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Research Report

Nitric oxide increases in the rat retina after continuous illumination

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ABSTRACT

Continuous illumination (CI) of the retina induces an oxidative stress followed by the degeneration of photoreceptors. This phenomenon may be partially related to the excessive production of nitric oxide (NO). In order to confirm this hypothesis, the aims of this work are to determine NO levels during the illumination of the retina by electron paramagnetic resonance (EPR), and if an increase of NO is found, to characterize the NOS isoform responsible of the increment by using Western blot. Sprague–Dawley rats were continuously illuminated with white light (12,000 lux) for 2, 24, 48 h, 5 and 7 days while control rats were maintained at light/dark cycles of 12/12 h. Using EPR, an increase of NO signal was observed in the light exposed retinas peaking at 24 h of CI. Western blot analysis showed the expression of iNOS in the illuminated retinas with a peak after 24 h of CI, but did not show significant differences of nNOS among illuminated and control retinas. In summary, there is an increase of NO during CI. Further studies will reveal whether this mechanism is responsible for light induced photoreceptor degeneration.

1. Introduction

Continuous illumination (CI) is a widely used model to study degenerative diseases of the retina (Abler et al., 1996; La Vail et al., 1999; Marti et al., 1998; Reme et al., 1998a, 2003). Experimental CI induces the degeneration of photoreceptors (PR) both in adult and immature rats (Chader, 2002, 2005; Gorn and Kuwabara, 1967; Noell et al., 1966; Pecci Saavedra and Pellegrino de Iraldi, 1976).

Illumination generates the formation of free radicals in the retina (reactive oxygen species (ROS) and reactive nitrogen species (RNS)). As to counteract the oxidative insult there is a

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Abbreviations: AMD, age-related macular degeneration; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; CI, continuous illumination; DETC, diethyldithiocarbamate; EPR, electron paramagnetic resonance; ESR, electron spin resonance; GC, guanylate cyclase; GCL, ganglion cell layer; GPx, glutathione peroxidase; IL, illuminated; ip, intraperitoneal; LIRD, light induced retinal degeneration; L-NAME, L-arginine methyl ester; Mn-SOD, manganese superoxide dismutase; NO, nitric oxide; NOS, nitric oxide synthase; iNOS, inducible nitric oxide synthase; mNOS, macrophagic nitric oxide; PBN, phenyl-*N*-tertbutylnitrone; PR, photoreceptor; ROS, reactive oxygen species; RNS, reactive nitrogen species; RPE, retinal pigment epithelium; SNP, sodium nitroprusiate; SOD, superoxide dismutase

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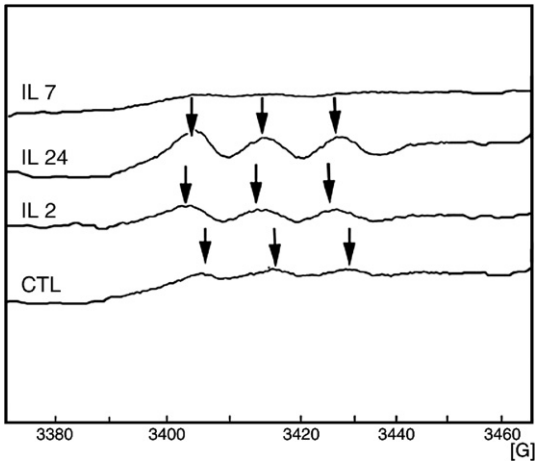

Fig. 1 – ESR spectra of the NO–$Fe^{2+}(DETC)_2$ complex in retinas from control rats (CTL) and rats illuminated for 2 h (IL2), 24 h (IL24), and 7 days (IL7). The paramagnetic signal of the NO–$Fe^{2+}(DETC)_2$ adduct (arrows) was detected by ESR. Instrumental settings were: center field 3420 G, sweep width 100 G, microwave power 20 mW, microwave frequency 9.62 GHz, conversion time 10.24 ms, time constant 40.96 ms, modulation frequency 50 kHz, modulation amplitude 1.00 G, gain 2.00×10^4, resolution 1024 points. All spectra were the accumulation of 20 scans.

