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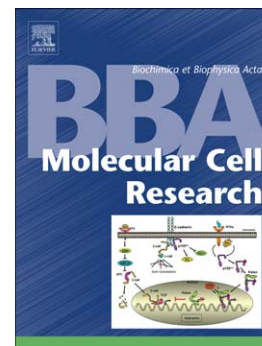

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PROTECTIVE EFFECTS OF RETINOID X RECEPTORS ON RETINA PIGMENT EPITHELIUM CELLS

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Abbreviations: AMD, age-related macular degeneration; RPE, retina pigment epithelium cells; ROS, reactive oxygen species; PUFA, Polyunsaturated fatty acids; DHA, docosahexaenoic acid; RXRs, retinoid x receptors; PPAR, peroxisome proliferator-activated receptor; LXR, liver X receptor; RAR, Retinoic acid receptor; Bclxl, B-cell lymphoma-extra large; Bcl-2 B-cell lymphoma-2; Bax, Bcl-2-associated X protein; NFκB, Nuclear factor kB; RA, Retinoic acid; H_2O_2, hydrogen peroxide, DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PBS, phosphate buffer saline.

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ABSTRACT

Age-related macular degeneration (AMD) is among the main pathologies leading to blindness in adults and has currently no cure or effective treatment. Selective apoptosis of retina pigment epithelial (RPE) cells results in the progressive loss of photoreceptor neurons, with the consequent gradual vision loss. Oxidative stress plays an important role in this process. We have previously determined that activation of RXRs protects rat photoreceptor neurons from oxidative stress- induced apoptosis. In this study we investigated whether RXR ligands prevented apoptosis in an RPE cell line, D407 cells, exposed to hydrogen peroxide (H_2O_2). H_2O_2 induced apoptosis of D407 cells, promoting p65NFκB nuclear translocation, increasing Bax mRNA expression, activating caspase-3 and altering cell morphology. We show, for the first time, that HX630, a RXR pan-agonist, protected D407 cells from H_2O_2-induced apoptosis, preventing p65NFκB nuclear translocation, increasing Bclxl and PPARγ mRNA levels and simultaneously decreasing Bax mRNA levels and caspase-3 activation. Pretreatment with a RXR antagonist blocked HX630 protection. LG100754, which binds RXRs but only activates heterodimers and is an antagonist of RXR homodimers, also had a protective effect. In addition, only agonists known to bind to RXR/PPARγ were protective. As a whole, our results suggest that RXR activation protects RPE cells from oxidative stress-induced apoptosis and this protection might involve signaling through a heterodimeric receptor, such as RXR/PPARγ. These data also imply that RXR agonists might provide potential pharmacological tools for treating retina degenerative diseases.

Keywords: retina pigment epithelium cells, retinoid x receptors, hydrogen peroxide, oxidative stress, peroxisome proliferator-activated receptor.

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1. Introduction

Age-related macular degeneration (AMD), an ocular neurodegenerative disease, is currently one of the major challenges in ophthalmological research. Despite extensive research, this disease has no cure or effective treatment so far, being the major leading cause of vision loss in the Western world.

Retinal pigment epithelium (RPE) cells, located between the choroid and the neural retina in the eye, interact closely with photoreceptor outer segments and have several functions that make them essential to preserve visual function. Their dysfunction is a fundamental, early event that leads to clinically relevant changes in AMD. In this disease, degeneration of RPE cells by apoptosis results in the progressive and irreversible loss of photoreceptor neurons with a consequent gradual loss of vision. [1].

Oxidative stress plays an important role in this process. The macula, the retina region essential for visual acuity, is particular susceptible to oxidative stress in RPE cells. Phagocytosis of photoreceptor outer segments, lipid peroxidation, and light induced-stress, together with the high oxygen tension in the macular and choroidal region lead to the generation of reactive oxygen species (ROS) [2-6]. H_2O_2 is a ubiquitous oxidant found in ocular tissues in vivo; it is produced in the RPE during phagocytosis of photoreceptor outer segments [7-8]. Different types of oxidative stress induce RPE cells degeneration through different pathways [9] and ROS cytotoxic effect is a key cause of cell death.

The effects of diets enriched in polyunsaturated fatty acids (PUFA)s, mainly docosahexaenoic acid (DHA) in patients having retinal degeneration such as Retinitis Pigmentosa and AMD are still under debate [10-16]. DHA, which has protective effects in some retinal degenerations, is an endogenous ligand for retinoid X, nuclear, receptors (RXR) [17]. We have recently determined that DHA activation of RXRs protects rat photoreceptor neurons from apoptosis induced by different types of oxidative stress [18]. We have also shown that two noncommercial, synthetic agonists, PA024 and HX630, have a similar protective effect, implicating RXR activation in retina cell survival.

RXRs, which are members of the superfamily of steroid / thyroid receptors, are involved in numerous cellular processes from cell proliferation to lipid metabolism and are essential for normal eye development [19]. These

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receptors act as transcription factors that bind to DNA response elements after forming homo or heterodimers with other members of this receptor family, and mediate the biological effects of many hormones, vitamins, and drugs. This complexity, together with the fact that there are three subtypes of RXRs, explains the pleiotropic effects of these receptors [20]. Work using mice with mutated genes for each of the RXR isoforms evidence that these receptors regulate several developmental processes, including neurogenesis [19]. The RXRα isoform is the most relevant during eye development as RXRα null mutants exhibit ocular abnormalities; these mutants die after E14.5, making it impossible to study its function at later developmental times. However, when the RXRα gene is overridden only in retinal epithelial cells (RPE RXRα - /-), the resulting failure in the functionality of these cells leads to degeneration of photoreceptors [21]. The functional role of this nuclear receptor in RPE cells is largely unknown and it is of great interest to investigate whether RXRs regulate apoptosis in RPE cells and whether they might prevent retinal neurodegeneration.

DHA, a RXR agonist as mentioned above, has also been shown to protect RPE cells from oxidative stress [9, 22]. However, PUFAs are easily peroxidized and metabolized to bioactive subproducts that lead to lipid dysregulation and accumulation in and under RPE cells and Bruch's membrane. AMD is characterized by the accumulation of lipid and protein rich extracellular deposits under the RPE including drusen, basal linear deposits and basal laminar deposits [23], collectively referred to as sub-RPE deposits; hence, DHA supplementation might exacerbate this process. In addition, DHA gives rise to bioactive metabolites such as Neuroprotectin D1, which has been shown to protect RPE cells from oxidative damage [24], so using DHA would make it difficult to discern whether the protection is due to DHA itself or to Neuroprotectin D1. Hence, we reasoned that testing agents more stable than DHA would be more convenient to analyze the potential role for specific RXR activation in the regulation of retinal degeneration.

In this work we investigated whether RXR ligands modulate apoptosis in a RPE human cell line, D407, exposed to hydrogen peroxide (H_2O_2), a well-established model to study RPE apoptosis associated with oxidative stress [25-27]. We show, for the first time, that RXR signaling protects RPE cells from oxidative stress. Our results demonstrate that the RXR synthetic agonist HX630 protected RPE cells from apoptosis induced by H_2O_2 by blocking the nuclear translocation of p65NFκB, increasing Bclxl and PPARγ mRNA levels and decreasing Bax

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mRNA levels and caspase-3 activation. Our data suggest that the RXR/PPARγ heterodimer or, less probably, RXR/RXR homodimer, but not RXR/RAR, are involved in this protective effect.

2. Materials and methods

2.1. Materials

Plastic 35- and 60-mm diameter culture dishes (Cellstar) were from Greiner Bio-One (Frickenhausen, Germany). Dulbecco's modified Eagle's medium (DMEM) and trypsin were from Invitrogen (Carlsbad, CA). 4,6-diamidino-2-phenylindole (DAPI), MTT reagent (M2128), Gentamicin, pan-cytokeratin antibody (c1801), Bovine Suerum Albumin (BSA), LG100754 (SML0771), LG100268 (SML0279), Bexarotene (SML0282), all-trans-retinoic acid (RA) (98% purity by HPLC) and Paraformaldehyde were from Sigma-Aldrich (St. Louis, MO). High-capacity cDNA Reverse Transcription Kit and primers for RQ-PCR, Terminal Deoxynucleotidyl Transferase, Recombinant, 5-bromo-2´deoxyuridine 5´-triphosphate (BrdUTP) and Terminal Deoxynuleotidyl Transferase (TdT) Buffer were from Molecular Probes, Invitrogen (Argentina). H_2O_2 was from Merck (Argentina). Antibody for CRALBP (GTX15051) was from GeneTex. Antibody for cleaved caspase-3 (D175) was from Cell Signaling. The secondary antibody Cy2-conjugated goat anti-mouse was from Jackson ImmunoResearch (West Grove, PA). Secondary antibodies, Cy2-conjugated-goat anti-mouse and Cy5-conjugated-goat anti-rabbit were from Molecular Probes (Eugene, Oregon). KAPPA SYBRs FAST qPCR Kit were from Biosystems S.A (Buenos Aires, Argentina). Monoclonal antibodies for pan-RXR (sc774), RXRα (sc553), and antibodies used for Western blot, goat anti-mouse IgG-horseradish peroxidase (HRP) and goat anti-rabbit IgG-HRP, antibody for p65NFκB (sc109) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibody for RPE65 (NB100-355) was from Novus bio (United States). RXR non-comercial pan-agonists, HX630 and PA024, and pan-antagonist HX531 were generous gifts from Dr. Kagechika (Tokyo Medical and Dental University, Japan). Solvents were HPLC grade, and all other reagents were analytical grade.

