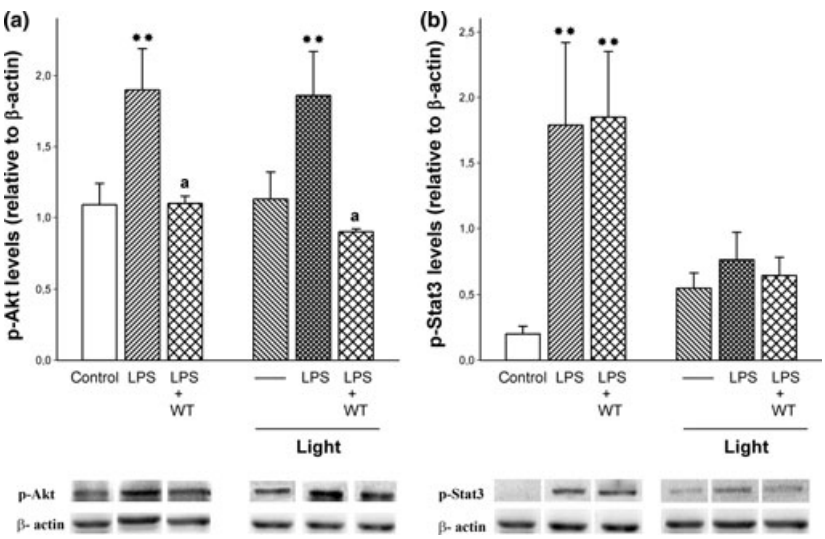

Fig. 9 Effect of LPS and light exposure on retinal p-Akt and p-Stat3 levels. (a) LPS induced a significant increase in p-Akt levels in non-exposed and light-exposed eyes which was prevented by WT. (b) LPS induced a significant increase in p-Stat3 levels in non-exposed eyes, and WT did not affect the effect of LPS on this parameter. No differences were observed in p-Stat3 levels in light-exposed eyes among groups. Data are mean ± SEM (n = 5 eyes/group). $^{**}p < 0.01$ vs. eyes injected with vehicle and under the same light regime, $^{a}p < 0.01$ vs. eyes injected with LPS and under the same light regime, by Tukey's test.

by WT. It was demonstrated that WT attenuates the neuroprotective effect of IPC on I/R damage through an Akt (a serine/threonine kinase also known as protein kinase B)-dependent pathway (Dreixler *et al.* 2009) and a role for Akt in the preservation of photoreceptors from light damage has been demonstrated (Li *et al.* 2007; Ueki *et al.* 2008). On the other hand, it was reported that Stat3 is the primary effector protein of LPS-mediated JAK-STAT signaling (Jang *et al.* 2007). As shown herein, LPS induced a significant increase in p-Akt and p-Stat3 levels, but WT only prevented the effect of LPS on p-Akt (but not p-Stat3) levels both in non-exposed and light-exposed eyes, supporting the involvement of PI3K/Akt pathway in the protective effect of LPS (Jang *et al.* 2007). It was demonstrated that dexamethasone inhibits PI3K-Akt signaling in several systems (Fujita *et al.* 2004; Gupta *et al.* 2007). Thus, this pathway could be involved in the inhibitory effect of dexamethasone on the retinal protection induced by LPS against intense light damage.

While all types of animal models can be criticized for their relevance to human physiology, people with inherited ocular diseases primarily live and work in light environments. The use of intense or prolonged light exposures and animal models can improve our understanding of basic mechanisms in retinal degeneration by exacerbating visual cell death with an acute insult superimposed on a chronic condition. At this time, we need to extrapolate our findings in animal models to human disease, but it may turn out that simple treatments developed to prevent retinal degeneration in animals may be the basis of therapeutic interventions in people (Organisciak and Vaughan 2010). In that context, the demonstration that a moderate dose of LPS protected the retina against light damage could constitute a future fertile avenue for promoting the survival of retinal cells.

Acknowledgments

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