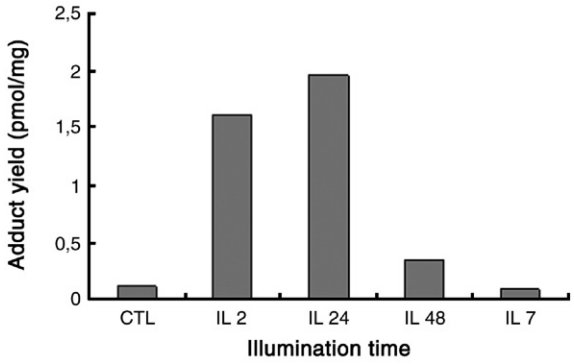

Fig. 3 – NO–$Fe^{2+}(DETC)_2$ adduct detected by ESR yields in retinas from control (CTL) and illuminated rats for 2 h (IL2), 24 h (IL24), 48 h (IL48) and 7 days (IL7). Adduct yields (pmol/mg tissue) were estimated by the double integration of the simulated signals using as a standard the stable radical TEMPOL.

complex defensive system in the retina composed by the following antioxidant enzymes: superoxide dismutase (SOD) (Rao et al., 1985), catalase (Atalla et al., 1987), and glutathione peroxidase (GPx) (Atalla et al., 1988). Certainly, light exposure induces the superoxide scavenger, mitochondrial manganese SOD (Mn-SOD) in the retina (Yamamoto et al., 1999) and increases the activity of GPx in different cells at the posterior retinal pole (Ohira et al., 2003).

However, if illumination persists, the excessive production of ROS overcomes the defensive antioxidant mechanisms causing an oxidative stress that generates a photic damage of the retina (Organisiak et al., 1998). This light-induced oxidative stress activates procaspase-3 in rat retina (Wu et al., 2002), increases caspase-1 in mice retina (Grimm et al., 2000) and triggers the apoptotic death of photoreceptors (Abler et al., 1996; Grimm et al., 2000, 2001; Reme et al., 1998b, 2000).

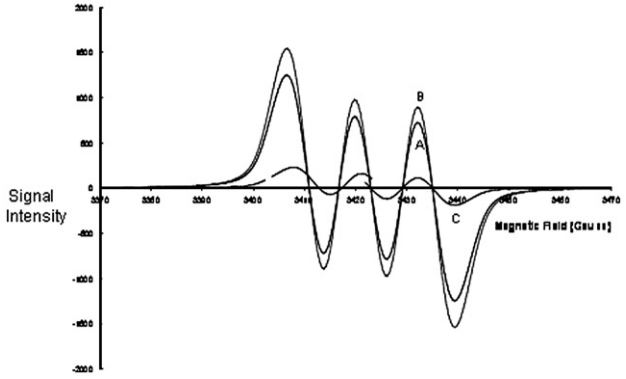

Fig. 2 – Adjusted simulated ESR spectra from experimental signals of the NO–$Fe^{2+}(DETC)_2$ complex in retinas from rats illuminated for (A) 2 h, (B) 24 h and (C) 48 h. Instrumental parameters in the simulated spectra were setting equally as the experimental ones. Control signal spectrum was subtracted from every spectrum. The adjusted experimental signals were obtained using a modification of the Levenberg–Marquardt algorithm.