2.2. Cell culture

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The human retinal pigment epithelium cell line D407 was a generous gift from Dr. E. Rodriguez-Bouland (Weill Medical College of Cornell University, New York, NY, USA). Cells were cultured in growth medium (DMEM) supplemented with 5% fetal bovine serum (FBS) and 0.5 % gentamicin and were incubated at 37°C in a humid atmosphere of 5% CO_2. Before treatment, cells were starved in DMEM without FBS for 18-21 h.

2.3. Agonist treatment and oxidative damage

All agonist treatments were performed by adding different agonists or vehicle 1 h before induction of apoptosis with H_2O_2, in DMEM without FBS. The agonists used were: HX630 [28-30] at 0.01 -5 μM final concentration, Bexarotene (LGD1069) [31] at 0.001 -10 μM, PA024 [28-30] at 0.001-10 μM, LG100268 [30-31] at 0.1-10 μM, LG100754 [32] at 0.01-10 μM, all-trans-RA [33] at 10^{-5}-10^{-11} μM. Stock solutions of all-trans-RA (1 mM in absolute ethanol, EtOH) were prepared, maintained in the dark at −70°C, and diluted further in DMEM at the time of supplementation; aliquots were added under dim light to reduce RA degradation. In some experiments, all-trans-RA (10^{-7} μM) plus Bexarotene (0.1 μM) or LG100268 (5 μM) were added at the same time. Alternatively D407 cells were treated with the antagonist HX531 [34-35] at (0.1-5 μM) 1 h before agonists treatments. One hour after agonist supplementation, the cultures were treated with H_2O_2 at final concentrations of 250, 500 and 750 μM for different times.

2.4. Immunocytochemical Methods

Cultures were fixed for 1 h with 4% paraformaldehyde (PF) in PBS, followed by permeation with 0.1% Triton X-100. Then the cultures were stained for 10 min with DAPI, TOPRO-3, or 30 min phalloidin at room temperature. Alternatively, immunostaining of cells was carried out using anti-RPE65 mouse antibody (1:250), anti-CRALBP mouse (1:100 dilution), pan-cytokeratin mouse (1:50 dilution), pan-RXR rabbit (1:50), RXRα rabbit (1:100), cleaved caspase-3 rabbit (1:400) , p65NFκB rabbit (1:100) and then with a secondary antibody anti specific specie conjugated with Cy2 or Cy3 (1:200 dilution) for 1 h at room temperature. Excess label was removed by washing with PBS prior to microscopy. Cultures were then analyzed by phase-contrast and fluorescence microscopy (Eclipse E600; Nikon, Tokyo, Japan, with a CC phase contrast turret condenser and a Y-FL epi-fluorescence

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attachment), and a laser scanning confocal microscope (DMIRE2; Leica, Wetzlar, Germany) with a X 63 water objective. Images were collected and processed (LCS software; Leica, and Photoshop Elements 7.0; Adobe Systems, San Jose, CA).

2.5. Quantitative image analysis

Fluorescence intensities of the 8- or 16-bit image were analyzed after manually outlining regions of interest (ROI) with the software Image J (NIH, Bethesda, MD), as previously described [36]. The average fluorescence intensity of a given ROI was measured within the stained-positive regions of the cell and the average fluorescence intensity of an area of the same size positioned over a region outside the cell was subtracted. These measurements were undertaken on randomly chosen cells, selected from phase-contrast images to avoid bias, for several experimental conditions. For illustration purposes, images were processed using Adobe Photoshop, scaled with identical parameters, and pseudo-colored according to a custom designed look-up-table (LUT).

2.6. Cellular apoptosis

Apoptosis was determined by different methods: terminal deoxynucleotide transferase dUTP nick-end labeling method (TUNEL) to evaluate DNA integrity and DAPI staining to assess the amount of fragmented or pyknotic nuclei. For TUNEL staining, the cells were fixed and stored in 70% ethanol for 72 hours at 20°C. Then, cells were incubated with 2% PF for 15 min, Triton X-100 for 10 min. They were pre-incubated with 1X TdT buffer for 15 minutes and incubated with the TdT reaction mixture (0.05 mM BrdUTP and 0.3 U/L TdT in TdT buffer) at 37°C in a humidified atmosphere for 1 hour. The reaction was stopped by 15-minute incubation with stop buffer (300 mM NaCl and 30 mM sodium citrate; pH 7.4) at room temperature. Negative controls were prepared by omitting TdT. BrdU uptake was determined with an anti-BrdU monoclonal antibody, according to a standard immunocytochemical technique. Nuclei integrity was evaluated after staining cell nuclei with DAPI; the cells were considered to be apoptotic when they showed either fragmented or condensed (pyknotic) nuclei. Nuclei integrity was evaluated after staining cell nuclei with DAPI and TOPRO-3, fluorescent dyes that bind to DNA. Briefly, cells were permeated with Triton X-100, washed with PBS and incubated with DAPI for 10 min or

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TOPRO-3 for 5 min. Cells were considered to be apoptotic when they showed either fragmented or condensed (pyknotic) nuclei, a characteristic feature of apoptotic cell death.

2.7. DNA ladder visualization

Genomic DNA was isolated from D407 cells using DNAZOL Reagent (Life technology). The extraction process was performed according to manufacturer's instructions. Samples were resuspended in 8 mM NaOH (DNAse free) at a final concentration of 2 µg/ml. DNA concentration in each sample was determined spectrophotometrically. DNA (~20 ng) was loaded onto each lane of a 1.2% agarose gel, and electrophoresis was performed in Tris-borate-EDTA buffer (45 mM Tris-borate, 1 mM EDTA, pH 8.0). Bands were visualized with ethidium bromide.

2.8. Analysis of cell viability

Half an hour before the end of each treatment, the supernatant was extracted (which was saved for processing) and 1 ml PBS 1X mixed with 10 µl MTT reagent (5 mg/mL) was added to each well. After 30 min incubation at 37°C, MTT was removed and solubilization buffer was added to dissolve the formazan crystals and the absorbance was measured at 590 nm using a spectrophotometer and normalized with proteins. Wells containing only solubilization buffer were used as blanks and were subtracted as background from each sample. Results were expressed as a percentage of the untreated cells. In parallel cells in the supernatant were counted with Neubauer chamber and used as a correction factor. Results were expressed as percentage of cell viability relative to controls, considering this value as 100%.

2.9. Western blot analysis

After treatments, cells were washed with PBS buffer plus 25 mM NaF and 1 mM Na3VO4, and lysed in buffer containing 50 mM Tris HCl (pH 7.4), 150 mM NaCl, 3 mM KCl, 1 mM EDTA, 1% Tween-20, 1% Nonidet P-40, 20 lg/ml aprotinin, 20 lg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF), 25 mM NaF and 1 mM Na3VO4. The lysates were incubated on ice for 10 min, vortexed for 45 s and maintained on ice for another 10

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min. After centrifugation at 13,000g and 4 °C during 15 min the supernatant was collected and proteins were quantified by the Bradford method [37]. Lysate proteins dissolved in Laemmli sample buffer were separated (35 µg/lane) on 10% SDS–polyacrylamide gels and electrotransferred to PVDF membranes. After blocking with 5% non-fat milk in PBST buffer (PBS 1X, 0.1% Tween-20), the membranes were incubated overnight with the appropriate dilution of primary antibody in PBST plus 5% BSA. After washing, the membranes were incubated with the appropriate dilution of horse radish peroxidase-conjugated secondary antibody in PBST plus 1% non-fat milk. Finally, the blots were developed by ECL.

2.10. Real-time quantitative polymerase chain reaction (RQ-PCR)

Total RNA was isolated using the Quick-Zol reagent (Kalium Technologies). The extraction process was performed according to manufacturer's instructions. The amount of RNA was measured by spectrophotometry. Reverse transcription of total RNA was performed using the High-Capacity cDNA Reverse Transcription Kit. Quantitative PCR was done by SYBR Green real-time PCR methods. PCR was performed as previously described [38]. The relative mRNA expression was calculated using the comparative threshold method (Ct-method) with 18s for normalization. All experimental conditions were processed in triplicate. RQ-PCR primers were specifically designed to amplify the following cDNAs (Table I). The efficiency was about 90% to each pair of primers.

