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Instituto de Investigación Sanitaria La Fe
Laboratorio de Biomedicina Molecular, Celular y Genómica

TESIS DOCTORAL

PAPEL DEL ESTRÉS OXIDATIVO Y LA INFLAMACIÓN EN LA RETINOSIS PIGMENTARIA. EFECTO DE LA INHIBICIÓN DEL TNFα EN LA PROGRESIÓN DE LA DEGENERACIÓN RETINIANA.

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La Dra. Regina Rodrigo Nicolás, Investigadora del Programa Miguel Servet en el Instituto de Investigación Sanitaria La Fe,

CERTIFICA:

Que la tesis doctoral titulada "**Papel del estrés oxidativo y la inflamación en la retinosis pigmentaria. Efecto de la inhibición del TNFα en la progresión de la degeneración retiniana**", ha sido realizada bajo su dirección en el Grupo de Biomedicina Molecular, Celular y Genómica del Instituto de Investigación Sanitaria La Fe, por Cristina Martínez Fernández de la Cámara, Licenciada en Biotecnología.

Para que conste, en cumplimiento de la legislación vigente, firman el presente certificado en Valencia, a 2 de febrero de 2015.

Fdo. Regina Rodrigo Nicolás

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"Al carro de la cultura española le falta la rueda de la ciencia"

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"One never notices what has been done; one can only see what remains to be done."

Marie Curie

RESUMEN

Las degeneraciones retinianas son la principal causa de ceguera hereditaria debida, principalmente, a la pérdida de los fotorreceptores, los conos y los bastones. La retinosis pigmentaria (RP) es una forma frecuente de degeneración retiniana que constituye la principal causa genética de ceguera en los países desarrollados. Las mutaciones genéticas son las responsables de la muerte de los bastones. Sin embargo, la muerte de los conos parece debida a los cambios metabólicos que provoca la degeneración de los bastones como la hiperoxia (estrés oxidativo y nitrosativo), la secreción de diversos factores (citoquinas, quimioquinas) por parte de los bastones y otras células del entorno, etc. La muerte de los conos provoca la pérdida de la visión central en RP.

El principal objetivo de esta tesis es profundizar en el papel del estrés oxidativo y la inflamación en la muerte de los fotorreceptores en la RP. Para ello, en primer lugar, se analizó la respuesta antioxidante, la presencia de marcadores de estrés oxidativo/nitrosativo y el contenido de citoquinas en humor acuoso y en sangre periférica de pacientes con RP, que pudiesen estar implicados en la progresión o en el retraso de la enfermedad. En segundo lugar, se desarrolló un modelo *ex vivo* de degeneración retiniana mediante la realización de cultivos organotípicos de retina porcina expuestos a Zaprinast, un inhibidor de fosfodiesterasas (PDEs), simulando una de las mutaciones típicas de RP. Por último, se evaluó el efecto de la inhibición de TNFα con anticuerpos anti-TNFα sobre la progresión de la degeneración retiniana en el modelo *ex vivo* de retina porcina y en un modelo *in vivo* de RP, el ratón *rd10*.

Los resultados obtenidos en este estudio muestran que los pacientes con RP presentan una deficiente respuesta antioxidante en el ojo y niveles elevados de algunos marcadores de estrés oxidativo/nitrosativo

en sangre periférica. A nivel ocular, estos pacientes tienen menor capacidad antioxidante total incluyendo una menor actividad del enzima superóxido dismutasa (SOD) 3. A nivel periférico, también presentan menor actividad del enzima SOD3, además de un incremento de indicadores de peroxidación lipídica y una activación de la vía óxido nítrico/GMP cíclico. Los pacientes con mejor respuesta antioxidante ocular mostraban mejor función visual sugiriendo un papel del estrés oxidativo en la progresión de la enfermedad. Por otro lado, se observó un incremento de la concentración de IL-6 y TNFα en el humor acuoso de estos pacientes corroborando resultados obtenidos por otros autores que sugieren que en RP existe una inflamación crónica sostenida.

El modelo experimental de degeneración retiniana *ex vivo*, reproduce algunas de las alteraciones que se han descrito en modelos murinos y en pacientes con RP. En este modelo la degeneración retiniana observada al inhibir el enzima PDE6 con Zaprinast, va acompañada de estrés oxidativo e inflamación. En este modelo la inhibición de TNFα con el anticuerpo Infliximab, reduce la degeneración retiniana, la activación de las células de Müller y normaliza la capacidad antioxidante total aunque no reduce el contenido de marcadores de estrés oxidativo.

En el ratón *rd10*, el tratamiento con Adalimumab, anticuerpo monoclonal anti-TNFα, reduce la muerte de los fotorreceptores y la gliosis reactiva a día postnatal 18. La inhibición de TNFα reduce la actividad de la poli(ADP) ribosa polimerasa (PARP), enzima implicada en el proceso de muerte celular, previene la sobreexpresión de las citoquinas TNFα y factor inhibidor de leucemia (LIF) y mejora la respuesta antioxidante.

En definitiva, estos resultados confirman la hipótesis de que alteraciones del sistema antioxidante y los procesos inflamatorios a nivel ocular están implicados en la patogénesis de la RP en pacientes. El

modelo *ex vivo* de degeneración retiniana en retina porcina puede ser un modelo alternativo útil para la búsqueda de dianas terapéuticas y el ensayo de diversas drogas que prevengan o retrasen la muerte de los fotorreceptores. Nuestros resultados sugieren que en RP el TNFα desempeña un papel importante en la muerte de las células de la retina activando diversas vías de muerte celular. El diseño de estrategias que promuevan su bloqueo pueden ser terapias prometedoras en pacientes con RP.

RESUM

Les degeneracions retinianes són la principal causa de ceguesa hereditària deguda, principalment, a la pèrdua dels fotoreceptors, els cons i els bastons. La retinosi pigmentària (RP) es una forma freqüent de degeneració retiniana que constitueix la principal causa genètica de ceguesa en els països desenvolupats. Les mutacions genètiques son les responsables de la mort del bastons. Però, la mort del cons pareix deguda als canvis metabòlics que provoca la degeneració dels bastons com la hiperoxia (estrès oxidatiu y nitrosatiu), la secreció de diversos factors (citoquines, quimioquines) per part del bastons i altres cèl·lules del entorn, etc. La mort del cons provoca la pèrdua de la visió central en RP.

El principal objectiu d'aquesta tesi es profunditzar en el paper de l'estrès oxidatiu i la inflamació en la mort del fotoreceptors en la RP. Per això, en primer lloc es va avaluar la resposta antioxidant, la presència de marcadors d'estrès oxidatiu/nitrosatiu i el contingut de citoquines en humor aquós y en sang perifèrica en pacients amb RP, que poguessin estar implicats en la progressió o en el retràs de la malaltia. En segon lloc, es va desenvolupar un model *ex vivo* de degeneració retiniana mitjançant la realització de cultius organotípics de retina porcina exposada a Zaprinast, un inhibidor de fosfodiesterases (PDEs), simulant una de les mutacions típiques de RP. Per últim, es va avaluar l'efecte de la inhibició del TNFα amb anticossos anti-TNFα sobre la progressió de la degeneració retiniana en el model *ex vivo* de retina porcina i en un model *in vivo* de RP, el ratolí *rd10*.

Els resultats obtinguts en aquest estudi mostren que els pacients amb RP presenten una resposta antioxidant deficient en el ull i nivells elevats d'alguns marcadors d'estrès oxidatiu/nitrosatiu en sang perifèrica. A nivell ocular, aquests pacients tenen menor capacitat antioxidant total

incloent una menor activitat de l'enzim superòxid dismutasa (SOD) 3. A nivell perifèric, també presenten menor activitat de l'enzim SOD3 a més d'un increment d'indicadors de peroxidació lipídica y una activació de la via òxid nítric/GMP cíclic. Els pacients amb millor resposta antioxidant ocular mostraven millor funció visual suggerint un paper de l'estrès oxidatiu en la progressió de la malaltia. Per altre costat, es va observar un increment de la concentració de IL-6 i TNFα en el humor aquós d'aquests pacients corroborant els resultats obtinguts per altres autors que suggereixen que en RP existeix una inflamació crònica sostinguda.

El model experimental de degeneració retiniana *ex vivo*, reprodueix algunes de les alteracions que s'han descrit en models murins i en pacients amb RP. En aquest model, la degeneració retinana observada al inhibir l'enzim PDE6 amb Zaprinast, va acompanyada d'estrès oxidatiu e inflamació. En aquest model, la inhibició de TNFα amb l'anticòs Infliximab redueix la degeneració retiniana, l'activació de les cèl·lules de Müller i normalitza la capacitat antioxidant total encara que no redueix el contingut de marcadors d'estrès oxidatiu.

En el ratolí *rd10*, el tractament amb Adalimumab, anticòs monoclonal anti-TNFα, redueix la mort del fotoreceptors i la gliosi reactiva a dia postnatal 18. La inhibició del TNFα redueix l'activitat de la poli(ADP) ribosa polimerasa (PARP), enzim implicada en el procés de mort cel·lular, prevé la sobreexpressió de les citoquines TNFα i el factor inhibidor de leucèmia (LIF) i millora la resposta antioxidant.

En definitiva, aquests resultats confirmen la hipòtesi de que alteracions del sistema antioxidant i els processos inflamatoris a nivell ocular estan implicats en la patogènesi de la RP en pacients. El model *ex vivo* de degeneració retiniana en retina porcina pot ser un model alternatiu útil per a la cerca de dianes terapèutiques i l'assaig de diverses drogues que previnguin o retarden la mort dels fotoreceptors. Els

nostres resultats suggereixen que en RP el TNFα exerceix un paper important en la mort de les cèl·lules de la retina activant diverses vies de mort cel·lular. El disseny d'estratègies que promoguin el seu bloqueig poden ser teràpies prometedores en pacients amb RP.

ABSTRACT

Retinal degenerations are the main cause of hereditary blindness mainly due to the loss of photoreceptors, cones and rods. Retinitis pigmentosa (RP) is a common form of retinal degeneration that constitutes the largest genetic cause of blindness in the developed world. Genetic mutations are responsible of rod death. However, the death of cones seems to be due to metabolic changes caused by rod degeneration as hyperoxia (oxidative and nitrosative stress), secretion of different factors (cytokines, chemokines) by rods and other cells of the environment, etc. The death of cones causes the loss of central vision in RP.

The main objective of this thesis is to examine the role of oxidative stress and inflammation in photoreceptors cell death in RP. For that, first, antioxidant response, the presence of oxidative/nitrosative stress markers and the content of cytokines were analysed in aqueous humor and peripheral blood in patients with RP that could be involved in the progression or delay of the disease. Second, we developed an *ex vivo* model of retinal degeneration by performing organotypic cultures of porcine retina exposed to Zaprinast, a phosphodiesterase (PDE) inhibitor, simulating a typical mutation of RP. Last, we evaluated the effect of TNFα inhibition with antibodies anti-TNFα on progression of retinal degeneration in the *ex vivo* model of porcine retina and in an *in vivo* model of RP, *rd10* mouse.

Results obtained in this study show that patients with RP have a deficient antioxidant response in the eye and elevated levels of some markers of oxidative/nitrosative stress in peripheral blood. At ocular level, these patients have less total antioxidant capacity including less activity of the superoxide dismutase (SOD) 3 enzyme. At peripheral level, they also have less SOD3 activity in addition to an increase of lipid peroxidation indicators and an activation of the nitric oxide/cyclic GMP pathway. Patients with better antioxidant response showed better visual function suggesting a role of oxidative stress on the progression of the disease. On the other side, an increase of IL-6 and TNFα content was observed in the aqueous humor of these patients confirming the results obtained by other authors and suggesting that in RP there is a sustained chronic inflammation.

The experimental *ex vivo* model of retinal degeneration reproduces some of the alterations described in murine models and in patients with RP. In this model, the retinal degeneration observed after PDE6 enzyme inhibition with Zaprinast, was accompanied by oxidative stress and inflammation. In this model, TNFα inhibition with the antibody Infliximab, reduces retinal degeneration, Müller cell activation and normalizes total antioxidant capacity although it does not reduce the content of oxidative stress markers.

In *rd10* mouse, treatment with Adalimumab, a monoclonal antibody against TNFα, reduces photoreceptor cell death and reactive gliosis at postnatal day 18. The inhibition of TNFα

reduces poly(ADP) ribose polymerase (PARP) activity, an enzyme involved in the process of cell death, prevents overexpression of TNFα and leukemia inhibitory factor (LIF) cytokines and improves antioxidant response.