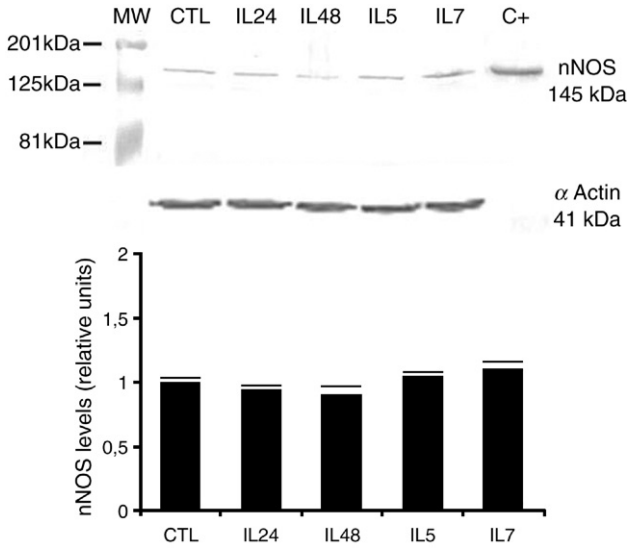

Fig. 4 – Levels of nNOS in rat retina after continuous illumination by Western blot. Representative immunoblotting of nNOS in extracts from retina of illuminated rats. CTL: control without exposure to continuous illumination (CI); IL: Rats submitted to CI for 24 h (IL24); 48 h (IL48); 5 days (IL5) and 7 days (IL7). Positive control (C+) was obtained from mouse pituitary. The illustrated blot is representative of 4 independent experiments, each using different animals. nNOS levels were determined by Western blot analysis on rat retina after CI by scanning densitometry. The relative expression of nNOS (140 kDa) was calculated, and the value for the control was arbitrarily defined as 1. Each bar represents the mean ± S.E.M. of enzyme activity for each group ($n=4$).

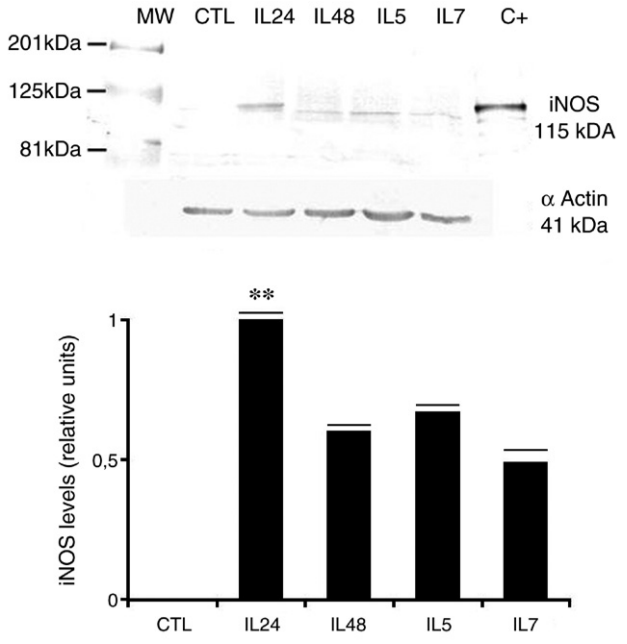

Fig. 5 – Levels of iNOS in rat retina after continuous illumination by Western blot. Representative Western blot of iNOS in extracts from retina of illuminated rats. CTL: control without exposure to continuous illumination (CI); IL: Rats submitted to CI for 24 h (IL24); 48 h (IL48); 5 days (IL5) and 7 days (IL7). Positive control (C+) was obtained from mouse macrophages. Observe a significant expression of retinal iNOS after 24 h of CI (IL24). The illustrated blot is representative of 4 independent experiments, each using different animals. iNOS levels determined by Western blot analysis on rat retina after continuous illumination by scanning densitometry. The relative expression of iNOS (115 kDa) was calculated, and the value for the IL24 h was arbitrarily defined as 1.Each bar represents the mean ± S.E.M. of enzyme activity for each group ($n=4$), iNOS band from IL24 was compared with the other reactive bands (IL48, IL5 and IL7), this difference was significant as $p<0.01$.

Supporting these findings, the experimental use of antioxidant therapies or of radical trapping agents, as phenyl-N-terbutylnitrone (PBN), have shown to be protective against light-induced retinal degeneration (LIRD) (Blanks et al., 1992; Kim et al., 2005; Organisciak et al., 1992, 1999; Ranchon et al., 2001, 2003). In human beings, oxidative stress has been postulated as an important factor in the pathophysiology of age-related macular degeneration (AMD) and retinitis pigmentosa (Ambati et al., 2003; Beatty et al., 2000); furthermore apoptosis is one of the final pathways involved in PR degeneration during AMD and pathologic myopia (Xu et al., 1996).