Target	Forward	Reverse
h-Bax	5´ TCCAGCTCTTTAATGCCCGT 3´	5´GCGTCCCAAAGTAGGAGAGG 3´
h-Bclxl	5´CTTCCCCTTCTCACCAGCTC 3´	5´ TTCTGTCAGAGCCCCTCCAT 3´
h-PPARγ	5´TAAAAGTTGGGCTGCTTTGCC 3´	5´ ACCTCCTTACCTGTCCGTGT 3´
h-18s	5´CGTCGTCCTCCTCGCTTG 3´	5´ TAGGTAGAGCGCGGCGA 3´

2.11. Statistical analysis

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The results represent the average of at least three separate experiments (± SD), unless specifically indicated, and each experiment was performed in triplicate. For cytochemical studies, 10 fields per sample were analyzed in each case. Statistical significance of data was evaluated using Student's t-test, and probability values below 0.05 ($p<0.05$) were considered significant. Quantitative data were expressed as means ± standard deviation (SD) from the indicated set of experiments.

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3. Results

3.1. Characterization of the retinal pigment epithelial progeny

To study the role of RXRs in RPE cells subjected to oxidative stress, we used the RPE cell line D407. We first confirmed the identity of D407 cells as retinal epithelial cells by analyzing the expression of different markers such as RPE65, CRALBP and Cytokeratin (Supplementary figure). RPE65 (retinal pigment epithelium-specific protein 65kDa), an all-trans-retinyl ester isomerohydrolase, is essential for the visual cycle [39] and is one of the retinal epithelial cells markers up-regulated in differentiated retinal pigment epithelial cells [40]. CRALBP, which participates in the transport of retinoids to the retina, is found in RPE and Muller cells of the retina in vivo. Cytoqueratin, an intermediate filament protein, is found in human RPE cells in vivo and in most other human epithelia [41]. Immunocytochemical analysis revealed D407 cells expressed RPE65, CRALBP and pan-cytokeratin on their cytoplasm (Supplementary figure).

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3.2. D407 retinal epithelial cells express RXR

We previously reported that RXRs are expressed in ARPE19 cells, another retinal pigment epithelial cell line [18]. Using a pan-RXR antibody we now determined that RXRs were expressed in D407 cells and this expression was mostly localized in the cell nuclei (Fig. 1A, upper line). Since RXRα isoform is the most relevant during eye development [21] we also investigated its expression. RXRα isoform was present in D407 cells (Fig. 1A, lower line), showing an intense labeling in the nuclei and in the peri nuclear region. Western blot analysis confirmed the expression of RXR and RXRα isoform in D407 cells (Fig. 1B) and also in the ARPE19 cell line.

3.3. H_2O_2 induces cell death in D407 cells

We first investigated the effect of different concentrations of H_2O_2 on cell viability in D407 cells, by the MTT assay. Cell survival was not affected by exposure to 250 μM H_2O_2 for 24 h (Fig. 2A). Survival of D407 cells was reduced upon treatment with 500 and 750 μM H_2O_2 for 24 h. A 53 ± 8.6% reduction of cell survival compared to controls (100 ± 7.1%) was observed with 500 μM H_2O_2. A 5 h treatment with 500 μM H_2O_2 led to a significant

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25 % reduction in cell viability compared to controls (Fig. 2B). Since these conditions reduced cell detachment, we chose them when performing inmunocytochemical analysis was required.

3.4. HX630 rescued D407 cells from apoptosis induced by oxidative stress

We previously demonstrated that DHA and HX630, a RXR agonist, protect photoreceptors from different types of oxidative stress through RXR activation [18]. To determine RXR involvement in RPE cell protection after oxidative damage, we investigated the effect of different concentrations of HX630 on preventing D407 cell death. MTT assays revealed that 1 µM HX630 efficiently prevented D407 cell death after both 24 and 5 h of H_2O_2 treatment (Fig. 3A). Apoptosis was tested by the DNA ladder formation assay, as the presence of the genomic DNA ladder is extensively used as a marker for apoptotic cell death. As shown in Fig. 3B, DNA laddering was observed after a 5 h treatment with H_2O_2 (500 µM) in D407 cell lysates. DNA ladder formation was reduced with HX630 addition, being similar to that observed in controls. H_2O_2 triggered apoptosis in D407 cells, increasing the number of cells having fragmented or pyknotic nuclei (Fig. 3C X) and of cleaved caspase-3 positive cells (Fig. 3C XI), compared to controls (Figs. 3 C II and III, respectively). As observed in Fig. 3 D and E, H_2O_2 induced 13-fold increase in the percentage of cells having fragmented or pyknotic nuclei and 10-fold increase in the percentage of caspase-positive cells, compared to controls, respectively ($p<0.05$). HX630 supplementation before H_2O_2 addition protected RPE cells, reducing the percentage of D407 cells with fragmented or pyknotic nuclei from 27% in H_2O_2–treated cultures to nearly 12% (Fig. 3C XIV, 3D) and the percentage of cleaved-caspase 3 positive cells (Fig. 3C XV, 3E), from 20% to nearly 9% ($p< 0.05$).

We also studied RXR involvement in apoptosis prevention after H_2O_2 treatment by measuring the mRNA levels of both a pro, Bax, and an anti-apoptotic, Bclxl, Bcl-2family protein, by RQ-PCR (Fig. 4), since these proteins are modulated by a bioactive subproduct of DHA in RPE cells upon oxidative stress [22]. The relative expression levels of Bax and Bclxl mRNA in D407 cells were tested following a 5 h treatment with 500 µM H_2O_2, with or without addition of the HX630 agonist. As shown in Figure 4, the mRNA expression level of pro-apoptotic Bax significantly decreased upon HX630 addition, from 1 ± 0.02 to 0.7 ± 0.1 (arbitrary units) compared to H_2O_2

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treated cells. The mRNA expression level of anti-apoptotic Bclxl was undetectable in cells treated with either HX630 or H_2O_2. However, when cells were treated with HX630 prior to H_2O_2, we were able to detect Bclxl mRNA and its level increased to 1.8 ± 0.18, compared with Bax mRNA level (Fig. 4). These results suggest that HX630 may act at the transcription level, being involved in the signaling that regulates promoters of Bcl-2 family genes.

3.5. HX531, a RXR antagonist, blocked HX630 protective effects on apoptosis

We have established that HX630, a RXR pan-agonist, protects D407 cells from apoptosis induced by oxidative stress. To investigate whether HX630 exerted its protective effect through specific and unique activation of RXRs, D407 cells were treated or not (control) with HX531, a RXR pan-antagonist, prior to the addition of HX630, and then exposed to oxidative damage for 5 h. We first analyzed the disruption of actin cytoskeleton, an early event in cell death. H_2O_2 disrupted actin filaments and significantly altered cellular shape, whereas pre-treatment with HX630, before H_2O_2 addition mitigated these changes (Fig. 5A and C). Supplementation with the antagonist HX531, either without or with the addition of the HX630 agonist did not affect the actin cytoskeleton (Fig. 5A). Pre-treatment with HX531 had no effect on H_2O_2-induced disruption of actin filaments; cytoplasmic retraction was clearly evident in this condition. Noteworthy, pre-treatment with HX531 completely blocked HX630 preservation of actin cytoskeleton upon H_2O_2 treatment (Fig. 5A and C).

We then evaluated the effect of this antagonist on H_2O_2 induced apoptosis of D407 cells. H_2O_2 treatment increased the amount of cells with pyknotic or fragmented nuclei (Fig. 5A and D) and of TUNEL-positive cells compared with controls (Fig. 5B and E), inducing a 13-fold increase in RPE cells with fragmented or pyknotic nuclei and 15-fold increase in TUNEL-positive, compared with controls, respectively ($p< 0.05$) (Fig. 5D and E). HX630 supplementation protected D407 cells from H_2O_2 induced apoptosis (Fig. 5A), reducing the percentage of cells with fragmented or pyknotic nuclei from 27% in H_2O_2 treated cultures to nearly 11% ($p<0.05$) (Fig. 5D) and the percentage of TUNEL-positive cells from about 17% to nearly 7% (Fig. 5E). However, when cultures were pretreated with HX531, before HX630 and H_2O_2 addition, the number of TUNEL-positive cells (Fig. 5B and E)

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and the percentage of RPE cells with fragmented or pyknotic nuclei were similar to those found in H_2O_2-treated cultures lacking HX630 ($p < 0.05$) (Fig. 5D). As a whole, these results suggest that the protective effect of HX630 on RPE cells was exclusively due to its activation of RXR.

3.6. HX630 prevented the nuclear translocation of p65NFκB in D407 cells

An increase in ROS, as that induced by H_2O_2 treatment, has been shown to play an important role in the activation of NFκB, which translocates to the nucleus in order to activate its target genes [42].