Ultimately, these results confirm the hypothesis that alterations in the antioxidant system and inflammatory processes at ocular level are involved in the pathogenesis of RP in patients. The *ex vivo* model of retinal degeneration in porcine retina can be a useful alternative model for the searching for therapeutics targets and for testing diverse drugs that prevent or delay the death of photoreceptors. Our results suggest that in RP, TNFα plays an important role in the death of retinal cells by activating different death pathways. The design of strategies that promote its blockade can be promising therapies in patients with RP.

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ABREVIATURAS

ADN	Ácido desoxirribonucleico
AIF	Factor inductor de apoptosis (*apoptotic inducing factor*)
CCG	Capa de células ganglionares
CNE	Capa nuclear externa
CNG	Canal iónico de nucleótidos cíclicos
CNI	Capa nuclear interna
CNTF	Factor neurotrófico ciliar (*ciliary neurotrophic factor*)
CPE	Capa plexiforme externa
CPI	Capa plexiforme interna
CSE	Capa de los segmentos externos de los fotorreceptores
DHA	Ácido docosahexanoico (*docosahexaenoic acid*)
DMAE	Degeneración macular asociada a la edad
EPR	Epitelio pigmentario retiniano
GFAP	Proteína glial fibrilar acídica (*Glial fibrillary acidic protein*)
GMPc	Guanosín monofosfato cíclico
HDAC	Histona deacetilasas
IL	Interleuquina
ipRGC	Células ganglionares intrínsecamente fotosensibles (*intrinsically photosensitive retinal ganglion cells*)
LIF	Factor inhibidor de leucemia (*leukemia inhibitory factor*)
MCP	Proteínas quimioatrayentes de monocitos (*monocyte chemoattractant proteins*)
MIP	Proteínas inflamatorias de macrófagos (*macrophage inflammatory proteins*)
MLE	Membrana limitante externa
MLI	Membrana limitante interna
NO	Óxido nítrico (*nitric oxide*)
NOS	Óxido nítrico sintasa (*nitric oxide synthase*)
PAR	Poli(ADP-ribosa)

PARP	Poli(ADP-ribosa) polimerasa
PDE	Fosfodiesterasa (*phosphodiesterase*)
PKG	Proteína quinasa G (*Protein kinase G*)
RCS	*Royal College of Surgeons*
RIP	Proteínas de interacción con receptores (*Receptor interacting proteins*)
RNS	Especies reactivas del nitrógeno (*Reactive nitrogen species*)
ROS	Especies reactivas del oxígeno (*Reactive oxygen species*)
RP	Retinosis pigmentaria
SOD	Superóxido dismutasa
TNF	Factor de necrosis tumoral (*Tumor necrosis factor*)

Capítulo I. Introducción general

I.1 ANATOMÍA Y FISIOLOGÍA DEL GLOBO OCULAR

El ojo o globo ocular humano tiene una morfología esférica ovoidea con un diámetro de 24 mm y un peso aproximado de 7g. Se sitúa en la mitad anterior de la órbita ósea. Como órgano visual, es capaz de percibir la radiación electromagnética del espectro visible (rango de longitudes de onda de 400 a 700 nm) y transformarla en impulsos electroquímicos que serán transmitidos al cerebro a través de las distintas estructuras que lo conforman.

En los mamíferos, el globo ocular está recubierto por tres capas (Figura 1) (Moore KL, 2007; Welsch, 2008):

· La capa externa o fibrosa, compuesta por la esclerótica y la córnea. La esclerótica es visible como la parte blanca del ojo. Es una cubierta de tejido conjuntivo que le aporta forma y resistencia al globo ocular. En la parte anterior del ojo, la esclerótica se continúa con la córnea, capa transparente que protege las estructuras situadas posteriormente y cuya superficie refracta la mayor parte de la luz incidente.

· La capa media o vascular, también llamada úvea, está compuesta principalmente por tres estructuras: la coroides, el cuerpo ciliar y el iris. La coroides es una capa rica en melanina que evita que la luz penetre por la esclerótica impidiendo así la reflexión de la luz. Es una capa con abundantes vasos sanguíneos que aportan oxígeno y nutrientes a la retina, situada en la parte más interna. El cuerpo ciliar conecta la coroides y el iris y permite que el cristalino varíe su morfología para enfocar objetos a distintas distancias. El iris se localiza delante del cristalino y funciona como un diafragma contráctil controlando la entrada de luz a través de una apertura central, la pupila.

· La capa interna o nerviosa compuesta por la retina. En esta estructura nerviosa tiene lugar la fototransducción, mecanismo complejo

por el que la información recibida del exterior en forma de luz se transmite al cerebro a través del nervio óptico. Debido a la relevancia de la retina en este trabajo, en el siguiente apartado se hará una descripción más detallada de su estructura y funcionamiento.

Estas estructuras delimitan tres espacios: (1) la *cámara anterior*, localizada entre la córnea y el iris, (2) la *cámara posterior*, delimitada por un lado por el iris y el cuerpo ciliar y por otro lado por el cristalino y (3) el *cuerpo o humor vítreo*, localizado entre el cristalino y la retina. Estos tres espacios están compuestos de dos medios acuosos que intervienen también en la refracción de la luz: el humor acuoso y el humor vítreo. El *humor acuoso*, localizado en la cámara anterior y posterior, es una solución compuesta principalmente por agua que aporta nutrientes a las estructuras avasculares con las que está en contacto, la córnea y el cristalino. Es segregado por el cuerpo ciliar en la cámara posterior desde donde fluye a la cámara anterior a través de la pupila, para drenar finalmente en el seno venoso escleral (canal de Schlemm). Es importante que exista un equilibrio entre la producción y el drenaje de humor acuoso para evitar alteraciones fisiológicas, como un aumento de presión, que podrían desencadenar graves problemas de visión (Chowdhury *et al.*, 2010). El *humor vítreo* es una solución viscosa que contribuye a mantener la forma del ojo y facilita que la luz llegue a la retina de forma uniforme. Se genera durante el desarrollo embrionario y permanece sin renovarse durante toda la vida (Murthy *et al.*, 2014).

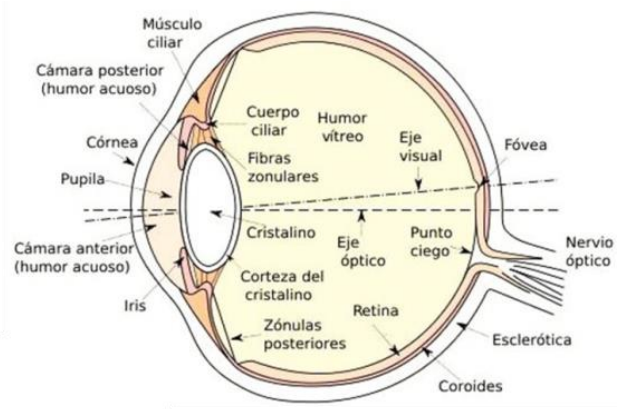

Figura 1. Anatomía del globo ocular. Corte sagital en el que se muestran las estructuras macroscópicas del ojo humano (Fuente: Wikipedia).

I.2 LA RETINA

I.2.1 Estructura de la retina en mamíferos

La retina de mamíferos es una estructura compleja que recibe los estímulos luminosos y los transforma en estímulos nerviosos que serán interpretados en el cerebro. Existen diferencias en su estructura según la especie. En el caso del ratón, modelo animal muy utilizado en el estudio de degeneraciones retinianas y en este trabajo, su retina carece de fóvea, tiene una densidad de conos menor que la retina humana y presenta una visión dicromática. Sin embargo, en otros aspectos como el desarrollo, la fisiología y la estructura general de la retina y el nervio óptico se asemeja a la retina de primates superiores (Jeon *et al.*, 1998; Fox *et al.*, 2006). Por su parte, la retina de cerdo, utilizada también en este trabajo, presenta mayor similitud con la retina humana que la de los ratones. Estas similitudes incluyen el tamaño, la estructura, la densidad de los fotorreceptores y el patrón vascular de la retina (Fernandez-Bueno *et al.*, 2008).

Macroscópicamente, se distinguen varias estructuras según se localicen en la parte central o periférica de la retina:

- En el área central de la retina destaca el punto ciego, la mácula y la fóvea (Figura 1):

· El *punto ciego* o papila del nervio óptico es la zona donde los axones de las células ganglionares abandonan la retina formando el nervio óptico. Es una zona no sensible a la luz.

· La *mácula* es una zona localizada en el polo posterior del eje óptico del globo ocular, con aspecto amarillento por su elevado contenido en pigmentos xantofílicos.

· La f*óvea* es una zona deprimida en la mácula que posee mayoritariamente conos, que son las células perceptoras de los colores. En condiciones luminosas, es la zona de mayor nitidez visual.

- El área periférica de la retina posee un bajo número de conos por lo que es menos sensible a la luz que el área central de la retina. En esta zona destaca la *ora serrata*, que constituye la estructura más periférica de la retina y conecta ésta con el cuerpo ciliar.

La retina posee un doble sistema de irrigación que proporciona los nutrientes a las distintas estructuras. Por un lado, la arteria retiniana central entra en el globo ocular por el nervio óptico y se divide para irrigar toda la superficie retiniana interna. Por otro lado, la coroides se encarga de nutrir y, en especial, de aportar oxígeno a la superficie externa de la retina (Guyton, 1992).

Microscópicamente, la retina está formada por distintos tipos celulares, básicamente neuronas, que se distribuyen a lo largo de ocho capas (Figura 2) (Guyton, 1992; Kolb, 2011b). Desde la parte más interna (en contacto con el humor vítreo) a la más externa (adyacente a la coroides) las capas que conforman la retina son: la *membrana limitante interna (MLI)*, situada entre el humor vítreo y la retina, está compuesta

por una lámina basal y las terminaciones de las células de Müller que se prolongan horizontalmente. La *capa de células ganglionares (CCG)* donde se localizan las células ganglionares y algunas células amacrinas desplazadas. La *capa plexiforme interna (CPI)* donde contactan las dendritas de las células ganglionares y amacrinas con los axones de las células bipolares. La *capa nuclear interna (CNI)* que contiene los cuerpos celulares de las células bipolares, amacrinas, horizontales y de las células de Müller. La *capa plexiforme externa (CPE),* capa donde contactan las dendritas de las células bipolares y horizontales con los axones de los fotorreceptores. La *capa nuclear externa (CNE)* que contiene los núcleos celulares de los fotorreceptores (conos y bastones). La *membrana limitante externa (MLE)*, situada entre los núcleos y los segmentos externos de los fotorreceptores, compuesta por uniones intercelulares entre las células de Müller y los fotorreceptores. La *capa de los segmentos externos de los fotorreceptores (CSE)*, que engloba los elementos sensibles a la luz de los fotorreceptores. Y por último, el *epitelio pigmentario retiniano (EPR)*, capa más externa localizada en la parte posterior de la coroides que no forma parte de la neuroretina pero es imprescindible para su correcto funcionamiento (Kolb, 1994).

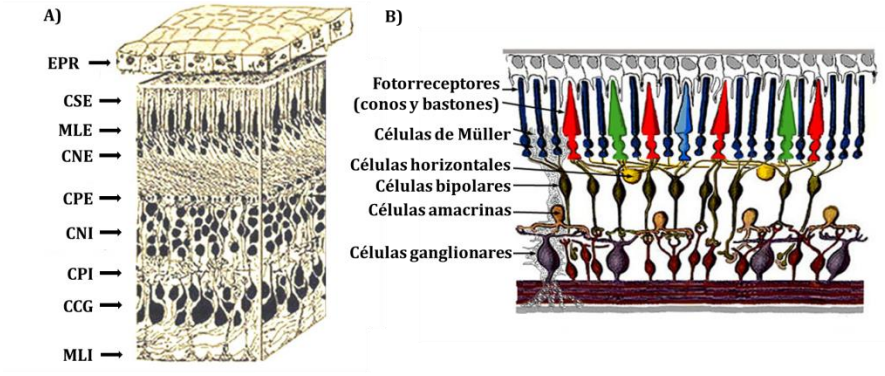

Figura 2. Estructura microscópica de la retina. Capas celulares de la retina (A). Tipos celulares que componen la retina (B). EPR: epitelio pigmentario retiniano, CSE: capa de los segmentos externos de los fotorreceptores, MLE: membrana limitante externa, CNE: capa nuclear externa, CPE: capa plexiforme

externa, CNI: capa nuclear interna, CPI: capa plexiforme interna, CCG: capa de células ganglionares, MLI: membrana limitante interna. (Imagen adaptada de Kolb, 2011b).