Light neurotoxicity and the apoptotic pathway are closely related with glutamate in the retina (Sucher et al., 1997) where it is one of the main neurotransmitters (Barnstable, 1993). Excessive glutamate release triggers NMDA-induced apoptosis in the retina (Kwong and Lam, 2000; Lam et al., 1999) and both NMDA receptor antagonists and Ca^{2+} channel blockers protect retinal cells (Morizane et al., 1997; Toriu et al., 2000). NMDA stimulation increases intracellular calcium which activates Nitric Oxide Synthase (NOS). Different NOS isoforms were reported in the normal retina by using nNOS immunocytochemistry (Haberecht et al., 1993; Yamamoto et al., 1993) and iNOS immunocytochemistry (Goureau et al., 1994; López-Costa et al., 1997).

Indirect evidence of the role of NO in photic injury was obtained as NOS inhibitors, N(G)-nitro-L-arginine methyl ester (L-NAME) or 7-nitroindazole, prevented LIRD in albino rats and mice (Goureau et al., 1993; Kaldi et al., 2003). After intense light exposure, nitrotyrosine immunoreactivity, an index of protein nitration, decreased in outer segments and increased in the PR inner segments and retinal pigment epithelium (RPE) suggesting that light modulates protein nitration (Miyagi et al., 2002). Up to the moment, there is no direct evidence showing an increase of NO in the retina during CI. Due to the short half life of NO, it has been hard to determine the presence of NO in animal tissues. However, the use of the trapping agent iron diethyldithiocarbamate (DETC) $_2$ has enabled the direct detection of NO in animal tissues in vivo by cryogenic electron paramagnetic resonance (EPR), also known as electron spin resonance (ESR) spectroscopy (Kikuchi et al., 1993; Mulsch et al., 1995; Shen et al., 1998). In the ischemic/reperfused diabetic retina, EPR was previously used to measure oxygen free radical formation (Szabo et al., 1997). The strategy of using EPR and $(DETC)_2$ as a trapping agent to measure NO was employed in myocardial tissue after ischemia/reperfusion (Shen et al., 1998), and in the brain of septic shock animals (Fujii and Berliner, 1999), but this experimental approach is completely original in the study of the illuminated retina.

The aim of this work is to demonstrate directly the increase of NO in the rat retina during CI by using EPR/ESR and to detect specifically the NOS isoform involved in the excessive production of NO by Western blot analysis.

2. Results

2.1. *Electron paramagnetic resonance determination (EPR)*

Most of the free radicals of interest in biological systems have very short half-lives, which makes direct detection virtually impossible. The spin-trapping technique is based on the reaction between the free radical and a spin-trap giving a stable complex, called an adduct, which has a large half-life allowing its detection by EPR/ESR. NO has a half-life range of 0.09 to < 2 s in hepatocytes (Thomas et al., 2001) but, when it is captured by $Fe^{2+}(DETC)_2$, a stable paramagnetic mononitrosyl iron complex $NOFe^{2+}(DETC)_2$ is formed (Mulsch et al., 1995).

In every case, $NOFe^{2+}(DETC)_2$ complex spectrum was a triplet characteristic signal in our work. The experimental ESR spectra of the NO–$Fe^{2+}(DETC)_2$ complex in retinas was obtained from control rats and from rats illuminated for 24 h (IL24), 48 h (IL48), 5 days (IL5) and 7 days (IL7). Control rats (CTL) showed a small characteristic ESR signal of NO–$Fe^{2+}(DETC)_2$ adduct (Fig. 1). A strong signal was observed at IL24 (Fig. 1). In order to know if there was an earlier NO peak, a shorter illumination period of 2 h (IL2) was added. Although there was an increase of the NO signal at this time point (compared with CTL), the maximal NO signal was detected at IL24 condition (Fig. 1). The signal intensity decreased after 48 h (IL48) (Figs. 2 and 3) and 5 days of illumination and was similar to control values in rats which