To determine p65NFκB nuclear translocation in D407 cells treated with H_2O_2 and the effect of RXR activation on this translocation, we evaluated the relative fluorescence intensity determined in cell nuclei in each experimental condition by immunocytochemistry, establishing as 100 arbitrary units the fluorescence intensity measured in the nuclei in controls. H_2O_2 treatment led to an increase in p65 NFκB fluorescence in nuclei, compared to that observed in controls (Fig. 6A) and pre-treatment with HX630 markedly reduced this increase. Treatment with H_2O_2 resulted in about a 5-fold (500 arbitrary units) increase in fluorescence intensity, i.e., in nuclear p65NFκB translocation compared to controls (Fig. 6A, arrowheads and Fig. 6B). When the cultures were supplemented with HX630 before H_2O_2 treatment, nuclear translocation of p65NFκB significantly decreased from about 500 to nearly 345 arbitrary units (p 0.05) (Fig. 6A and B). This suggests that HX630 prevented p65NFκB nuclear translocation in RPE cells to exert its protective effect.

3.7. Peroxisome proliferator-activated receptor (PPAR) γ, a novel target of HX630 in D407 cells

PPARs are permissive partner receptors of RXRs. To determine whether RXR stimulation modified the expression levels of PPAR genes, cells were treated with HX630 prior to H_2O_2 addition and PPARγ mRNA levels were then quantified by RQ-PCR analysis (Fig. 7). PPARγ mRNA was undetectable in D407 cells lacking RXR agonists whereas stimulation with the agonist HX630 slightly increased PPARγ mRNA levels (1 ± 0.4 relative units). Noteworthy, D407 cells treated with HX630 and exposed to oxidative stress markedly increased PPARγ mRNA levels (17 ± 1.9 relative units) (Fig. 7). These results suggest PPARγ is a possible HX630 gene target that might be involved in preventing apoptosis induced by oxidative stress.

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3.8. Several agonists of RXRs prevent apoptosis in D407 cells

We here demonstrate that the RXR pan-agonist, HX630, protected RPE cells upon oxidative stress (Figures 3-5). RXRs can form homodimers or heterodimers with other nuclear receptors. To narrow down the identification of their possible partners, we evaluated the protective effect of RXR agonists with different affinities for RXR homo or heterodimers. D407 cells were incubated with different doses of several RXR agonists (Bexarotene, PA024, LG100754 and LG100268) or all-trans-retinoic acid (RA), an agonist for retinoic acid receptor (RAR), prior to H_2O_2 addition, and either cell viability was evaluated by MTT assay 24 h later, or cells were analyzed by inmunocytochemistry 5 h later (data not shown). First we analyzed the involvement of RXR homo or permissive heterodimers using Bexarotene, PA024, LG100754 and LG100268, which are RXR pan-agonists of different chemical structure and with different affinity to the RXRs binding site. These ligands exhibit heterodimer selectivity. However, it is not yet possible to predict a correlation between ligand structure and physiological response [43]. Bexarotene and its variant, LG100268, are potent pan-retinoid agonists. HX630 and PA024 show different patterns of activation of RXR/PPAR and RXR/LXR heterodimers. Our results show that 0.01 μM PA024 and 0.1 μM Bexarotene have protective effects comparable to that of 1 μM HX630 (Table IIA). A similar protective effect was found with LG100754, an RXR agonist that exhibits antagonist activities toward RXR homodimers, but acts as an agonist for RXR heterodimers. In contrast, LG100268, with similar binding affinity to Bexarotene but more RXR specific, showed no protection at any concentration analyzed (Table IIA). These results suggest that RXR permissive heterodimers were undoubtedly involved, although we cannot rule out RXR/RXR participation.

We also studied RXR non permissive involvement using all-trans-RA, which is an agonist for RAR but not for RXR [44]. All-trans-RA alone did not prevent cell death at the doses tested. We reasoned that RAR activation by itself might be insufficient and that activation of RXR might also be required to protect RPE cells from oxidative stress. To investigate this hypothesis, we supplemented the cultures with all-trans-RA plus two different RXR agonists, Bexarotene, which promoted RPE cell survival by itself, and LG100268, which had no protective effect, as we showed above. Only Bexarotene promoted RPE cell survival together with all-trans-RA, as had been

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established when Bexarotene was added alone (Table IIB). These results suggest that RXR/RAR heterodimers did not participate in the protective effects.

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4. Discussion

The results of the present work provide, for the first time, evidence that RXR agonists can protect RPE, D407 cells from H_2O_2-induced apoptosis. Our work shows that RXR agonists prevented RPE cell death upon H_2O_2 treatment, upregulating Bclxl and decreasing Bax transcription levels, decreasing caspase-3 activation and preventing the nuclear translocation of p65NFκB. Pretreatment with a RXR antagonist blocked this protection, suggesting that RXR activation was essential for the protective effect.

RXRs can form homodimers or heterodimers with many nuclear receptors to mediate the biological effects of many hormones, vitamins, and drugs acting as transcription factors [45], recruiting co-regulator proteins that modify chromatin and the associated transcriptional complex [46]. RXR signaling is involved in nervous system development [47] and is essential for normal development [48], being the RXRα isoform the most relevant for eye development [21]. Our data evidence that RXR nuclear receptors were expressed in D407 RPE cells. We demonstrated, using a pan-RXR antibody, that RXRs were present in D407 cells, mainly with a nuclear localization, whereas the RXRα isoform had both nuclear and perinuclear localization. The discrepancy in localization might arise in the differential sensitivity of the two antibodies, since the RXR alpha-antibody binds to a longer region of the alpha epitope than the pan-RXR antibody.

Oxidative stress in the RPE and retina is triggered by several factors, such as light exposure, and has been implicated in the pathogenesis of age-related macular degeneration (AMD). Generation of intracellular ROS has been related with a cytotoxic effect leading to cell death, and different types of oxidative stress, such as H_2O_2, Paraquat and hypoxia induce degeneration of ARPE19 cells, an extensively used RPE cell line, through different pathways [9]. In these cells, death occurs by necrosis or apoptosis, depending on H_2O_2 concentration, and the mitochondrial pathway is involved in apoptotic death [49-50]. Pintea and coworkers [51] have shown that treatment of D407 cells with H_2O_2 increased ROS, simultaneously decreasing the activities of several antioxidant enzymes and reduced glutathione concentration. In this study, we provide the first evidence of the apoptotic mechanism induced by H_2O_2 treatment of D407 cells. Generation of an acute oxidative stress decreased cell viability, led to remarkable changes in cell morphology and increased the levels of apoptotic markers, such as cleaved caspase-3.

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The protective effects of the pharmacological activation of RXRs upon H_2O_2 treatment in several cells types were recently demonstrated. Activation of RXRs exerts a protective effect against H_2O_2 -induced apoptosis in H9c2 rat ventricular cells through antioxidant and mitochondria-protective mechanisms [52]. In human mesangial cells, 9-cRA, an endogenous RXR agonist prevented H_2O_2-induced cell death; this protection was not due to an increase in H_2O_2 catabolism and persisted even when both catalase and glutathione synthesis were inhibited [53]. We have recently shown that micromolar concentrations of HX630 have an anti-apoptotic effect on retina photoreceptors [18]. Noteworthy, RXR activation prevented H_2O_2-induced apoptosis of D407 cells. HX630 pretreatment, which had no cytotoxic effects in the absence of H_2O_2, partially protected D407 cells from apoptosis induced by H_2O_2 (Fig. 3). HX630 treatment decreased the number of apoptotic nuclei and reduced the alterations in the cytoskeleton induced by H_2O_2 (Fig 5). This RXR agonist also reduced the activation of caspase-3, which was triggered by H_2O_2. This activation leads to the cleavage of several vital molecules downstream in the apoptotic cascade, among them the cleavage of DNA into oligonucleosomal fragments, which allows the association between morphological events and the underlying molecular mechanisms [54]. In this work, we show that HX630 reduced DNA fragmentation, as evidenced by the TUNEL method, and by agarose gel electrophoresis. Pretreatment of D407 cells with the RXR antagonist HX531 completely blocked HX630 protective effect (Fig. 5). Although a direct antioxidant activity of RXR agonists and antagonists cannot be ruled out, it is highly unlikely. H_2O_2, a non-radical species, is extremely reactive and produces ROS, especially hydroxyl radical [55]. RXR agonists and antagonists could act as hydroxyl radical scavengers, and even react directly with H_2O_2, since they have an aromatic structure in addition with carboxylic groups, and some of them, such as HX630, also have a sulfur group. However, in our experimental system the relative amount of H_2O_2 compared to that of RXR agonists or antagonists was at least 500 fold higher, implying that the contribution of a hypothetical scavenging of ROS to their protective effect would be negligible. As a whole, our data suggest that the protective effect of HX630 resulted exclusively from RXR activation.