I.2.2 Tipos celulares de la retina

En 1892, Santiago Ramón y Cajal y posteriormente, en 1941, Stephen Polyak describieron en detalle la estructura de los componentes celulares de la retina de vertebrados (Figura 3). Estos estudios siguen teniendo hoy en día su relevancia ya que sentaron las bases de la anatomía de la retina. Con el tiempo, el desarrollo de técnicas más sofisticadas como la microscopía electrónica, las técnicas histológicas, etc. han permitido avanzar en el conocimiento de la estructura y funcionamiento de la retina (Kolb, 2011a).

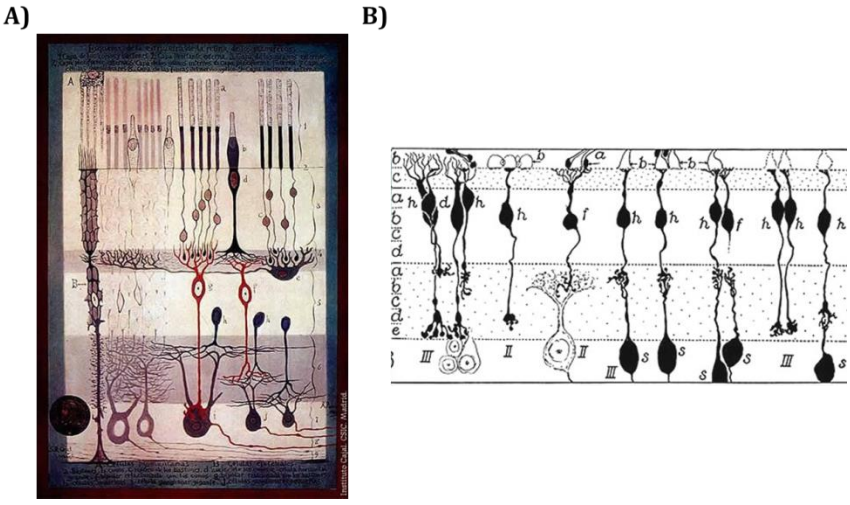

Figura 3. Dibujo de la estructura de la retina según Ramón y Cajal (1900) (A) y Polyak (1941) (B).

I.2.2.1 Fotorreceptores: conos y bastones

Los fotorreceptores son neuronas especializadas sensibles a la luz que constituyen el tipo celular más abundante de la retina, estimándose unos 130 millones de fotorreceptores en la retina humana (110-125 millones de bastones y 6 millones de conos, aproximadamente) (Kolb,

2013b). Los bastones se concentran en la retina periférica mientras que los conos predominan en la zona central, especialmente en la fóvea, donde la densidad media es de 161.900 conos/mm^2 (Curcio *et al.*, 1987). No obstante, el número y distribución de los fotorreceptores varía dependiendo de la especie. En ratones se estima que hay 6'4 millones de bastones y 180.000 conos cuya densidad media es de 12.400 conos/mm^2 (Jeon *et al.*, 1998). La retina porcina, en cambio, presenta una distribución más similar a la retina humana ya que en la parte central hay una mayor densidad de conos que en la periferia (39.000 conos/mm^2 en el centro *versus* 8.500 conos/mm^2 en la periferia) (Chandler *et al.*, 1999).

Los conos presentan una estructura cónica, con sus núcleos alineados en una sola capa justo por debajo de la membrana limitante externa. Por su parte, los bastones, como su propio nombre indica, tienen aspecto de bastones delgados y sus cuerpos celulares se disponen en el resto de la capa nuclear externa (Guyton, 1992).

En ambas células se distinguen cuatro partes bien diferenciadas: el *segmento externo* que contiene los pigmentos visuales, el *segmento interno* encargado del metabolismo celular, el *cuerpo celular* que contiene el núcleo, y la *terminal sináptica* denominada pedículo en el caso de los conos y esférulas en el caso de los bastones (Figura 4) (Germain *et al.*, 2010; Lamb, 2011). Los segmentos externos de los bastones están formados por discos membranosos derivados de la membrana plasmática pero que no están en contacto con ella. En esta zona hay un alto contenido en rodopsina, fotopigmento característico de los bastones. Los discos se renuevan constantemente siendo las células del epitelio pigmentario las encargadas de fagocitar los discos antiguos. Los segmentos externos de los conos, en cambio, están formados por invaginaciones de la membrana plasmática y pueden contener distintas opsinas. Los segmentos internos de ambos tipos celulares son ricos en

mitocondrias ya que es esta parte de la célula la encargada del metabolismo celular y donde se efectúa la síntesis de macromoléculas y el aporte de la energía necesaria para la fototransducción. Por último, las terminales sinápticas constituyen la zona desde donde los fotorreceptores despolarizados, en condiciones de oscuridad, liberan el neurotransmisor glutamato que estimula a las neuronas de la capa nuclear interna (Kolb, 2013b).

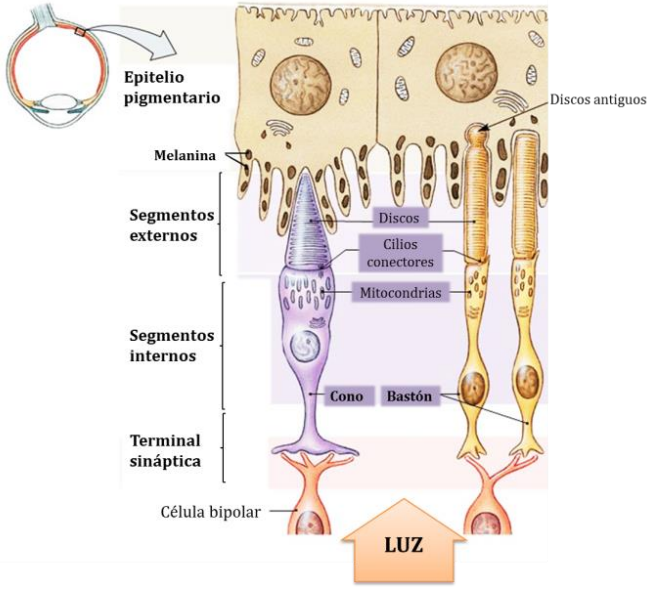

Figura 4. Estructura de los fotorreceptores (imagen adaptada de (Slide-Player, Chapter 10c)).

Los bastones son células muy sensibles que se encargan de la visión cuando las condiciones son de baja luminosidad (visión escotópica), no siendo capaces de percibir colores ni detalles espaciales. Su pigmento, la rodopsina, es sensible a longitudes de onda cercanas a 500 nm (luz verde-azul). Los conos son los encargados de la visión en condiciones de luminosidad (visión fotópica) y son capaces de percibir los colores así como los detalles espaciales. En humanos y muchos primates existen tres tipos de conos en función de la opsina que contienen: conos L (*large*

wavelength) sensibles a la luz de longitud de onda larga (luz roja), conos M (*medium wavelength*) sensibles a la luz de longitud de onda media (luz verde) y conos S (*short wavelength*) sensibles a longitudes de onda cortas (luz azul) (Kolb, 2013b).

I.2.2.2 Células bipolares

Las células bipolares se localizan en la capa nuclear interna y contactan a través de su árbol dendrítico con los fotorreceptores a nivel de la capa plexiforme externa, y a través de su axón con las células amacrinas y ganglionares en la capa plexiforme interna. En la retina humana existen dos tipos de células bipolares: las células bipolares que establecen sinapsis con conos y las que establecen sinapsis con bastones. A nivel de la capa plexiforme externa, las células bipolares pueden responder al glutamato liberado por los fotorreceptores hiperpolarizándose (bipolares tipo ON) o despolarizándose (bipolares tipo OFF). Las células bipolares de conos (tipo ON y OFF) establecen sinapsis glutamatérgicas con las células ganglionares y amacrinas, sin embargo, las células bipolares de bastones (únicamente de tipo ON) solo establecen sinapsis con las células amacrinas que actúan como intermediarias (Kuffler, 1953; Nelson, 2012). Estas células amacrinas se comunican con los dos tipos de células bipolares de conos, OFF y ON, a través de la liberación de glicina y uniones de tipo gap (sinapsis eléctrica), respectivamente (Kolb, 2012; Nelson, 2012).

I.2.2.3 Células horizontales

Los cuerpos celulares de las células horizontales se localizan en el límite exterior de la capa nuclear interna y establecen conexiones sinápticas con los fotorreceptores y las dendritas de las células bipolares en la capa plexiforme externa. Morfológicamente, se clasifican en células horizontales de tipo A o sin axón y de tipo B o con axón corto. Las células de tipo A son grandes neuronas estrelladas cuyo árbol dendrítico

conecta conos con conos. Sin embargo, las células de tipo B son neuronas más pequeñas cuyas dendritas contactan con conos y también con bastones a través del axón, dependiendo del tipo de célula horizontal (axón corto tipo I o axón corto tipo II). Las células horizontales son neuronas gabaérgicas de interconexión laterales que contribuyen a integrar y regular los impulsos de entrada enviados por varias células fotorreceptoras (Perlman *et al.*, 2012).

I.2.2.4 Células amacrinas

Las células amacrinas son interneuronas cuyo soma se localiza en la capa más interna de la capa nuclear interna y establecen conexiones sinápticas con las células ganglionares, bipolares y también con otras células amacrinas en la capa plexiforme interna. Por su morfología, tamaño y extensión, las células amacrinas constituyen el grupo más heterogéneo de las células de la retina, estimándose alrededor de 40 tipos distintos, muchas de ellas con función todavía desconocida (Germain *et al.*, 2010).

I.2.2.5 Células ganglionares

Las células ganglionares son neuronas de axón mielinizado localizadas cerca de la superficie interna de la retina. Reciben la información visual de los fotorreceptores a través de interneuronas bipolares y amacrinas y transmiten esta información desde la retina a través del nervio óptico a varias regiones en el tálamo, hipotálamo y el mesencéfalo o cerebro medio. Se estima que en la retina humana hay aproximadamente 1'5 millones de células ganglionares, pudiendo diferenciarse, al menos, 30 tipos distintos (Germain *et al.*, 2010).

Se ha descubierto un nuevo tipo de células ganglionares, denominadas intrínsecamente fotosensibles o ipRGC (*intrinsically photosensitive retinal ganglion cell*) (Berson, 2003; Sand *et al.*, 2012).

Estas células contienen un pigmento sensible a la luz, la melanopsina que, aunque es menos fotosensible que los pigmentos presentes en los fotorreceptores (conos y bastones), dota a estas células de la capacidad de responder a estímulos lumínicos del exterior. Estas células mantienen las funciones típicas de las células ganglionares y además, están implicadas en la sincronización de los ritmos circadianos con la luz solar, el reflejo de la pupila a la luz y otras respuestas fisiológicas (Wong *et al.*, 2005).

I.2.2.6 Células gliales

Las células gliales de la retina no sólo desempeñan funciones de sostén de las neuronas comentadas anteriormente sino que participan activamente en la fisiología de la retina. En la retina de mamíferos se han identificado tres tipos de células gliales: astroglía, microglía y células de Müller, siendo estas últimas las más abundantes (Kolb, 2013a).

- Astrocitos

Los astrocitos o astroglía se localizan en la parte más interna de la retina. Desempeñan funciones de protección, nutrición y mantenimiento de la homeostasis participando, posiblemente, en el metabolismo de los neurotransmisores. Tienen una morfología característica con cuerpos celulares aplanados y una serie de ramificaciones fibrosas. Estas células envuelven los vasos sanguíneos y los axones de las células ganglionares formando unas vainas axonal-glial-vascular que contribuyen al establecimiento de la barrera hemato-retiniana. Son células ricas en glucógeno que pueden suministrar glucosa a las neuronas (Kolb, 2013a).

- Microglía

La microglía son macrófagos residentes en la retina y sistema nervioso central que se encuentran inhibidos por el cortisol endógeno en condiciones normales. En esta situación, la microglía presenta un tamaño

relativamente pequeño, con morfología ramificada y se encuentra en las capas más internas de la retina, en ocasiones asociada a vasos sanguíneos (Gupta *et al.*, 2003; Wang *et al.*, 2011). Si se produce alguna alteración en la retina, la microglía se activa, adquiriendo una morfología ameboide y migrando a las capas más externas de la retina donde desempeña una función principalmente fagocítica (Gupta *et al.*, 2003; Lewis *et al.*, 2005).