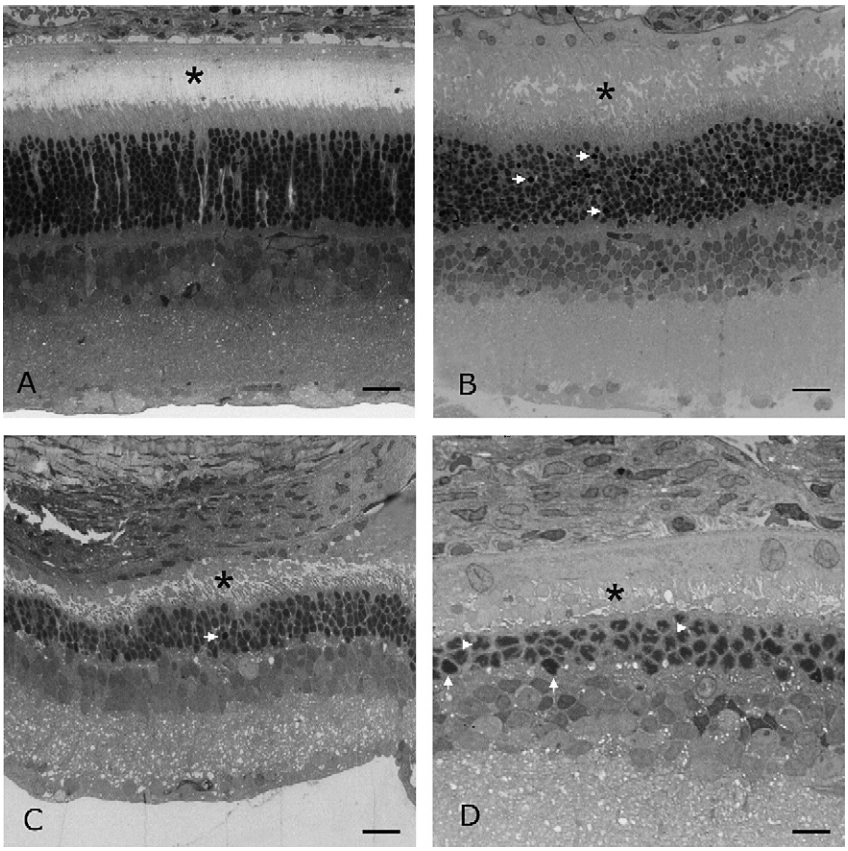

Fig. 6 – Semithin sections of retinas stained with toluidine blue. (A) Retina of a non-illuminated rat (CTL). All retinal layers are clearly observed. Outer photoreceptor segments are labeled with an asterisk. (B) Retina of a rat illuminated for 24 h (IL24). Observe the disorganization of outer photoreceptor segments (*). Pycnotic nuclei (white arrows) may be observed in outer nuclear layer (ONL). Scale Bar: 30 μm. (C and D) Retinas of a rat illuminated for 7 days (IL7). (C) A severe degeneration of photoreceptors and the presence of cellular debris corresponding to outer and inner photoreceptor segments may be observed (*). There is a decrease of ONL thickness (compare with A) and a pycnotic nucleus is shown in ONL (white arrow). In D, a higher magnification shows pycnotic nuclei (white arrow) and irregular nuclei (arrow heads) in ONL. Retinal pigment epithelial cells appear swollen. C: Scale Bar: 30 μm; D: Scale Bar: 12 μm.

were illuminated for 7 days (IL7) (Figs. 1 and 3). The signal was abolished by administration of the NOS inhibitor L-NAME confirming that the signal of $NOFe^{2+}(DETC)_2$ complex is originated from NO.

In order to obtain the concentration of the adducts, the adjusted simulated ESR signals were calculated for 2, 24 and 48 h of illumination (Fig. 2). The hyperfine coupling constant (a^N) calculated from fitting was 12.6 Gauss. The $NO–Fe^{2+}(DETC)_2$ adduct concentrations were calculated from the respective simulated spectra (Fig. 3). The excessive formation of NO during the first hours of illumination could be responsible of the cytotoxic effects by means of the reaction of NO with superoxide anions and the consequent formation of peroxynitrites (ONOO–).

2.2. *Western blot analysis*

2.2.1. *Expression of nNOS and iNOS in the retina after continuous illumination*

The expression of nNOS and iNOS isoforms in the retina of rats after CI was examined by Western blot. nNOS immunoreactivity was detected in retinal extracts from every time point along the illumination period. There were no significant differences in retinal nNOS levels from illuminated animals compared with retinas from control rats (Fig. 4). Positive immunoreactivity to iNOS was detected in retinal extracts of illuminated animals, but not in the control retinas (Fig. 5). Expression of iNOS was maximum in the retinas of animals illuminated for 24 h (IL24), and the difference with the other illumination periods was significant ($p < 0.01$) (Fig. 5).