In both the RPE and retina, oxidative stress shifts the balance of Bcl-2 family protein expression toward that favoring cell damage [56-57]. We here show that H_2O_2 increased the mRNA level of Bax, a pro-apoptotic protein, suggesting that the early RPE response to oxidative stress included transcriptional modulation added to the

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canonical posttranslational events upstream of the mitochondrial apoptotic step. HX630 decreased Bax mRNA level and induced the transcription of Bclxl, an anti-apoptotic protein, leading to an increase in the anti-apoptotic/pro apoptotic protein ratio. Similarly, treatment with RXR agonists of naive T lymphocytes decreases apoptosis by increasing the expression of Bcl2a1, another anti-apoptotic protein [58]. Our results emphasize the relevance of the relative levels of Bclxl and Bax in RPE cell survival upon oxidative stress and imply that HX630 protective effects arised, at least in part, from shifting this balance to an increased Bclxl/Bax ratio.

The role of RXRs on the transcription of Bax and Bclxl might be direct or mediated through another signal. A RXR homodimer or heterodimer might bind to an appropriate response element in the Bclxl and Bax promoter and thereby enhance or diminish transcription, respectively. However, to our knowledge, direct binding of RXRs to the Bax or Bclxl promoter has not been reported up to date. Moreover, in the absence of oxidative stress HX630 did not increase Bclxl mRNA level. A plausible explanation might be that in order to upregulate Bclxl mRNA transcription, HX630 requires either the presence of specific co-activators or the absence of specific co-repressors that are only modulated upon oxidative stress. An alternative explanation is that rather than binding to a response element in Bcl-2 family proteins, RXRs might interact directly with other transcription factors in order to affect transcription. A plausible candidate might be NFκB, one of the most complex transcription factors [59], as pro-oxidative molecules can activate NFκB and this factor has been involved in apoptosis [60]. Other transcription factors such as several members of the steroid receptor family can physically interact with NFκB *in vitro* and have been shown to inhibit NFκB activity [61]. Interestingly, PPARγ, a nuclear receptor, may bind to both p50 and p65NFκB subunits, directly resulting in NFκB inactivation [62-64]. In most cells, NFκB exists in an inactive form in the cytoplasm, bound to inhibitory, IκB, proteins. Different stimuli affecting cells result in the degradation of IκB proteins, resulting in the release and nuclear translocation of NFκB, to activate specific target genes. Our results show that H_2O_2 induced nuclear translocation of the p65NFκB subunit whereas pretreatment with HX630 blocked this translocation. Na and coworkers showed that retinoid receptors form a transcriptionally inhibitory complex with NFkB, which may involve both inhibition of the NFκB-DNA interactions and competitive recruitment of transcription integrators between NFκB and RXR [61]. Our results evidencing the inhibition of NFκB nuclear translocation by RXR activation support an indirect inhibition of NFκB by RXRs.

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This might involve the upregulation of IκB to sequester NFκB [65-66], or, more indirectly, the activation of the Nrf2 transcription factor, in order to reduce the generation of the pro-oxidative molecules required for NFκB activation. Establishing the precise mechanisms involved in the prevention of NFκB nuclear translocation demands further research.

We then investigated which nuclear receptor might be the partner forming dimers with RXRs to achieve the observed protective effect. RXR can function as homodimers or as the obligated partner for other nuclear receptors, forming heterodimers. Some heterodimer partners, such as the PPARγ, are 'permissive' for RXR activity, with ligands for either partner strongly activating these heterodimers [67-68]. On the other hand, other partners such as the retinoic acid receptor (RAR) or thyroid hormone receptor (TR) are 'non-permissive' for RXR, since their heterodimers with RXR mostly do not respond to RXR ligands [44]. To narrow down the possible partner alternatives we tested diverse RXR agonists with different ability to distinguish between homo or heterodimer partners. Our finding that HX630 protected RPE cells in the absence of other agonists and in serum-free media led us to reason that the receptor involved in this protection would probably be either a RXR homodimer or a permissive heterodimer. When using RXRs agonists with differential binding affinity and specificity for RXRs, and differential selectivity for heterodimers, we established that while Bexarotene and PA024 protected RPE cells from oxidative stress, LG100268, which is more RXR specific, did not. Noteworthy, LG100754, which binds RXRs but only activates heterodimers and antagonizes RXR homodimers, also had a protective effect. Hence, although we cannot discard the participation of RXR homodimers, our data support the hypothesis that a heterodimeric receptor was involved in the protective effect.

The puzzling finding that LG100268 did not prevent RPE cell death might be explained by the fact that different RXR agonists do not necessarily exhibit the same biological activities due to the complex protein-protein interactions in the RXR signaling, which include the interaction with various factors, including heterodimers partners, co-repressors, and co-activators. Hence, LG100268 might interact on different heterodimers or require other cofactors that those present in these cells.

Our results also demonstrated that HX630 supplementation increased PPARγ mRNA levels in cells subjected to oxidative stress, suggesting that PPARγ might be a downstream target of RXR activation and/or signaling.

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Activation of PPARγ is known to participate in protection from oxidative stress [69-71]. Interestingly, LG100754, has been reported to be more selective than LG100268 for RXR/PPARs heterodimers in vitro and activation of RXR/PPARγ by both agonists occurs through different mechanisms, as has been shown in transient cotransfection assays [72]. Moreover, LG100754 also functions as a transcriptionally active agonist of RXR/PPARγ heterodimers, enhancing the potency of PPAR ligands [32]. In addition, PA024 and HX630, which protect D407 cells from oxidative stress, have been shown to activate RXR/PPARγ in macrophage cell lines, with PA024 also activating RXR/LXRα [73]. Collectively, these data support the hypothesis that RXR/PPARγ might be actively participating in RPE (D407) cells protection from oxidative damage. HX630 activation of RXR/PPARγ might then lead to increased PPARγ mRNA transcription as a positive retroactive regulation. A possible connection between RXR/PPARγ heterodimers and our findings showing the inhibition of NFκB nuclear translocation and the regulation of PPARγ, Bax and Bclxl mRNA levels would be the involvement of transcription factor Nrf2, which might regulate multiple mechanisms [74-76]. The link between Nrf2 and HX630 will be the aim of future studies.

Retinoic acid (RA) signaling through RXR/RAR is very important during embryonic eye development [45] and for photoreceptor differentiation [33]. Despite RXR/RAR being "non-permissive' heterodimers, some compounds might escape suppression by RAR in certain contexts, as is the case of LG100754 [77]. Hence, we investigated whether RAR receptors were involved in the RPE cell protection from oxidative stress. Our results show that all-trans-RA, the RAR agonist, only had a protective effect when combined with Bexarotene, which is protective by itself, but not alone or combined with LG100268, which has no protective effect. This implies that the RXR/RAR heterodimer is not involved in the protective effects resulting from RXR activation.

5. Conclusion

Our results demonstrate that pan-RXR agonists protected RPE cells from oxidative stress by blocking the nuclear translocation of p65NFκB, increasing Bclxl and PPARγ mRNA levels, decreasing Bax mRNA levels and caspase-3 activation. Our data suggest that the RXR/PPARγ heterodimer or, less probably, RXR/RXR homodimer are

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involved in this protective effect, whereas RXR/RAR is not. Identification of the RXR isoforms and the specific RXR partner involved in this protection is still a complex and exciting task that might contribute to adding potential pharmacological tools for treating devastating retinal diseases.

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Fig. 1. RXRs are expressed in D407 cells. A) Phase (first column) and fluorescence (second-fourth columns) photomicrographs of D407 cells showing labeling of nuclei with DAPI (second column), pan-RXR or RXRα antibodies (second column) and the merge image (fourth column) to visualize the nuclei and different proteins-cell distribution respectively. Scale bar represents 18 μm. B) The levels of RXR and the RXRα isoform were determined by Western blot analysis of protein lysates obtained from D407 and ARPE19 cells (used as a positive control), using pan-RXR and RXRα antibodies.

Fig. 2. Effect of H_2O_2 on the viability of D407 cells. A) D407 cells were treated with 250, 500 and 750 μM H_2O_2 for 24 h, or B) with H_2O_2 (500 μM) for 5 and 24 h and cell viability was evaluated by the MTT assay. *$p < 0.05$ and **$p < 0.01$, statistically significant differences as compared with the control group. Six separate experiments with three samples for each condition were used.

Fig. 3. Effect of HX630 on the prevention of D407 cell apoptosis. A) Bars depict the cell viability (percentage compared to controls) of D407 cells treated for 24 h with 500 μM H_2O_2 (left graph) in the presence or absence of HX630 at 0.1, 1.0, 2.5 μM or for 5 h with (+) or without (-) 1 μM HX630 (right graph). Cell viability was determined by MTT assay. B) Agarose gel electrophoresis of DNA extracted from D407 cells after treatment with HX630 (1 μM) (lane 2), vehicle (lane 3), HX630 (1 μM) plus H_2O_2 (500 μM) (lane 4), or only with H_2O_2 (500 μM) (lane 5) for 5 h; a MW marker (lane 1) was processed in parallel. Bands were visualized with ethidium bromide staining under ultraviolet light. C) Phase and fluorescence photomicrographs show nuclei labeled with DAPI (II, VI, X, XIV) and the levels of cleaved caspase-3 (III, VII, XI, XV) identified using anti- cleaved caspase-3 rabbit polyclonal antibody followed by mouse anti-rabbit Cy2-conjugated secondary antibody in D407 cells treated with 500 μM H_2O_2 for 5 h in the presence or absence of HX630 (1 μM). Six separate experiments with three samples for each condition were used in DAPI analysis. D) Bars depict the percentage of cells having pyknotic or fragmented nuclei and E) the percentage of cells showing cleaved caspase-3 expression, determined by immunocytochemistry, as described in (C). * $p < 0.05$ with respect to control values.