- Células de Müller

Son las principales células gliales de la retina. El cuerpo celular se localiza en la capa nuclear interna y sus ramificaciones se extienden a lo largo de todas las capas de la retina constituyendo los límites de la retina a nivel de la membrana limitante interna y externa como se ha comentado anteriormente. Esta células desempeñan distintas funciones, entre las cuales destacan proporcionar nutrientes a las neuronas por su alto contenido en glucógeno, regular el flujo sanguíneo de la retina contribuyendo al mantenimiento de la barrera hemato-retiniana, mantener la homeostasis extracelular, liberar D-serina y glutamato, etc. (Bringmann *et al.*, 2006).

I.2.3 Fototransducción

La fototransducción o transducción visual es el proceso por el cual un fotón genera una respuesta eléctrica en las células fotorreceptoras de la retina. En presencia de luz, la estimulación en los discos del segmento externo de los pigmentos visuales (rodopsina en los bastones y opsinas en los conos) activa una compleja cascada de reacciones enzimáticas y bioquímicas, induciendo el cierre de los canales catiónicos de la membrana del fotorreceptor y la inhibición de la liberación de glutamato. Los fotopigmentos están formados por una parte proteica, la opsina, que está unida covalentemente a un cromóforo llamado retinal, derivado de la vitamina A.

La rodopsina contiene el isómero 11-cis retinal que cuando llega el fotón, se fotoisomeriza a trans-retinal cambiando la conformación de la opsina y activando una transducina. La transducina activada aumenta la actividad de la fosfodiesterasa 6 (PDE6) que degrada el GMP cíclico (GMPc). La disminución de GMPc cierra los canales iónicos de sodio y calcio regulados por GMPc e hiperpolariza la membrana disminuyendo la liberación de glutamato, iniciándose así la respuesta neural a la luz (Figura 5). Tras la isomerización y liberación de la opsina, el trans-retinal generado se reduce a trans-retinol en el epitelio pigmentario y regresa de nuevo al segmento externo del fotorreceptor donde se conjuga con la opsina para formar el pigmento visual funcional (Fu, 2010). Las alteraciones en cualquier paso de esta cascada pueden provocar la muerte del fotorreceptor (Nakao *et al.*, 2012).

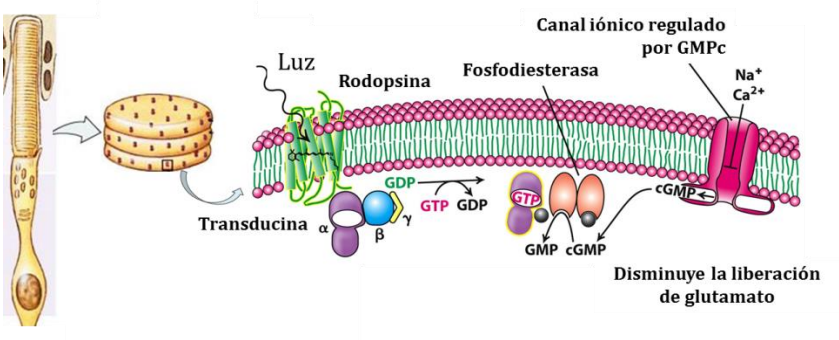

Figura 5. Proceso de transducción visual (adaptado de (Freeman and company., 2012)).

I.3 RETINOSIS PIGMENTARIA

I.3.1 Definición de retinosis pigmentaria

La degeneración retiniana se define como el deterioro de la retina causado por la muerte progresiva de sus células. Existen diversas causas que pueden conducir a esta degeneración como la diabetes mellitus, el envejecimiento, la exposición a la radiación solar, la prematuridad en el

nacimiento, la hipertensión o una enfermedad, generalmente hereditaria, como es el caso de la retinosis pigmentaria. La retinosis pigmentaria (RP) es la principal causa genética de ceguera en los países desarrollados constituyendo el 85-90 % de las distrofias hereditarias de retina (Ayuso and Millan, 2010) y tiene una prevalencia mundial de 1 por cada 4.000 individuos (Orphanet; Anasagasti *et al.*, 2012).

I.3.2 Fisiopatología de la retinosis pigmentaria

La RP es una enfermedad con un desarrollo lento y progresivo en el que la función de los fotorreceptores va disminuyendo con los años. Comienza con la degeneración de los bastones debido al defecto genético y con el tiempo degeneran también los conos que no pueden sobrevivir mucho tiempo sin el microambiente dominado por los bastones. La muerte de estos conos es el principal problema porque provoca la pérdida de la visión central. Aunque es una enfermedad de origen genético, en los últimos años se ha puesto de relevancia la implicación del estrés oxidativo y la inflamación en la progresión de la enfermedad (Punzo *et al.*, 2012; Yoshida *et al.*, 2012a; Athanasiou *et al.*, 2013).

Clínicamente, existe gran variabilidad en cuanto a la edad de inicio, la progresión de la enfermedad y las manifestaciones clínicas secundarias. La primera manifestación clínica, que a menudo tiene lugar en la adolescencia, es la ceguera nocturna con escasa capacidad de adaptación a la oscuridad (nictalopía) causada por la pérdida funcional de los bastones. La muerte de los bastones reduce el campo visual y provoca la pérdida de la visión periférica, manteniéndose la visión central (visión en túnel) (Figura 6).

Figura 6. Visión en túnel característica de pacientes con RP. Tras la pérdida de los bastones, dispuestos mayoritariamente en la periferia de la retina, se pierde la visión periférica manteniéndose la visión central.

La patología prosigue pudiendo llegar incluso en edades más avanzadas a la pérdida total de la agudeza visual, alteración en la percepción de los colores y fotopsias (Orphanet; Valverde-Pérez, 2001). Durante los exámenes oftalmológicos se puede observar pigmentación periférica del fondo de ojo con aspecto en espículas óseas, estrechamiento de los vasos y palidez del nervio óptico (Figura 7) (Kalloniatis and Fletcher, 2004).

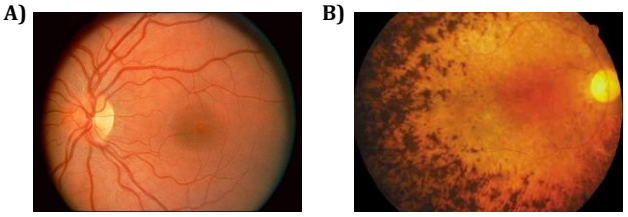

Figura 7. Imagen del fondo de ojo de un individuo sano (A) y de un paciente con retinosis pigmentaria (B). En la Figura B se observa pigmentación periférica, atenuación de la vasculatura y palidez del nervio óptico, características de la RP.

La RP es una enfermedad con una elevada heterogeneidad genética habiéndose descrito más de 3.000 mutaciones en alrededor de 60 genes o loci (Orphanet; Daiger *et al.*, 1996-2014). El 50% de los casos son esporádicos, y el resto presentan patrones dominantes, recesivos o ligados al cromosoma X. Se han descrito casos de herencia digénica, en la que interaccionan dos mutaciones en dos genes funcionalmente relacionados, y casos de herencia mitocondrial (Valverde-Pérez, 2001).

El 70-80 % de los casos de RP son de naturaleza no sindrómica y el 20-30 % restante están asociados a diversos síndromes como el síndrome de Usher o de Bardet-Biedl. El síndrome de Usher, el más frecuente, provoca también sordera neurosensorial y disfunción vestibular. El síndrome de Bardet-Biedl lleva asociado obesidad, polidactilia y retraso mental. Otros síndromes menos frecuentes son el síndrome de Senior-Locken, el síndrome de Alport, el síndrome de Älmstron y el síndrome de Joubert (Ayuso and Millan, 2010).

I.3.3 Modelos experimentales de retinosis pigmentaria

El modelo animal mejor caracterizado de RP es el ratón *rd1* o *Pde6brd1* (Pittler and Baehr, 1991), modelo de RP humana autosómica recesiva. El ratón *rd1* lleva una mutación espontánea *nonsense* en el gen de la subunidad β de la PDE6 que causa la muerte masiva de los bastones en las primeras semanas de vida. Con el tiempo también mueren los conos como en una RP típica (LaVail *et al.*, 1997). Otro modelo murino muy utilizado es el ratón *rd10* (*Pde6brd10*). Esta cepa lleva una mutación *missense* en el mismo gen que provoca un comienzo de la degeneración de los fotorreceptores más tardío que la del ratón *rd1* (Chang *et al.*, 2007). La progresión más lenta de la degeneración hace que el ratón *rd10* sea un modelo más apropiado de RP humana para diseñar terapias de rescate de los fotorreceptores (Otani *et al.*, 2004; Pang *et al.*, 2011). Los modelos de roedores han sido de gran ayuda en la comprensión de los mecanismos moleculares implicados en la muerte de los fotorreceptores. Sin embargo, como ya hemos comentado anteriormente, presentan ciertas diferencias estructurales con respecto a la retina humana como la ausencia de fóvea o el menor porcentaje de conos.

Para solventar este problema se ha tratado de buscar modelos alternativos de RP en animales superiores como es el caso de los modelos de RP en perros (Suber *et al.*, 1993; Tuntivanich *et al.*, 2009) y

gatos (May *et al.*, 2005). Sin embargo, estos modelos requieren mayores inversiones para su mantenimiento y tiempos de experimentación más largos.

En el año 2012 Ross y colaboradores establecieron un modelo de RP autosómico dominante en cerdo transgénico que presentaba la mutación P23H en el gen de la rodopsina (Ross *et al.*, 2012). Aunque la retina porcina se asemeja más a la retina humana, el uso del cerdo como modelo de RP no está exento de inconvenientes. El manejo de estos animales, su elevada susceptibilidad al estrés y el elevado coste de mantenimiento hace que no sea una tarea fácil disponer de ellos como modelo de RP. Una alternativa al trabajo con animales *in vivo*, es establecer modelos *ex vivo* (Lipinski *et al.*, 2011; Fernandez-Bueno *et al.*, 2012) o *in vitro* (Carmody and Cotter, 2000; Kaempf *et al.*, 2008). En estos modelos lo que puede ser un inconveniente en determinados casos como por ejemplo, la pérdida de conexión con otros órganos o tejidos que puedan influir en los mecanismos de degeneración de la retina, por otro lado, puede resultar una ventaja al permitirnos disponer de unas condiciones controladas en las que evaluar los mecanismos moleculares o celulares que están ocurriendo en presencia de distintos fármacos, etc.

I.3.4 Tratamientos en retinosis pigmentaria

En la actualidad no existe un tratamiento efectivo, sin embargo, se están llevando a cabo diversas aproximaciones terapéuticas en humanos (ensayos clínicos) y en modelos animales (ensayos preclínicos):

· Manipulación genética: se han probado distintas tecnologías con el objetivo de corregir la mutación genética causante de la enfermedad. La terapia génica ha resultado efectiva en algunos modelos murinos de RP (Chadderton *et al.*, 2009; Pang *et al.*, 2011; Petrs-Silva and Linden, 2014). Sin embargo, presenta algunos problemas que hacen difícil su aplicación en humanos como el uso de vectores virales, la disminución de la

efectividad con el avance de la enfermedad, siendo más efectiva en las primeras fases, etc (He *et al.*, 2014).

Se han ensayado tecnologías dirigidas a reprimir la transcripción del gen mutado en modelos de RP autosómica dominante mediante el uso de *zinc-fingers* o el sistema Cre-lox P (Mussolino *et al.*, 2011; Davis *et al.*, 2013). Uno de los principales problemas de estas técnicas es la necesidad de llevarlas a cabo en las etapas iniciales de la enfermedad para que resulten efectivas.

· Uso de células madre (*stem cells*) o células madre pluripotentes inducidas (*induced pluripotent stem cells*, iPSC): el uso de células madre es una terapia prometedora aunque presenta algunos inconvenientes que deben ser resueltos como por ejemplo, la respuesta inmune, la supervivencia celular, la migración al lugar diana, etc. y otras cuestiones de bioseguridad (He *et al.*, 2014; Tucker *et al.*, 2014; Yoshida *et al.*, 2014). El uso de células madre mesenquimales ha tenido resultados positivos en dos modelos de roedores de RP (Arnhold *et al.*, 2007; Wang *et al.*, 2010) y cada vez son más los ensayos clínicos que sugieren que la inyección de estas células es factible, no altera la estructura, ni resulta tóxica a largo plazo (He *et al.*, 2014). Recientemente, se ha iniciado un ensayo clínico en el que se va a evaluar la seguridad de la inyección intravítrea de células madre autólogas de médula ósea en 10 pacientes con RP en el Hospital Universitario Virgen de la Arrixaca de Murcia (ClinicalTrials.gov, NCT02280135).