2.3. *Retinal histology*

The observation of the retinas of IL24 rats showed disorganization of outer photoreceptor segments and pycnotic nuclei in outer nuclear layer (ONL) (Fig. 6B). After 7 days of CI (IL7) the degeneration of photoreceptors was severe and there was a decrease of retinal thickness due to cell death which occurred mainly in ONL (Figs. 6C and D). Irregular nuclei and nuclear fragmentation were observed in ONL at this illumination timepoint.

3. Discussion

In the present work, the increase of NO was demonstrated in the rat retina after CI, an animal model of retinal degeneration (Abler et al., 1996; La Vail et al., 1999; Marti et al., 1998; Reme et al., 1998a, 2003). The use of EPR technique rendered a $NOFe^{2+}(DETC)_2$ complex spectrum with a triplet characteristic signal which corresponded to NO. This signal was previously reported in other biological models by the use of the present spin trapping agent (Fujii and Berliner, 1999; Kikuchi et al., 1993; Mulsch et al., 1995; Shen et al., 1998). The original technique described the i.p. administration of DETC followed by the subcutaneous administration of ferrous sulphate to detect NO signal. However to obtain a working concentration of the trapping agent in the retina we needed to perform an additional intravitreous administration of both (DETC and ferrous sulphate) after anesthesia and before the sacrifice. This modification let us to detect NO signal in the retina by EPR.

It may be argued that ferrous sulphate induces chemical changes and leads to retinal degeneration but these events take 3 and 7 days respectively (Homan et al., 2000). In our experimental condition, the short time that ferrous sulphate was in the eye (2–5 min), was insufficient to induce retinal degeneration but enough to form detectable levels of $NOFe^{2+}(DETC)_2$ complex. In addition, the injection of ferrous sulphate always followed the administration of the trapping agent DETC. Finally, the administration of the NOS inhibitor L-NAME suppressed the spectrum adding a major support to the present results and confirming the specificity of the technique.

As was mentioned previously, the biggest signal was found after 24 h of CI (IL24). The signal decreased thereafter being similar to control values at IL7. Although it is difficult to indicate causality, the excessive formation of NO during the first hours of illumination could be one factor involved in light-induced cytotoxic effects. Although NO plays a physiological role, the excessive production of NO is neurotoxic because it reacts with superoxide anion forming peroxynitrite anion ($ONOO^-$), which is a highly reactive molecule, harmful per se, and responsible for the formation of other highly reactive radicals (Dawson and Dawson, 1994). These ROS/RNS peroxidate fatty acids, induce nitration of proteins impairing protein functions, such as tyrosine kinase signal transduction pathways, and alter DNA strands (Specht et al., 1999), triggering high energy requiring repair mechanisms and apoptotic pathways that participate in LIRD (Beatty et al., 2000; Organisiak et al., 1998). An alternative explanation is that NO could also release cytochrome *c* from mitochondriae triggering apoptosis by an intrinsic pathway. Whichever is the apoptotic pathway involved, certainly NO plays a cytotoxic role in the retina during LIRD as NOS inhibitors protect against light damage in albino rats and mice (Goureau et al., 1993; Kaldi et al., 2003).

Although, free radical formation was suggested during CI there were no direct demonstrations of NO formation during light exposure. The present work is the first direct demonstration of the increase of NO production along illumination in the retina as ESR directly detects NO. Electron paramagnetic resonance was previously used to demonstrate the formation of ROS in the retina of diabetic induced animals (Szabo et al., 1997), however this is the first time that the technique was used to demonstrate NO in the rat retina using the model of CI.