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Fig. 4. HX630 treatment increased Bclxl and decreased Bax mRNA levels in D407 cells. D407 cells were treated with 500 µM H_2O_2 for 5 h in the presence or absence of 1 µM HX630. The levels of Bclxl and Bax mRNA levels was performed by real-time PCR. The expression levels of target genes were standardized by 18s mRNA level in each sample and normalized with respect to Bax-mRNA expression in H_2O_2 condition. UD, undetectable mRNA levels. Results are shown as average value ±SD of data from at least two separate experiments, each sample processed in triplicate. Means with * are significantly different ($p < 0.05$).

Fig. 5. Effect of HX531, an antagonist of RXR, in D407 cells. Epithelial retinal cells were treated with 1 µM of HX630 with or without 500 µM of H_2O_2 in the presence or absence of HX531 (2 µM), a specific RXR antagonist. A) Florescence confocal micrographs show D407 cells with their actin cytoskeleton visualized with phalloidin (red) and nuclei stained with TOPRO-3 (blue); arrowheads indicate fragmented or pyknotic nuclei. B) Phase (first column) and fluorescence photomicrographs show nuclei labeled with DAPI (second column), TUNEL-labeled cells (third column) and merge (fourth column). C) Bars represent the percentage of cells showing cytoplasmic retraction. D) Bars depict the percentage of cells showing fragmented or pyknotic nuclei. E) Bars represent the percentage of TUNEL positive-cells. Scale Bar: 40 µM. * $p <0.05$, ** $p <0.01$ with respect to control values.

Fig. 6. Effect of HX630 on p65NFκB in D407 cells. A) D407 cells were treated with 500 µM of H_2O_2 for 5 h in the presence or absence of HX630 (1 µM) and then inmunolabeled with anti-p65NFκB antibody followed by mouse anti-rabbit Cy3-conjugated secondary antibody. Arrowheads show nuclear translocation of p65NFκB. B) Bars represent the relative fluorescence intensity, measured in the nuclei of treated cells, in arbitrary units, compared to that determined in the nuclei of control cells. * $p <0.05$ with respect to control values.

Fig. 7. Treatment with HX630 increases PPARγ mRNA levels in D407 cells. D407 cells were treated with 500 µM H_2O_2 for 5 h in the presence or absence of 1 µM HX630 and PPARγ mRNA level was quantitated by real-

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time PCR. The expression levels of target genes were standardized by 18s mRNA level in each sample and normalized with respect to HX630-H_2O_2 condition. UD, undetectable levels. Results are shown as average value ±SD of data from at least two separate experiments, each sample processed in triplicate. Means with * are significantly different ($p < 0.05$).

Table II. Effect of different RXR agonists on prevention of D407 cells apoptosis. D407 cells, in the presence or absence of different concentrations (µM) of the indicated agonists (Table IIA), or with the combination of all trans retinoic acid (RA) and either Bexarotene or LG100268 (Table II B), were treated with 500 µM H_2O_2 for 24 h. Cellular viability was measured by MTT assay and was qualitatively quantified in accordance to the degree of protection (+ or ++) or the absence of protective effect (-), compared with the control group. Nd, not determined.

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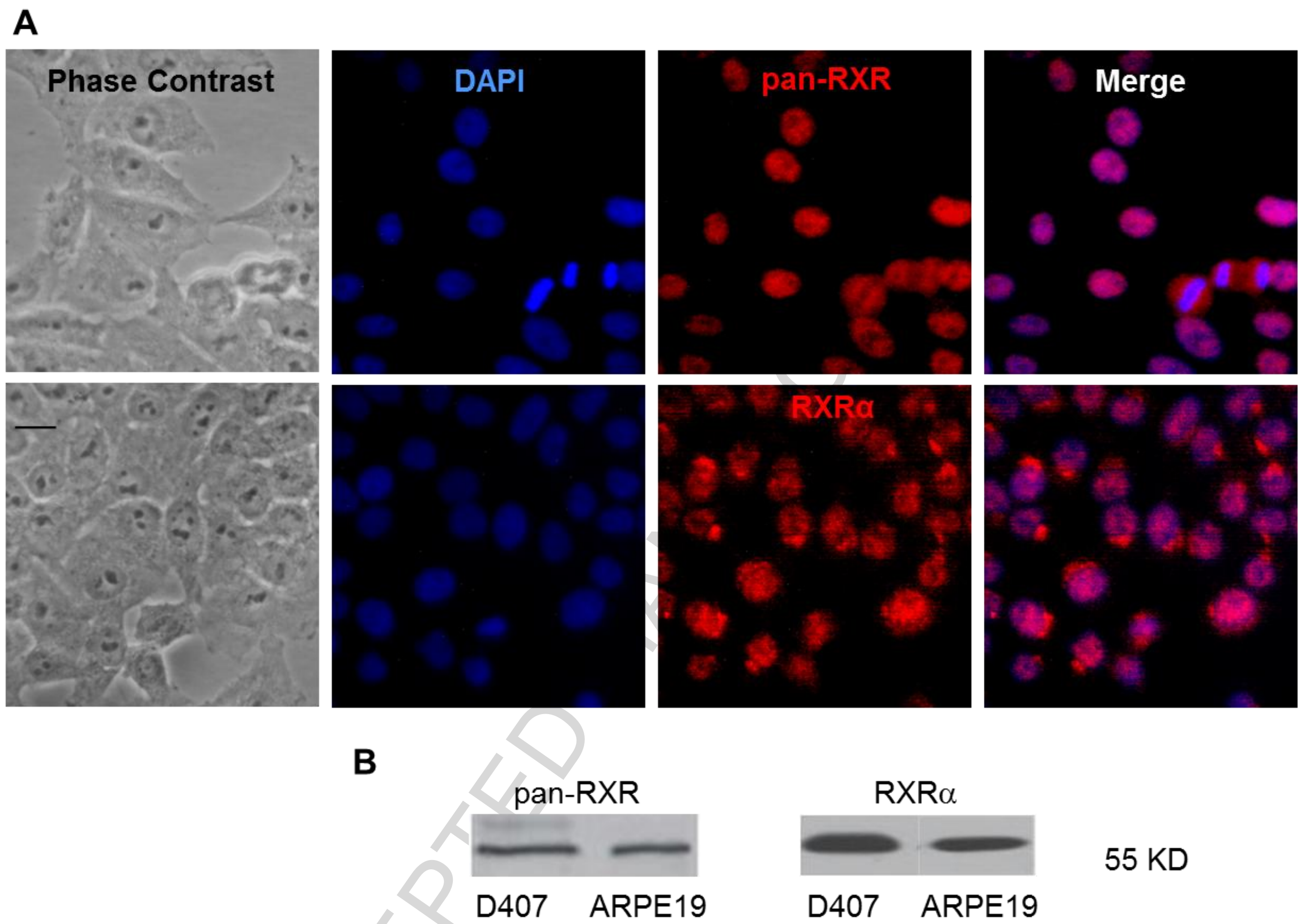

Fig. 1

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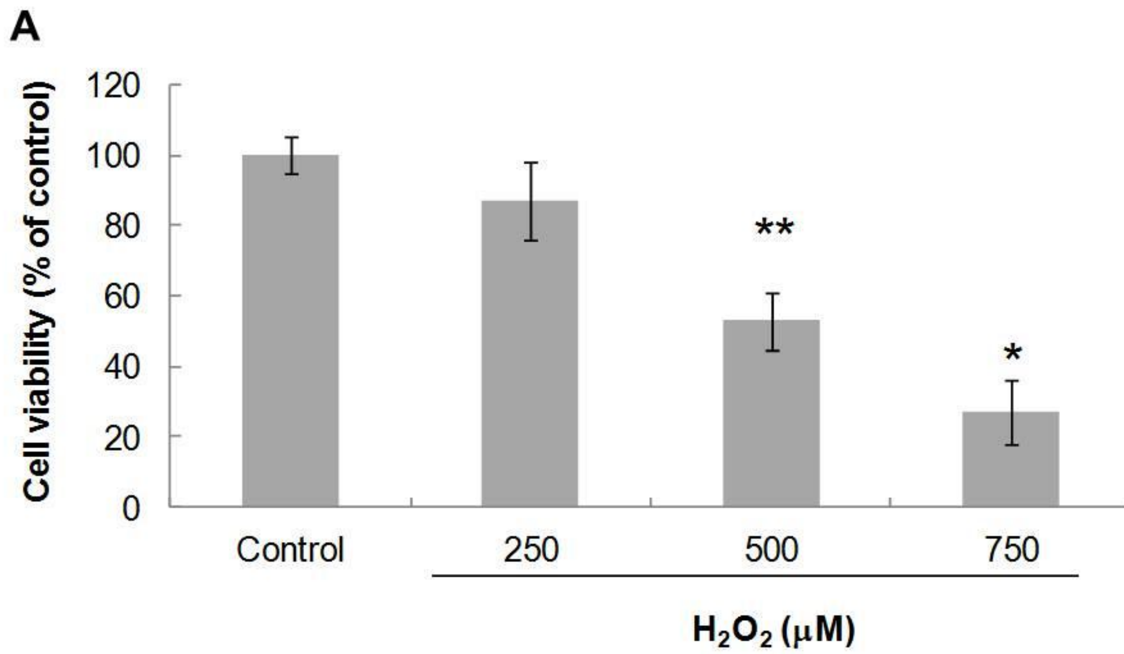