· Manipulación farmacológica: el uso de fármacos es una estrategia ampliamente utilizada tanto en modelos animales como en humanos. En este sentido, en RP se han probado distintas drogas para disminuir la muerte celular (Doonan and Cotter, 2004; Nakazawa, 2011), mejorar el estado antioxidante de la retina (Komeima *et al.*, 2006; Umapathy *et al.*,

2013), mejorar la supervivencia con la administración de factores neurotróficos (Lipinski *et al.*, 2011; Xia *et al.*, 2011), etc.

· Optogenética: es una técnica de ingeniería genética que consiste en introducir en las neuronas de las capas internas de la retina un gen (denominado 'optogen') que exprese una proteína fotosensible (*channelrhodopsin-2*, *halorhodopsin*,…) en la superficie celular. Cuando dicha proteína recibe luz, se activa y modula la actividad neuronal iniciando una señal nerviosa equivalente a la generada por los fotorreceptores (Busskamp *et al.*, 2012; Roska *et al.*, 2013). Esta estrategia restaura la función visual en modelos murinos de RP (Busskamp *et al.*, 2010).

· Implantes electrónicos: pueden insertarse en la superficie interna de la retina para estimular las células ganglionares (implantes epiretinales) o en la superficie externa, entre la capa de los fotorreceptores y el epitelio pigmentario (implantes subretinales). Los implantes de retina estimulan eléctricamente las células supervivientes de la retina en pacientes con RP que han perdido gran parte de la visión, permitiendo restaurar habilidades visuales específicas como la percepción de la luz y el reconocimiento de objetos (Kusnyerik *et al.*, 2012; Stingl *et al.*, 2013).

La adopción de la estrategia terapéutica idónea depende en gran medida de la fase en que se encuentre el proceso degenerativo (Figura 8).

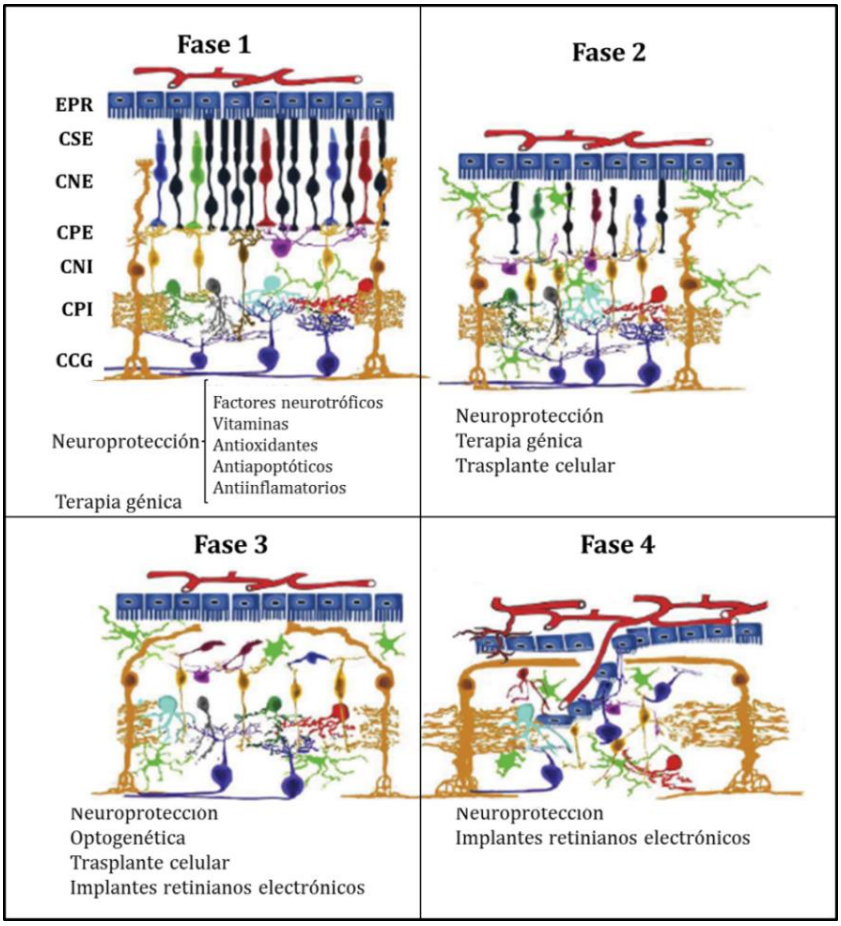

Figura 8. Fases de la degeneración retiniana y posibles terapias en cada una de ellas (Figura adaptada de (Cuenca *et al.*, 2014)). EPR: epitelio pigmentario retiniano, CSE: capa de los segmentos externos de los fotorreceptores, CNE: capa nuclear externa, CPE: capa plexiforme externa, CNI: capa nuclear interna, CPI: capa plexiforme interna, CCG: capa de células ganglionares.

Las intervenciones terapéuticas dirigidas contra el defecto genético primario serían muy específicas, sin embargo las terapias dirigidas contra los efectos secundarios de estas mutaciones (estrés oxidativo, inflamación, etc) tienen la ventaja de superar la heterogeneidad genética asociada a esta patología. Una aproximación combinada podría ser la estrategia terapéutica óptima.

I.4 PAPEL DEL ESTRÉS OXIDATIVO EN RETINOSIS PIGMENTARIA

I.4.1 Estrés oxidativo y sistema antioxidante en la retina

El estrés oxidativo se define como un desequilibrio entre la producción de especies reactivas de oxígeno (*reactive oxygen species*, ROS) y de nitrógeno (*reactive nitrogen species*, RNS) y los sistemas antioxidantes existentes en las células (Betteridge, 2000). Estas especies reactivas son una parte esencial de la inmunidad innata que protege a las células de las infecciones pero también contribuyen a la patogénesis de enfermedades degenerativas. Las ROS son moléculas químicamente reactivas derivadas del oxígeno cuyos principales miembros son el anión superóxido (O_2^-), el peróxido de hidrógeno (H_2O_2) y el radical hidroxilo (OH·). Las RNS son un grupo de moléculas químicamente reactivas derivadas del óxido nítrico (NO) cuyos principales miembros son los peroxinitritos ($ONOO^-$), el dióxido de nitrógeno (NO_2) y los S-nitrosotioles.

En condiciones fisiológicas, la retina es especialmente sensible al estrés oxidativo ya que las membranas de sus células son muy ricas en lípidos poliinsaturados (riesgo de peroxidación lipídica) y está expuesta a agentes pro-oxidantes (exposición a la luz o a elevadas concentraciones de oxígeno) (De La Paz and Anderson, 1992; Glickman and Lam, 1992). Por ello dispone de sustancias antioxidantes como los pigmentos visuales o las vitaminas C y E que contrarrestan estos efectos nocivos (Garland, 1991; Stoyanovsky *et al.*, 1995; Rozanowska *et al.*, 2012). Los pigmentos visuales actúan como filtros capaces de absorber la radiación de elevada energía como la melanina presente en los melanosomas de las células del epitelio pigmentario (Tokarz *et al.*, 2013), o los carotenoides abundantes en los fotorreceptores como la luteína, predominante en la

retina periférica, y la zeaxantina, concentrada en la mácula (Widomska and Subczynski, 2014).

En humor acuoso hay elevados niveles de ácido ascórbico o vitamina C (1 mM) y de glutatión (GSH) (2 μM) (Umapathy *et al.*, 2013) que protegen al ojo del daño oxidativo, neutralizando los radicales libres y las ROS (Ringvold, 1996). La carencia de ácido ascórbico en el organismo puede producir o acentuar la aparición de cataratas y degeneración macular asociada a la edad (DMAE) (Filas *et al.*, 2013). El α-tocoferol o vitamina E está presente en los segmentos externos de los fotorreceptores donde ayuda a prevenir la peroxidación lipídica. Las vitaminas C y E bloquean los radicales libres y la oxidación y degradación de la zeaxantina (Rozanowska *et al.*, 2012).

Las principales enzimas antioxidantes son la superóxido dismutasa (SOD), la catalasa (CAT) y las enzimas relacionadas con el metabolismo del glutatión: la glutatión peroxidasa (GPX) y la glutatión reductasa (GR). La SOD cataliza la conversión de O^{-}_{2} en H_2O_2. Según su localización subcelular y los metales del centro activo se distinguen tres tipos de SOD: la Cu/Zn-SOD citoplásmica (SOD1), la Mn-SOD mitocondrial (SOD2) y la Cu/Zn-SOD extracelular (SOD3) (Usui *et al.*, 2011). La CAT es una proteína tetramérica que convierte el H_2O_2 en agua y oxígeno. La GPX cataliza la oxidación de glutatión reducido (GSH) a glutatión oxidado (GSSG) mientras que la GR cataliza la conversión de GSSG a GSH, ayudando ambas al mantenimiento de los niveles de glutatión (Umapathy *et al.*, 2013).

I.4.2 Daño oxidativo en retinosis pigmentaria

Los bastones, además de ser más numerosos que los conos, son células metabólicamente muy activas con un elevado consumo de oxígeno. Durante la progresión de la RP, cuando estos bastones mueren como consecuencia de una mutación genética, el consumo de oxígeno en

la retina disminuye y se produce una situación de hiperoxia que originaría daño oxidativo. Este daño afectaría a la supervivencia de las células de la retina, entre ellas los conos, y además exacerbaría la muerte de los bastones. Los elevados niveles de oxígeno favorecerían la acumulación de radicales O_2^- que, junto con otras ROS y RNS como el H_2O_2 y los radicales OH·, causarían importantes daños a lípidos, proteínas y al ADN (Usui *et al.*, 2011). Esto es lo que se conoce como hipótesis oxidativa de la RP (Figura 9).

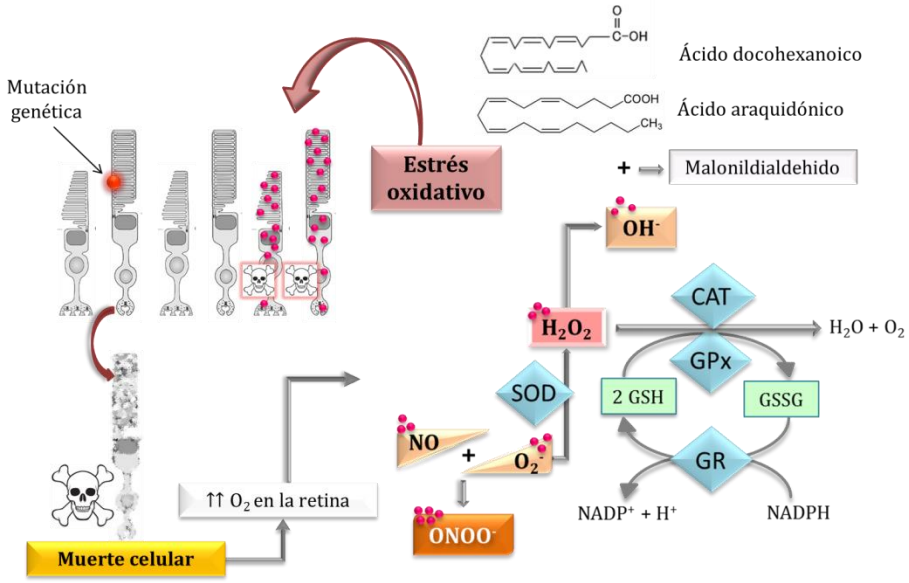

Figura 9. Representación de la hipótesis oxidativa de la RP. Tras la muerte de los bastones por mutaciones genéticas, el oxígeno en la retina aumenta provocando una acumulación de radicales libres, ROS y RNS (•) que el sistema antioxidante (◐) no sería capaz de neutralizar, provocando así la muerte de otras células de la retina.

En modelos animales de RP se ha asociado el estrés oxidativo con la muerte de los conos. En 2005, Shen y colaboradores demostraron en un modelo de cerdo transgénico de RP que tras la muerte de los bastones por mutaciones genéticas la hiperoxia generada causaba peroxidación lipídica y daño oxidativo a proteínas y ADN que afectaba a la supervivencia de los conos (Shen *et al.*, 2005). Posteriormente, Komeima

y colaboradores confirmaron estos resultados en el ratón *rd1*, y observaron, además, que la inyección de vitamina E y ácido α-lipoico reducía la muerte de los conos al prevenir el daño oxidativo tras la muerte de los bastones (Komeima *et al.*, 2006). En ratas P23H, un modelo autosómico dominante de RP, la muerte de los fotorreceptores inducida por la hiperoxia, incrementaba el estrés oxidativo y aceleraba la muerte de los fotorreceptores supervivientes (Yu *et al.*, 2004). Estos estudios apoyan la hipótesis oxidativa de la RP sugiriendo que los marcadores de estrés oxidativo, así como las enzimas antioxidantes pueden ser importantes dianas terapéuticas.