Western blot is a specific method that is based on the specificity of the antibodies. Previously characterized commercial monoclonal antibodies were used in the present work to demonstrate the different enzyme isoforms. Western blot analysis showed that nNOS and iNOS are expressed in the retina along CI. As was mentioned before, both NOS isoforms were previously reported in the normal rat retina. Neuronal NOS is localized in amacrine cells, in horizontal cells, in some bipolar cells, in a small group of neurons localized in GCL and in fibres at IPL (Haberecht et al., 1993; Yamamoto et al., 1993); while iNOS is localized mainly in Müller cells and RPE (Goureau et al., 1994; López-Costa et al., 1997). The present Western blot results showed an important expression of the iNOS during CI with a maximal peak at 24 h of CI. The increase was coincident with the peak of $NOFe^{2+}(DETC)_2$ complex signal detected by ESR. The reduced levels of iNOS at later exposure times would be explained by cell loss. It must be remarked that iNOS was not detected in control retinas with this technique and that nNOS did not show differences among control and illuminated retinas at any time point. In consequence, the increase of NO levels during LIRD is due to an induction of iNOS expression.

In summary, this work clearly shows the direct increase of NO during CI and also demonstrates that iNOS is the enzyme isoform involved in NO production during LIRD. The histological study confirmed that retinal damage appears under the present illumination conditions. The increased NO levels correlate with photoreceptor breakdown but further studies are needed to clarify the role of NO in this process.

4. Experimental procedures

4.1. *Chemicals and antibodies*

Mouse monoclonal antibodies to iNOS and nNOS were bought from BD Transduction Laboratories, Lexington, KY, USA; Goat anti mouse antibody conjugated with alkaline phosphatase was obtained from Santa Cruz Biotechnology Inc., CA, USA. Sodium diethyldithiocarbamate (DETC) was purchased from J.T.Baker, Mallinckrodt Baker Inc., Phillipsburg, NJ, USA; N(G)-nitro-L-arginine methyl ester (L-NAME), sodium nitroprusside (SNP), 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and all other chemical reagents were obtained from Sigma Chemical Co., St. Louis, MO, USA.

4.2. *Animals and illumination procedure*

All the animals were kept with food and water ad libitum. Animal care was performed in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals and the principles presented in the Guidelines for the Use of Animals in Neuroscience Research by the Society for Neuroscience.

Male Sprague–Dawley rats (weight: 100–120 g) were obtained from the animal house of the Instituto de Biología y Medicina Experimental (IBYME). Before the experiments rats were kept at 12/12 h light/dark cycles. Four to five rats were simultaneously 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. Lighting level (12,000 lx) was determined using an analog Gossen illuminance meter. Temperature was monitored and the air-conditioned room was maintained at 24 °C approximately to avoid high temperature which may accelerate retinal damage (Organisciak et al., 1995). In order to obtain the previously mentioned number of rats per time point, this procedure was repeated three times for NO determination by EPR and 4 times for Western blot.

The rats were continuously illuminated for 24 h, 48 h, 5 and 7 days (referred as IL24, IL48, IL5 and IL7, respectively). In addition to these exposure times, a shorter period of illumination (2 h referred as IL2) was also performed for NO determination by EPR. This time point was added in order to observe if there was an earlier NO peak to that observed at IL24 as is shown in Results. Control (CTL) rats were not illuminated and were kept at 12/12 h light/dark cycles until their sacrifice. Three animals per time point were used for NO determination by EPR, another four animals per time point were used for Western blot.

4.3. *NO detection by electron paramagnetic resonance*

NO production was detected by EPR/ESR using diethyldithiocarbamate (DETC) as a spin-trap. In order to detect NO production, all the illuminated rats and controls were pretreated intraperitoneally (i.p.) with sodium nitroprusside (SNP) (1 mg/kg) 10/15 min prior to the spin-trap injection. This procedure was performed to obtain NO levels in the range of sensitivity of the equipment. DETC (500 mg/kg) was injected i.p. followed by the administration of a solution containing $FeSO_4{\cdot}7H_2O$ (37.5 mg/kg) plus sodium citrate 250 mg/kg subcutaneously. After 5 min, rats were anaesthetized i.p. with chloral hydrate (350 mg/kg). Once the animals were anaesthetized, DETC (0.125 g/ml) followed by ferrous sulphate (9.4 mg/ml)–sodium citrate (46.9 mg/ml) solutions were injected intra-vitreous in both eyes. This local procedure is unable to lead to retinal degeneration in the few minutes of treatment and is necessary to detect a signal. After 2 to 5 min, retinas were removed and frozen at −20 °C in a quartz tube. The paramagnetic signal of the NO–$Fe^{2+}(DETC)_2$ adduct was recorded at 20 °C using a Bruker ECS106 spectrometer. Instrumental settings were: center field 3420 G, sweep width 100 G, microwave power 20 mW, microwave frequency 9.62 GHz, conversion time 10.24 ms, time constant 40.96 ms, modulation frequency 50 kHz, modulation amplitude 1.00 G, gain 2.00×10^4, resolution 1024 points. All spectra were the accumulation of 20 scans. Negative controls were performed by injecting L-NAME (100 mg/kg i.p.) 30 min before an illumination period of 2 h.