B

	5 h	24 h
Control	100.0 ± 5.2	100.0 ± 7.1
H_2O_2	75.0 ± 4.6 *	53.0 ± 8.6 **

Fig. 2

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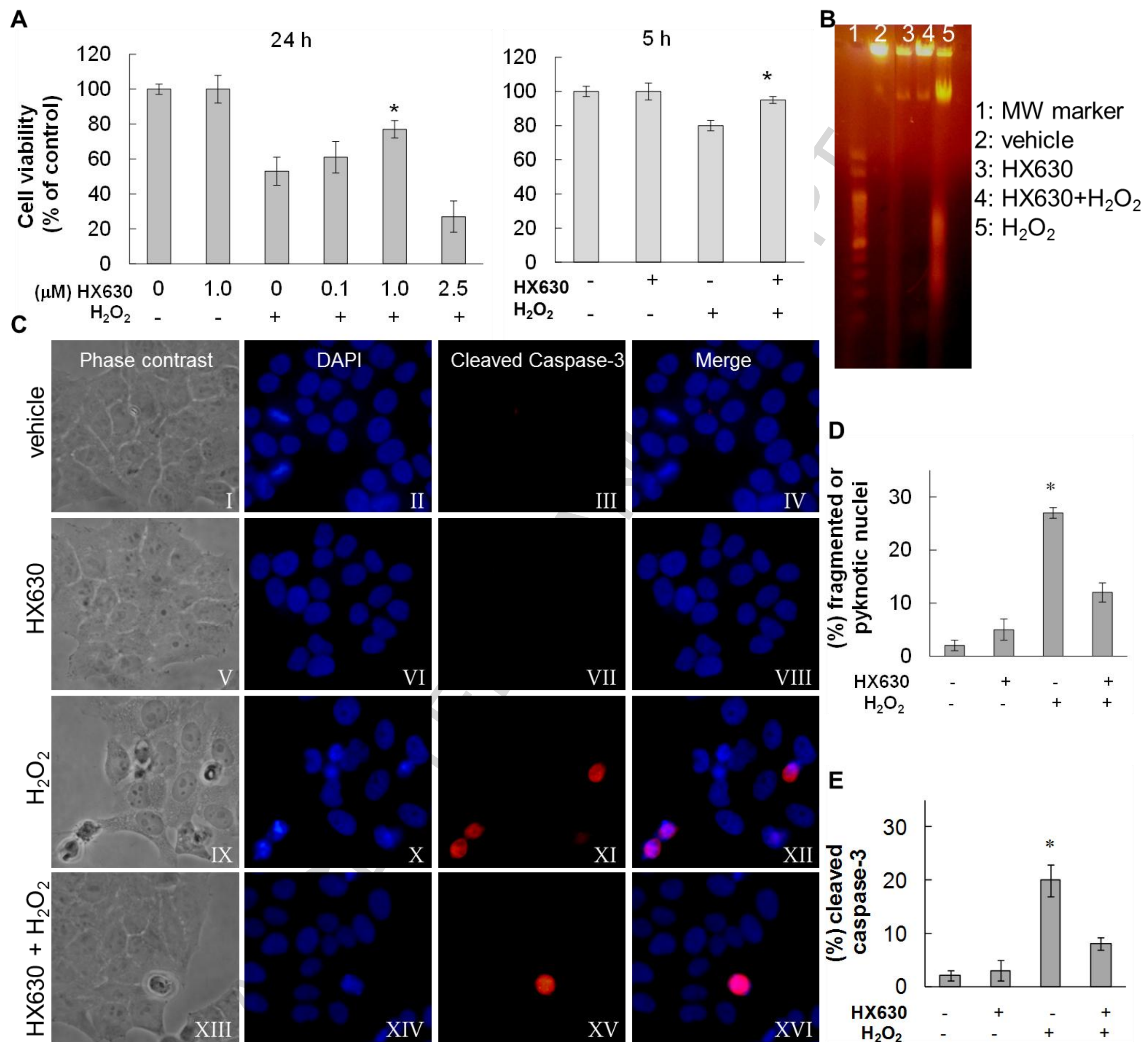

Fig. 3

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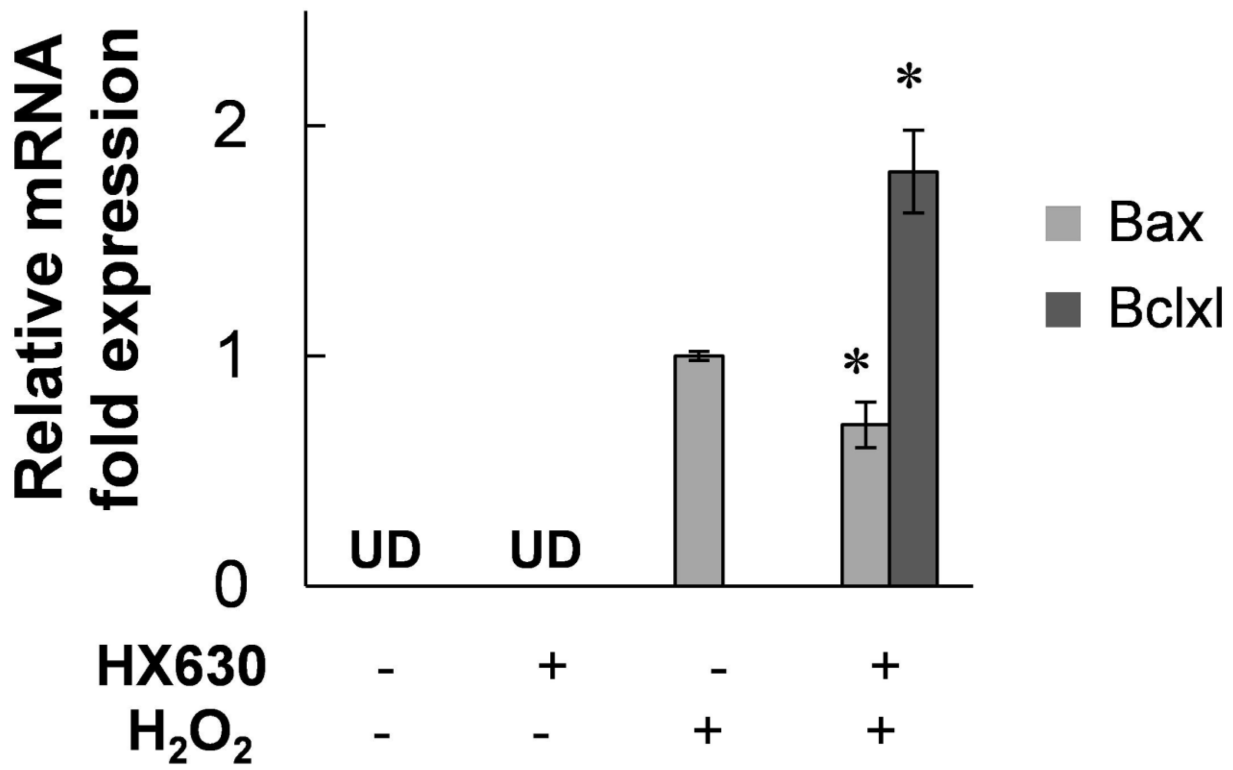