I.4.3 Terapias antioxidantes en retinosis pigmentaria

En los últimos años se han realizado varios ensayos en pacientes y en modelos animales de RP que evalúan la efectividad de terapias antioxidantes como la suplementación con antioxidantes o la modulación de la maquinaria antioxidante endógena. A continuación se comentan algunos ejemplos.

· Suplementación con antioxidantes: la *curcumina* es un polifenol procedente de la cúrcuma con propiedades antitumorales, antioxidantes y antiinflamatorias. La administración de curcumina mejora la morfología y fisiología de la retina en el modelo de rata P23H (Vasireddy *et al.*, 2011). El *ácido tauroursodeoxicólico (TUDCA)*, compuesto presente en el ácido biliar especialmente de los osos, preserva la función y el número de los fotorreceptores en el ratón *rd10* (Phillips *et al.*, 2008; Oveson *et al.*, 2011) y disminuye el estrés oxidativo y la actividad caspasa en un modelo de rata de desprendimiento de retina (Mantopoulos *et al.*, 2011). La *N-acetilcisteína*, derivado de la cisteína con propiedades antioxidantes, reduce la muerte de los fotorreceptores y preserva su función en ratones *rd1* y *rd10* (Lee *et al.*, 2011; Yoshida *et al.*, 2012b). En pacientes con RP se han probado diversos compuestos como

la *luteína* (Berson *et al.*, 2010), la *nilvadipina*, antagonista de los canales de calcio (Nakazawa *et al.*, 2011) o el *ácido clorogénico* (Shin and Yu, 2014) que parecen tener efectos beneficiosos al retrasar la pérdida de campo visual en pacientes con RP. También se está estudiando el efecto de la administración del *ácido docosahexanoico* (DHA) aunque, por el momento, no parece mejorar el estado antioxidante en sangre periférica de pacientes con RP (Hughbanks-Wheaton *et al.*, 2014).

· Modulación de la maquinaria antioxidante endógena: el aumento de la expresión de componentes de la defensa antioxidante endógena es una posible estrategia para el tratamiento de degeneraciones retinianas en las que el estrés oxidativo juega un papel importante. Por ejemplo, el aumento de la expresión de la glutatión peroxidasa tipo 4 en fotorreceptores en un modelo de ratón doble-transgénico reduce el contenido de proteínas carboniladas, preserva la función de la retina y reduce la muerte de los fotorreceptores inducida por hiperoxia o paraquat (Lu *et al.*, 2009). En RP, Usui y colaboradores (2009 y 2011) han demostrado que el incremento simultáneo de la expresión de SOD2 y de catalasa en el mismo compartimento subcelular previene de la muerte celular en ratones *rd10* (Usui *et al.*, 2009; Usui *et al.*, 2011).

I.5 PAPEL DE LA INFLAMACIÓN EN RETINOSIS PIGMENTARIA

I.5.1 Inmunoprivilegio y respuesta inflamatoria en la retina

La retina, junto con la córnea y la cámara anterior, constituyen las estructuras inmunoprivilegiadas del ojo. El inmunoprivilegio es una característica de algunos órganos que constituye un conjunto de procesos moleculares complejos que controlan la inflamación para prevenir el daño en tejidos especiales. El inmunoprivilegio se consigue a través de tres mecanismos: (a) barreras físicas como la hemato-acuosa y

la hemato-retiniana; (b) un microambiente intraocular inmunosupresor y (c) la tolerancia periférica (Taylor, 2009; Zhou and Caspi, 2010).

Diversos factores pueden desencadenar la activación de la respuesta inflamatoria: mutaciones genéticas, agentes biológicos (bacterias, virus,…), radiación UV, alteraciones vasculares, estrés oxidativo, etc. Esta respuesta está convenientemente regulada mediante el correcto equilibrio entre mecanismos proinflamatorios y antiinflamatorios en los que participan diversos mediadores como citoquinas, quimioquinas, factores de transcripción y de crecimiento, etc. El desarrollo de la respuesta inflamatoria tiene como principal propósito la protección celular, pero una respuesta exacerbada y mantenida en el tiempo puede convertirse en deletérea para las células.

Las citoquinas son proteínas de bajo peso molecular que median las comunicaciones intercelulares al interaccionar específicamente con receptores en la membrana de la célula diana (macrófago, célula T, célula B,…). Una de las principales citoquinas proinflamatorias que desencadena la respuesta inmunológica es el factor de necrosis tumoral alfa (TNFα), sintetizada como una molécula transmembrana de 26 kDa (memTNFα) que se escinde generando una molécula soluble de 17 kDa (sTNFα). Ambas son activas como homotrímeros pudiendo unirse a dos tipos de receptores en la superficie celular, TNFR1 (p55) y TNFR2 (p75), que activan distintas rutas de transducción de señales intracelulares. Mientras que el sTNFα se une preferentemente al receptor TNFR1, el memTNFα tiene mayor afinidad por el receptor TNFR2 (Nakazawa *et al.*, 2006). El TNFα desencadena la producción de otras citoquinas contribuyendo al proceso inflamatorio global. En el ojo, el TNFα interviene en la permeabilización de la barrera hemato-retiniana y parece estar implicado en la patogénesis de enfermedades oculares como la uveítis (Valentincic *et al.*, 2011) y en concreto en degeneraciones retinianas como la retinopatía diabética, la degeneración macular

asociada a la edad y recientemente en RP (Joussen *et al.*, 2009; Yoshida *et al.*, 2012a; Nassar *et al.*, 2014).

La interleuquina 6 (IL-6), está implicada en procesos inflamatorios, hematopoyesis, angiogénesis, diferenciación celular y supervivencia neuronal pudiendo actuar como citoquina proinflamatoria o antiinflamatoria. La IL-6 está aumentada en retinopatía diabética, degeneración macular asociada a la edad, glaucoma y RP (Seddon *et al.*, 2005; Cvenkel *et al.*, 2010; Cheung *et al.*, 2012; Yoshida *et al.*, 2012a). El receptor de la IL-6 consiste en dos glicoproteínas unidas a la membrana: una subunidad de 80 kDa (IL-6R, CD126) y un elemento de transducción de señales de 130 kDa (gp130, CD130). La IL-6 junto con el factor inhibidor de leucemia (LIF, *leukemia inhibitory factor*), el factor neurotrófico ciliar (CNTF, *ciliary neurotrophic factor*), la oncostatina M (OSM) y la cardiotropina 1 (CT-1) forman una familia de mediadores inflamatorios (*IL-6-type cytokines*) que ejercen su acción, generalmente neuroprotectora, a través de su unión al receptor gp130 (Samardzija *et al.*, 2006a). Algunos miembros de esta familia están alterados en distintas degeneraciones retinianas y se han propuesto como dianas terapéuticas como es el caso del CNTF (Wen *et al.*, 2012), la oncostatina (Xia *et al.*, 2011) o LIF debido a su papel neuroprotector (Joly *et al.*, 2008; Lange *et al.*, 2010; Agca *et al.*, 2013).

La interleuquina 1β (IL-1β) es una citoquina proinflamatoria secretada por macrófagos activados. Provoca neurotoxicidad, alteraciones vasculares y parece modular la angiogénesis por interacción directa con células endoteliales vasculares o promoviendo la producción de factores proangiogénicos de forma paracrina (Rivera *et al.*, 2013). Se han detectado niveles elevados de IL-1β en el humor vítreo de pacientes con retinopatía diabética y con RP (Kowluru and Odenbach, 2004; Yoshida *et al.*, 2012a).

La interleuquina 10 (IL-10) es una citoquina antiinflamatoria que inhibe la síntesis de citoquinas proinflamatorias como IFN-γ, TNFα e IL-1β, y activa rutas de protección celular como la vía Jak/STAT a través de la activación de STAT3, proteína antiapoptótica (Riley *et al.*, 1999; Boyd *et al.*, 2003). Se han observado niveles elevados de IL-10 en pacientes con degeneración macular asociada a la edad, glaucoma y RP (Chua *et al.*, 2012; Yoshida *et al.*, 2012a; Nassar *et al.*, 2014).

Las quimioquinas son citoquinas quimiotácticas que actúan a través de receptores acoplados a proteínas G. Algunos ejemplos de quimioquinas son las proteínas quimioatrayentes de monocitos (MCP), proteínas inflamatorias de macrófagos (MIP)-1α, MIP-1β, la fractalquina, etc (Graves and Jiang, 1995; Charo and Ransohoff, 2006). Estas moléculas causan daño secundario durante el proceso inflamatorio agravando la neurodegeneración. Se ha sugerido en diversos estudios que las degeneraciones retinianas inducidas por luz y en algunos casos hereditarios, los fotorreceptores que degeneran liberan quimioquinas que desencadenan la gliosis reactiva que se comenta más adelante. En ratones *rd,* con mutaciones en la subunidad β del gen de la PDE6, se ha visto que la expresión de diversas quimioquinas como MCP-1, MCP-3, MIP-1α y RANTES precede al inicio de la migración de la microglía (Zeng *et al.*, 2005).

Las células gliales (astrocitos, microglía y células de Müller) se vuelven reactivas en respuesta a un daño. Este proceso conlleva la proliferación y en algunos casos la hipertrofia de estos tipos celulares. Estudios realizados en modelos animales indican que la neuroinflamación retiniana mediada por la microglía participa en la muerte de los fotorreceptores (Thanos, 1992; Gupta *et al.*, 2003). Cuando hay daño celular, bacterias, lipopolisacáridos o ROS, la microglía se activa, migra al lugar del daño donde mata y fagocita a las células dañadas liberando TNFα, ROS y proteasas. El ambiente tóxico

extracelular que se produce destruye células sanas lo que produce un ciclo que amplifica el daño y acelera la muerte de los fotorreceptores (Zeng *et al.*, 2005; Peng *et al.*, 2014; Zeng *et al.*, 2014). A nivel tisular, la microglía presenta morfología ramificada y se localiza en las capas plexiformes y en las capas nucleares internas. Cuando se activa, migra hacia las capas más externas de la retina y adquiere morfología ameboide (Langmann, 2007).

Otro aspecto característico de la gliosis reactiva en la retina, además de la activación y migración de la microglía, es la activación de las células de Müller que en los primeros estadios proliferan e hipertrofian y en estadios más avanzados forman una cicatriz glial en el espacio subretiniano. Un marcador característico de esta activación es la sobrexpresión de la proteína fibrilar glial (GFAP) (Wang *et al.*, 2011; Roesch *et al.*, 2012). La activación de las células de Müller, a su vez, estimula el reclutamiento de microglía en la retina. Tras sufrir un daño, las células gliales de Müller liberan quimioquinas como las MCP-1 y citoquinas proinflamatorias como el TNFα, que promueven la infiltración de células inmunitarias (Figura 10) (Cuenca *et al.*, 2014).

I.5.2 Inflamación en retinosis pigmentaria

En los años 60-70 aparecieron las primeras publicaciones que sugerían que la inflamación podría estar implicada en RP. Diversos autores mostraron niveles elevados de autoanticuerpos en sangre de pacientes con RP e infiltración de linfocitos en humor vítreo (Viringipurampeer *et al.*, 2013). Sin embargo, los resultados eran variables probablemente debido a la heterogeneidad genética inherente a la RP. Años después, aparecieron las primeras evidencias de gliosis reactiva en modelos murinos y pacientes con RP. Se observó que la degeneración de los fotorreceptores iba acompañada de gliosis reactiva con activación y migración de la microglía de las capas internas de la

retina a las externas y al espacio subretiniano (Roque *et al.*, 1996; Gupta *et al.*, 2003; Zeng *et al.*, 2005; Peng *et al.*, 2014). La activación de las células gliales de Müller asociada a la degeneración de los fotorreceptores también se ha observado en varios modelos animales de RP con sobreexpresión de GFAP (Yu *et al.*, 2004; Cronin *et al.*, 2010; Roesch *et al.*, 2012; Chua *et al.*, 2013).