Simulated spectra were obtained by the calculation of the quantum density matrix using the Liouville equation with the incorporation of the modulated Zeeman effect (Kalin et al., 2003). The adjusted experimental signals were obtained using a modification of the Levenberg–Marquardt algorithm (Budil et al., 1996). The NO–$Fe^{2+}(DETC)_2$ adduct yields were estimated by the double integration of the fitted signals using as a standard the stable radical TEMPOL and related to the mass of the retina (Barr et al. in: www.bruker-biospin.com/brukerepr/appnotes.htm.). The adduct yield is the amount of NO–$Fe^{2+}(DETC)_2$ accumulated in the retina at a fixed time.

4.4. *Western blot analysis*

Retinal tissues from 4 different rats at each time point were homogenized in 200 μl of ice-cold lysis buffer (20 mM HCl pH 7.4, 150 mM NaCl, 1% Triton X-100 and 5 μl protease inhibitor cocktail containing 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), pepstatin A, E-64, bestatin, leupeptin and aprotinin (Sigma P8340)) and incubated on ice for 2 h. Tissue homogenates were centrifuged at 10,000 rpm for 10 min and the supernatant removed. Protein concentration was determined by the Bradford assay (Bradford, 1976). Equal amounts of tissue protein extract were subjected to SDS gel electrophoresis on 7.5% (w/v) polyacrylamide gel under reducing conditions. Proteins were transferred onto nitrocellulose membrane, blocked with 1% BSA (w/v) in TBST [0.01 M Tris–HCl, 0.15 M NaCl, and 0.05% (v/v) Tween-20, pH 7.6] for 1.5 h and subsequently incubated with a monoclonal mouse IgG antibody to iNOS (# 610432, BD Transduction Laboratories) (0.5 μg/μl) or nNOS (# 611852, BD Transduction Laboratories) (0.5 μg/μl) at room temperature for 1 h. After incubation, the membranes were washed four times with TBST (each time for 10 min), followed by a 1 h incubation with a second goat anti mouse antibody conjugated with alkaline phosphatase and revealed with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium to visualize the iNOS or nNOS bands. Macrophages and pituitary from mouse were used as positive control for iNOS and nNOS respectively. The relative intensity of protein signals was quantified by densitometric analysis using the Sigma Gel Program. Statistical analysis was performed using the Graph Pad Program.

4.5. *Histological procedure*

An additional group of 6 rats (2 CTL, 2 IL24 and 2 IL7) were processed to obtain semithin sections for light microscopy (LM) in order to show that illumination produces retinal degeneration.

After illumination, the rats were deeply anesthetized at the different timepoints with chloral hydrate (350 mg/kg) and their eyes were removed, the cornea and lenses were cut off and the remaining tissues with a shape of a cup were fixed by immersion in a solution containing 2.5% glutaraldehyde in 0.1 M phosphate buffer pH: 7.4 during 24 h. The eyes were cut in four pieces along two perpendicular meridians. The tissue blocks containing schlerotica, choroid and retina were post-fixed in 1% osmium tetroxide (O_4Os), dehydrated and embedded in Durcupan (Fluka Chemie AG). Semithin sections (thickness: 0.5–1 μm) were obtained with a Reichert–Jung ultramicrotome (model Ultracut E) and were stained with toluidine blue. Sections were mounted using DPX (Fluka-Chemie AG), and were observed and photographed using a Zeiss Axiophot LM (Zeiss, Oberkochen, Germany).

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