Fig. 4

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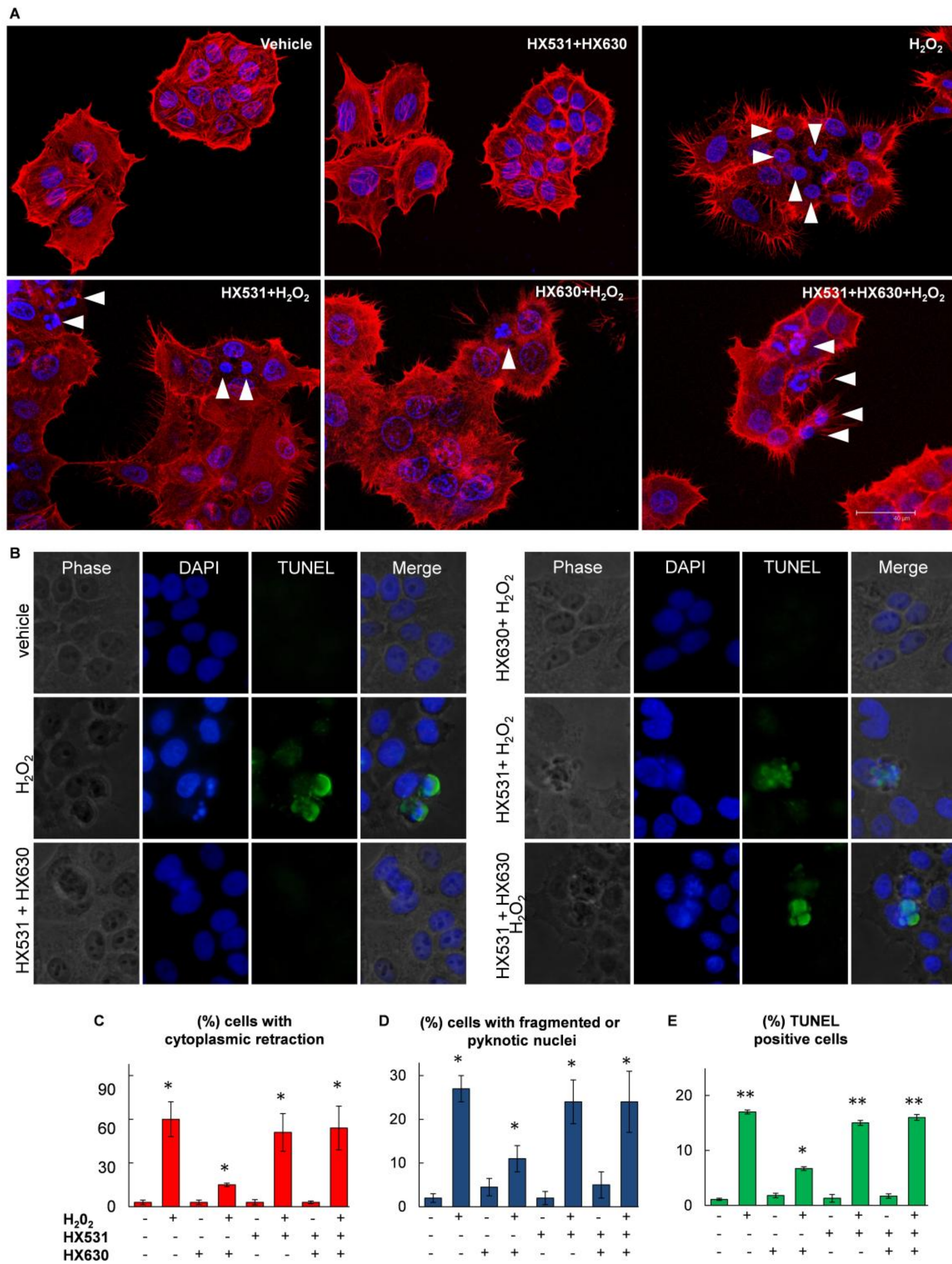

Fig. 5

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A

Phase contrast
p65NFκB
DAPI
Merge
vehicle
HX630
H_2O_2
HX630 + H_2O_2

B

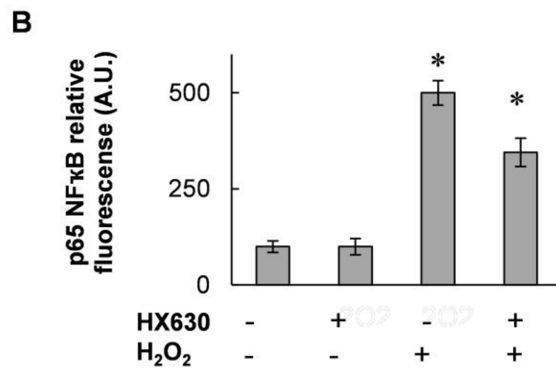

Fig. 6

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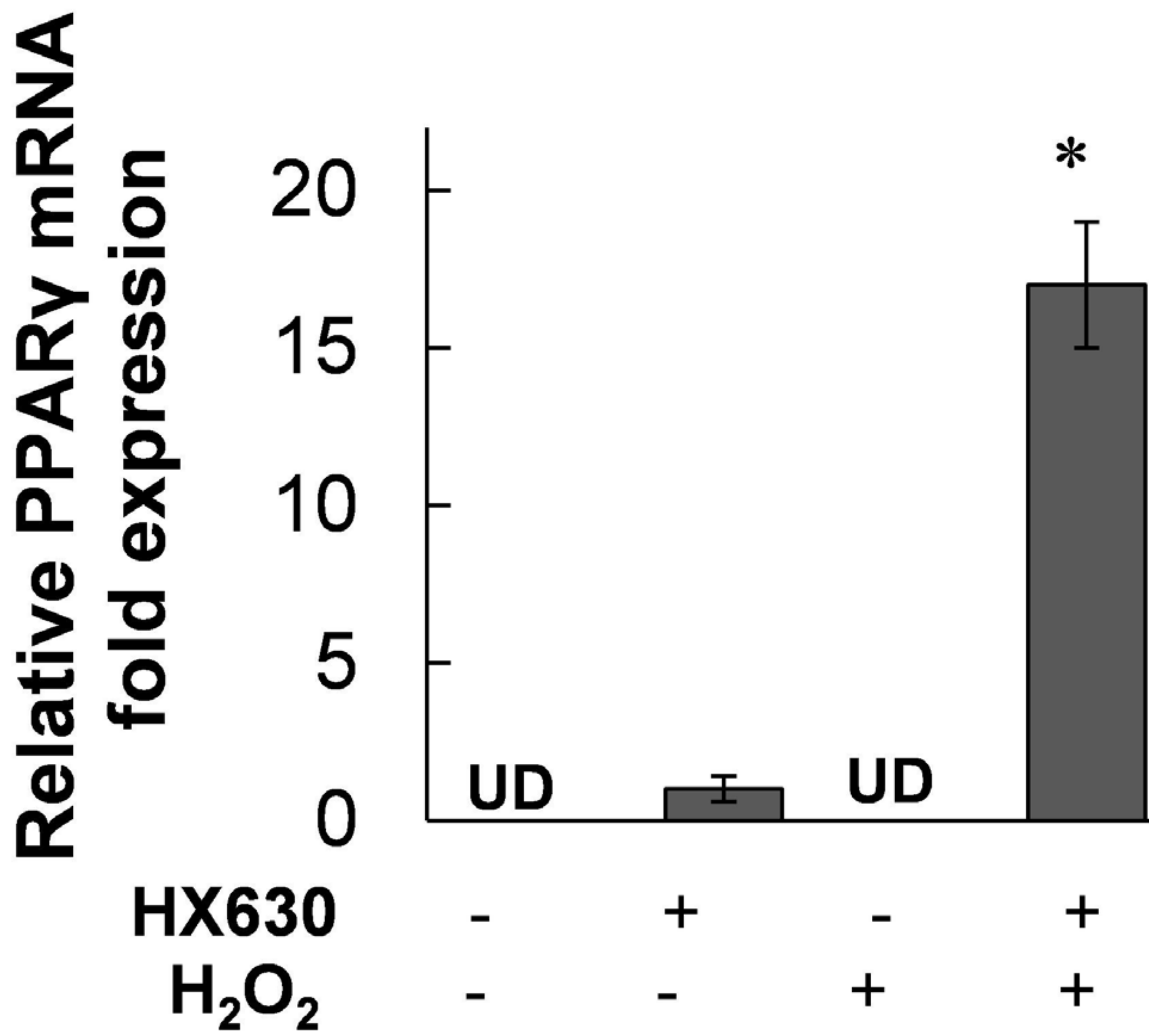

Fig. 7

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Table II

Table II A					
(µM)	10	1	0,1	0,01	0,001
HX630	-	++	+	-	-
Bexarotene	Tox	-	++	-	-
PA024	-	-	+	++	+
LG100754	-	+	++	+	Nd
LG100268	-	-	-	Nd	Nd
all-trans-RA (x10^{-6})	-	-	-	-	-

Table II B	
all-trans-RA (10^{-7}µM)+ Bexarotene (0,1 µM)	+
all-trans-RA (10^{-7}µM)+ LG100268 (5 µM)	-

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Highlights

- Pan-RXR agonists protected RPE cells from oxidative stress induced by H2O2.
- HX630, a RXR agonist, prevented apoptosis by regulating Bclxl, PPARγ and Bax mRNA levels.
- HX630 decreased nuclear translocation of p65NFκB and caspase-3 activation.
- Pan-RXR agonists acted through activation of RXR/PPARγ or, less probably, RXR/RXR.
- RXR/RAR was not involved in pan-RXR agonist protection on RPE cells.

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