En los últimos años, se ha descrito un aumento de mediadores inflamatorios como citoquinas (TNFα, IL-6, IL-1β,...) y quimioquinas (MCP-1, RANTES, IL-8,...) en retina de ratones *rd10* (Yoshida *et al.*, 2012b) y en humor acuoso y vítreo de pacientes con RP (Yoshida *et al.*, 2012a). Los autores sugieren que este incremento de mediadores inflamatorios contribuiría a una peor función visual en pacientes.

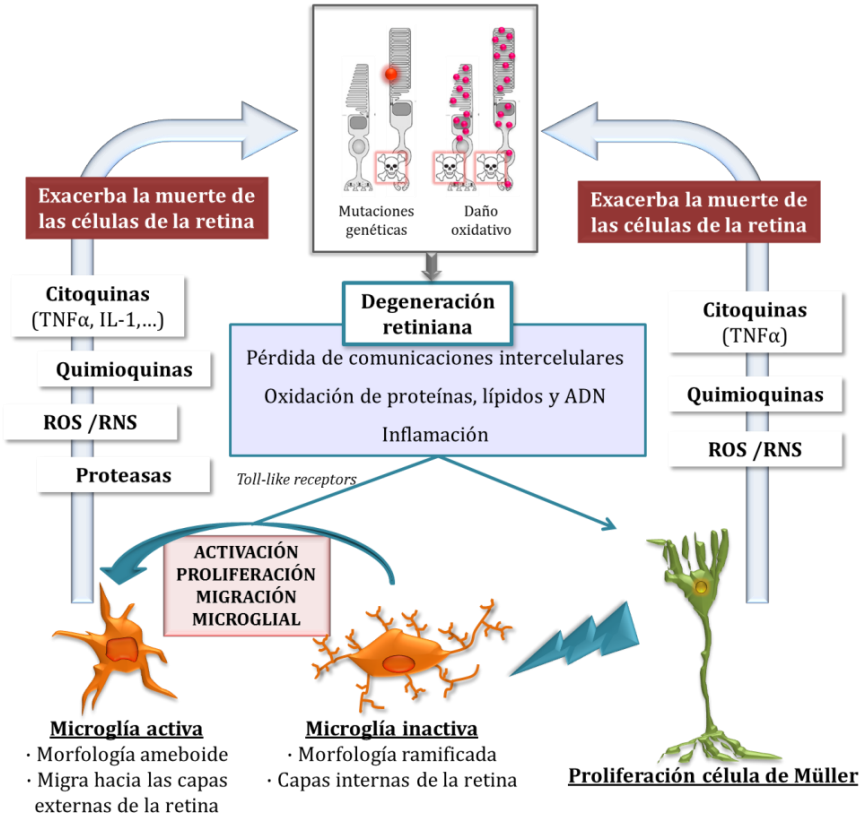

Figura 10. Proceso de gliosis reactiva en retinosis pigmentaria. La muerte de los fotorreceptores desencadena una serie de alteraciones como la pérdida de comunicaciones intercelulares, estrés oxidativo e inflamación que provocan la activación de la microglía. Cuando se activa, adquiere morfología ameboide y migra desde las capas más internas de la retina a las más externas, liberando citoquinas y especies reactivas del oxígeno (ROS) y del nitrógeno (RNS). En las primeras etapas de la degeneración, la microglía desencadena mecanismos de protección. Pero tras una excesiva o prolongada activación de la microglía se produce un proceso inflamatorio crónico. De forma similar actúan las células de Müller que, además, pueden activar a la microglía. En el proceso de gliosis proliferativa, las células de Müller secretan citoquinas proinflamatorias como el TNFα, quimioquinas como la MCP-1 o RNS como el óxido nítrico promoviendo la muerte celular neuronal. En definitiva, la muerte de los fotorreceptores genera un bucle de procesos inflamatorios que amplifican el daño, y con ello, la muerte de las células supervivientes de la retina.

I.5.3 Terapias antiinflamatorias en retinosis pigmentaria

Como se ha descrito anteriormente, la activación microglial es un hito del proceso inflamatorio que, si se mantiene de forma prolongada puede tener efectos deletéreos. Es por ello que la inhibición microglial se ha propuesto como diana terapéutica en RP. En ratones *rd10* y ratas con retinopatía isquémica el tratamiento con *minociclina*, que inhibe la activación de la microglía, reduce la muerte de los fotorreceptores y preserva la estructura y función de la retina (Abcouwer *et al.*, 2013; Peng *et al.*, 2014). El tratamiento con *corticoides*, como la acetonida de fluocinolona, también suprime la respuesta microglial y preserva significativamente la morfología y la función de los fotorreceptores (Glybina *et al.*, 2010). Los corticoides también se utilizan rutinariamente en el tratamiento del edema macular asociado a estadios avanzados de RP con más o menos éxito (Ozdemir *et al.*, 2005; Srour *et al.*, 2013).

Por otro lado, se ha comprobado que la activación del receptor gp130, al que se unen citoquinas neuroprotectoras como IL-6 o LIF, podría incrementar la supervivencia de los fotorreceptores (Rhee *et al.*, 2013). En estudios recientes se ha probado la efectividad de la administración del CNTF en pacientes con RP y en modelos murinos. Sin

embargo, no se ha observado un beneficio terapéutico destacable (Birch *et al.*, 2013; Pilli *et al.*, 2014). Además, parece que induce la expresión de genes proinflamatorios en las células de Müller en ratones (Talcott *et al.*, 2011).

El antioxidante N-acetilcisteína, comentado anteriormente, también reduce significativamente la expresión de IL-1β, TNFα y MCP-1 en ratones *rd10* (Yoshida *et al.*, 2012b). Una aproximación terapéutica todavía no ensayada en RP sería la administración de anticuerpos contra mediadores inflamatorios elevados como el TNFα. Los anticuerpos anti-TNFα se emplean en Oftalmología como tratamiento alternativo en pacientes con uveítis no infecciosa (Diaz-Llopis *et al.*, 2012), edema macular diabético (Wu *et al.*, 2011) y degeneración macular asociada a la edad (Wu *et al.*, 2012). En modelos animales de uveítis, glaucoma o retinopatía diabética, estos anticuerpos anti-TNFα mejoran la supervivencia de las células de la retina (Diaz-Llopis *et al.*, 2008; Joussen *et al.*, 2009; Roh *et al.*, 2012).

Actualmente existen varios tipos de inhibidores del TNFα disponibles en el mercado (Mirshahi *et al.*, 2012). El Infliximab (Remicade®), es una inmunoglobulina quimérica humana (IgG1) con un fragmento variable murino que tiene gran afinidad por el TNFα neutralizándolo. El Adalimumab (Humira®) es una anticuerpo totalmente humanizado contra TNFα. El Etarnecept (Enbrel®) es una proteína recombinante dimérica formada por una porción del TNFR2 humano unido a un fragmento cristalizable de la IgG1. El Certolizumab pegol (Cimzia®) es un fragmento Fab de anticuerpo monoclonal PEGylado. El Golimumab (Simponi®) es un anticuerpo humano IgG1k.

I.6 MECANISMOS DE MUERTE CELULAR EN RETINOSIS PIGMENTARIA

Se han propuesto varios mecanismos de muerte celular durante la progresión de la RP como la apoptosis (Cottet and Schorderet, 2009), necrosis (Trichonas *et al.*, 2010) y muerte celular no apoptótica (Sancho-Pelluz *et al.*, 2008). Recientemente, Sahaboglu y colaboradores (2013) han sugerido la existencia de otros mecanismos alternativos a la apoptosis y la necrosis mediados por la actividad de la proteína quinasa G (PKG) o de las histona deacetilasas (HDAC) (Sahaboglu *et al.*, 2013).

I.6.1 Apoptosis

La apoptosis es una forma de muerte celular controlada por múltiples vías de señalización. Morfológicamente, la apoptosis se asocia con encogimiento celular, condensación de la cromatina, alteraciones del citoesqueleto, condensación y fragmentación nuclear y formación de cuerpos apoptóticos que son fagocitados evitando así desencadenar una reacción inflamatoria.

Las caspasas, una familia de cisteína-aspártico proteasas, son las principales responsables de la regulación de la apoptosis en muchos sistemas. Las caspasas apoptóticas pueden dividirse en iniciadoras (caspasa 8, 9, 10) y efectoras (caspasa 3, 6, 7). Todas las caspasas se expresan como zimógenos inactivos (pro-caspasas) y se activan a través de distintos mecanismos: activación por otra caspasa previamente activada (es el caso de las caspasas efectoras), activación inducida por proximidad (por ejemplo la caspasa 8) y activación por asociación con alguna subunidad reguladora como es el caso de la caspasa 9.

Hay dos rutas apoptóticas principales: la ruta extrínseca que se activa en respuesta a factores externos como el ligando Fas (FasL) y el TNFα, y la ruta intrínseca que puede activarse en respuesta a varios estímulos

endógenos como daño en el ADN, niveles elevados de ROS, estrés celular, etc. y está mediada por la mitocondria (Figura 11) (Berridge, 2012).

En diversos modelos de RP se ha observado una implicación directa de la acción de las caspasas en la degeneración de la retina (Sanges *et al.*, 2006). El incremento de la actividad de la caspasa 3, una de las principales caspasas efectoras que se activa en ambas rutas, se ha relacionado con una rápida degeneración de los fotorreceptores en ratas con la mutación S334ter en la rodopsina (Cottet and Schorderet, 2009). No obstante, en los últimos años se ha reforzado la idea de que existen mecanismos alternativos a la apoptosis mediada por caspasas implicados en la degeneración de lo fotorreceptores (Sancho-Pelluz *et al.*, 2008; Nakajima *et al.*, 2011).

Las calpaínas, enzimas proteolíticas dependientes de calcio, son mediadoras de la ruta intrínseca de la apoptosis. Desempeñan un papel importante en la muerte de los fotorreceptores en ratones *rd1* y en ratas Royal College of Surgeons (*RCS,* modelo con una mutación en el gen *Mertk*) y, al igual que las caspasas, pueden constituir posibles dianas terapéuticas (Nakazawa, 2011).

I.6.2 Necroptosis

La necrosis programada o necroptosis es uno de los principales mecanismos de muerte celular independientes de caspasas. La necroptosis depende de la actividad de las proteína-quinasas RIP1 y RIP3 (Christofferson and Yuan, 2010).

Los dos mecanismos más estudiados a través de los cuales se inicia la necroptosis son el inducido por el TNFR1 (p55) tras la unión del TNFα y el mediado por la ruta de la poli(ADP-ribosa) polimerasa (PARP) (Sosna *et al.*, 2014). PARP es una enzima que cataliza la conversión de NAD^+ a polímeros de poli(ADP-ribosa) (PAR). En presencia de un daño celular

leve, la acumulación de PAR en el núcleo recluta proteínas reparadoras con el fin de evitar la muerte celular y proteger a la célula. Sin embargo, si el daño celular es grave, PAR actúa sobre la mitocondria directamente para inducir la muerte celular estimulando la liberación del factor inductor de apoptosis (AIF, *apoptosis-inducing factor*) que migra de la mitocondria al núcleo para formar un complejo con la histona H2AX y la ciclofilina A que degrada el ADN (Figura 11) (Heeres and Hergenrother, 2007). Paquet-Durand y colaboradores (2007) demostraron que tanto la actividad de PARP como la acumulación de PAR en la capa nuclear externa está implicada en la degeneración retiniana en ratones *rd1* (Paquet-Durand *et al.*, 2007).

I.6.3 Mecanismos alternativos: actividad HDAC

Las histona deacetilasas (HDACs) son enzimas involucradas en distintos procesos celulares como la regulación de la expresión génica, el reordenamiento del citoesqueleto y la división y diferenciación celular. Sancho-Pelluz y colaboradores (2010) describieron que la actividad de las HDAC, concretamente de las HDAC de tipo I y II, estaba implicada en la muerte de los fotorreceptores en el ratón *rd1* (Sancho-Pelluz *et al.*, 2010; Sancho-Pelluz and Paquet-Durand, 2012). El incremento de la actividad de HDAC parece ser responsable de la activación de PARP en la degeneración de los fotorreceptores en este modelo murino de RP (Figura 11) (Arango-Gonzalez *et al.*, 2014).

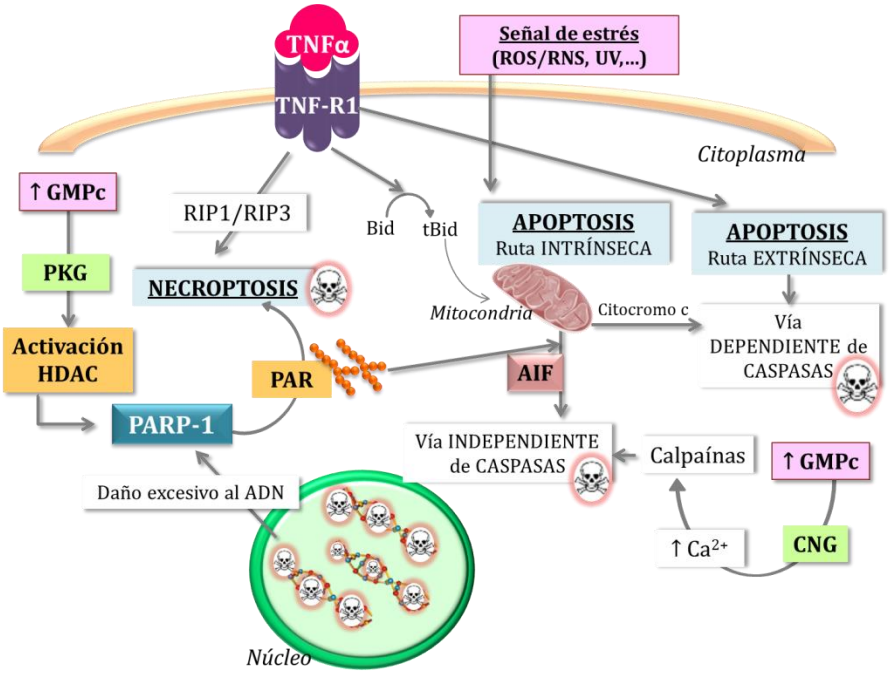

Figura 11. Representación de los posibles mecanismos que conducirían a la muerte celular a través de diversas vías: apoptosis, necroptosis y vía mediada por la actividad de PARP o de HDAC, en modelos de RP. El TNFα puede activar la vía extrínseca e intrínseca de la apoptosis y la necroptosis. Un excesivo daño al ADN provocaría una sobreactivación del enzima PARP que sintetiza PAR, el cual estimula la liberación de AIF y, en última instancia, la degradación del ADN. En modelos animales y pacientes con RP cuyas mutaciones provocan la acumulación de GMPc, pueden intervenir otras moléculas en el proceso de muerte celular. El GMPc provoca la apertura de los canales iónicos (CNG), causando la acumulación excesiva de calcio intracelular. El incremento del calcio intracelular activaría la vía intrínseca de la apoptosis mediada por calpaínas. Por otro lado, el GMPc activa las proteínas-quinasa G (PKG) que fosforilan proteínas. La fosforilación dependiente de PKG, activaría las HDAC, las cuales pueden activar la enzima PARP contribuyendo a la muerte celular.

I.6.4 Terapias para prevenir la muerte celular en retinosis pigmentaria

Como se ha descrito en los apartados I.4.3 y I.5.3, las terapias antioxidantes y antiinflamatorias son terapias dirigidas a prevenir la muerte de los fotorreceptores. Además de compuestos antioxidantes, neurotróficos o antiinflamatorios se han ensayado diversos compuestos

que actúan específicamente sobre componentes de las vías de señalización de muerte celular.

Uno de los tratamientos tradicionales ha sido el uso de compuestos antiapoptóticos. La inyección intravítrea de inhibidores de la caspasa 3 reduce drásticamente la muerte de los fotorreceptores en ratones *tubby*, modelo animal para el síndrome de Usher tipo I (Bode and Wolfrum, 2003). En dos modelos murinos de RP, los ratones *rd1* y VPP (ratones con una triple mutación en el gen de la rodopsina), se han observado niveles elevados de caspasa 1 en el inicio de la muerte celular y su ausencia solo tiene efecto protector en el ratón VPP (Samardzija *et al.*, 2006b). En ratas RCS la transferencia génica del factor derivado del epitelio pigmentario (PEDF) previene la migración al núcleo de AIF, una de las principales moléculas que desencadena la rotura del ADN, previniendo la muerte de los fotorreceptores (Murakami *et al.*, 2008). Otro ejemplo, en este caso un compuesto natural obtenido del azafrán, el *Safranal*, ejerce una función antiapoptótica disminuyendo la degeneración de los fotorreceptores y preservando la funcionalidad y la vasculatura de la retina en un modelo de rata de RP (Fernandez-Sanchez *et al.*, 2012).

Otra aproximación ha ido dirigida a inhibir la activación de calpaínas, mediadoras también de la ruta intrínseca de la apoptosis. En este sentido se han empleado antagonistas de los canales de calcio en modelos animales de RP, como el ratón *rd1*, pero los efectos han sido controvertidos (Nakazawa, 2011). La *calpastatina*, enzima inhibidora de las calpaínas, inhibe la activación de la proteína proapoptótica Bax reduciendo la muerte celular en ratones *rd1* (Comitato *et al.*, 2014).

Dirigido a reducir la muerte celular mediada por procesos necróticos, el bloqueo de PARP parece rescatar los fotorreceptores en el modelo de ratón *rd1* y en un modelo *in vitro* indicando que la actividad de PARP

contribuye a la muerte de los fotorreceptores (Paquet-Durand *et al.*, 2007; Sancho-Pelluz *et al.*, 2008).

El uso de inhibidores de HDAC, como el *ácido valproico*, parecen tener resultados prometedores en modelos animales y en pacientes con RP (Clemson *et al.*, 2011; Sancho-Pelluz and Paquet-Durand, 2012; Shanmugam *et al.*, 2012; Kumar *et al.*, 2014). Estos compuestos podrían actuar a través de la regulación de la transcripción, promoviendo la regeneración y la proliferación celular, la supervivencia celular, el efecto antiinflamatorio, la atenuación de la muerte celular y el aumento la expresión de factores neurotróficos.

Capítulo II. Hipótesis y Objetivos

HIPÓTESIS

La retinosis pigmentaria (RP) es una enfermedad genética que provoca degeneración retiniana y ceguera. Actualmente no existen terapias efectivas que retrasen la progresión de la enfermedad. Es por ello de gran interés profundizar en el conocimiento de los mecanismos moleculares y celulares que participan en su patogénesis así como el descubrimiento de nuevas dianas terapéuticas. Las mutaciones genéticas son las responsables de la muerte de los bastones. Sin embargo, la muerte de los conos y consiguiente pérdida de la visión central, parece debida a los cambios metabólicos que provoca la degeneración de los bastones. Aunque es una enfermedad de origen genético, en los últimos años se ha puesto de relevancia la implicación del estrés oxidativo y la inflamación en la progresión de la enfermedad.

Partimos de las siguientes hipótesis:

1. La determinación de factores inflamatorios, de respuesta antioxidante y de estrés oxidativo en el humor acuoso y en sangre periférica de pacientes con RP nos permitirá conocer el microambiente que rodea a los fotorreceptores, identificar posibles biomarcadores periféricos y encontrar nuevas dianas terapéuticas.
2. El cultivo organotípico de retina porcina expuesta a agentes elevadores de GMP cíclico podría ser un buen modelo para estudiar ciertos aspectos de la degeneración de los fotorreceptores en RP.
3. El TNFα puede ser una molécula implicada en la degeneración de los fotorreceptores en RP. Su inhibición con anticuerpos específicos podría reducir el proceso inflamatorio y con ello, la muerte de los fotorreceptores.
4. El estudio de la inhibición del TNFα con anticuerpos en un modelo *ex vivo* y en un modelo *in vivo* nos permitirá profundizar en el papel de la inflamación sobre la progresión de la RP. Asimismo podría suponer

el establecimiento de una nueva diana terapéutica para el desarrollo de tratamientos que previniesen o retrasasen el avance de la enfermedad en humanos.

OBJETIVOS

1. Determinar la presencia de factores solubles implicados en la progresión o en el retraso de la degeneración retiniana en pacientes con retinosis pigmentaria.

1.1 Determinar la respuesta antioxidante y el estrés oxidativo/nitrosativo en humor acuoso y en sangre periférica de pacientes con retinosis pigmentaria.

1.2 Analizar el contenido de mediadores inflamatorios (IL-10, IL-1β, IL-6 y TNFα) en humor acuoso y en sangre periférica de pacientes con RP.

1.3 Evaluar la relación entre el grado de afectación visual y el nivel de estrés oxidativo/nitrosativo e inflamación.

2. Desarrollar un modelo experimental de degeneración retiniana *ex vivo:* cultivos organotípicos de retina porcina expuestos a Zaprinast, un inhibidor de fosfodiesterasas.

2.1 Puesta a punto de cultivos organotípicos de retina porcina.

2.2 Caracterizar la degeneración retiniana en cultivos tratados con Zaprinast:

a) Procesos inflamatorios

b) Generación de estrés oxidativo

c) Rutas de muerte celular

3. Estudiar el efecto de la inhibición del TNFα con anticuerpos anti-TNFα sobre la progresión de la retinosis pigmentaria.

3.1 Estudiar el efecto de la inhibición del TNFα *ex vivo* en el modelo experimental de retina porcina.

a) Efecto sobre la progresión de la degeneración retiniana.

b) Efecto sobre la progresión de la inflamación.

c) Efecto sobre la respuesta antioxidante y el estrés oxidativo/nitrosativo.

3.2 Estudiar el efecto de la inhibición del TNFα *in vivo* en ratones *rd10*.

a) Efecto sobre la progresión de la degeneración retiniana.

b) Efecto sobre la progresión de la inflamación.

c) Efecto sobre la respuesta antioxidante.

Capítulo III. Artículo

Altered antioxidant-oxidant status in the aqueous humor and peripheral blood of patients with retinitis pigmentosa

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ALTERED ANTIOXIDANT-OXIDANT STATUS IN THE AQUEOUS HUMOR AND PERIPHERAL BLOOD OF PATIENTS WITH RETINITIS PIGMENTOSA

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ABSTRACT

Retinitis Pigmentosa is a common form of hereditary retinal degeneration constituting the largest Mendelian genetic cause of blindness in the developed world. It has been widely suggested that oxidative stress possibly contributes to its pathogenesis. We measured the levels of total antioxidant capacity, free nitrotyrosine, thiobarbituric acid reactive substances (TBARS) formation, extracellular superoxide dismutase (SOD3) activity, protein, metabolites of the nitric oxide/cyclic GMP pathway, heme oxygenase-I and inducible nitric oxide synthase expression in aqueous humor or/and peripheral blood from fifty-six patients with retinitis pigmentosa and sixty subjects without systemic or ocular oxidative stress-related disease.

Multivariate analysis of covariance revealed that retinitis pigmentosa alters ocular antioxidant defence machinery and the redox status in blood. Patients with retinitis pigmentosa present low total antioxidant capacity including reduced SOD3 activity and protein concentration in aqueous humor. Patients also show reduced SOD3 activity, increased TBARS formation and upregulation of the nitric oxide/cyclic GMP pathway in peripheral blood.

Together these findings confirmed the hypothesis that patients with retinitis pigmentosa present reduced ocular antioxidant status. Moreover, these patients show changes in some oxidative-nitrosative markers in the peripheral blood. Further studies are needed to clarify the relationship between these peripheral markers and retinitis pigmentosa.

INTRODUCTION

Retinal degenerations are the major cause of incurable blindness characterized by loss of retinal photoreceptor cells. Retinitis Pigmentosa (RP) is a common form of retinal degeneration, constituting the largest Mendelian genetic cause of blindness in the developed world. It has a prevalence of 1 in 4000, and it has been estimated that about two million people are affected worldwide. Patients with RP typically loose night vision in adolescence, peripheral vision in young adulthood, and central vision later in life due to the progressive loss of rod and cone photoreceptor cells by apoptosis [1]. About 20% of patients have an associated hearing loss and the combination is called Usher syndrome. There are approximately 60 genes implicated in the pathology of RP [2, 3].

Photoreceptor cell death starts with rod photoreceptor degeneration and eventually cone cell death, which is the major problem affecting RP patients because it leads to loss of central vision. Cone cells die possibly as a consequence of progressive oxidative damage [4-8], metabolic dysregulation, loss of trophic support [9] and, toxicity due to rod cell death [10]. Photoreceptor cells are especially susceptible to oxidative stress because of their high metabolic rate and their environmental risks such as exposition to ultraviolet radiation or high oxygen tension. The endogenous antioxidant machinery, which includes the mitochondrial antioxidant enzymes superoxide dismutases (SOD), glutathione peroxidases and catalases [11], contributes to reduce oxidative stress in photoreceptor cells. Rods represent 95% of all photoreceptors in humans [12] and are the main consumers of oxygen in the outer retina. Rods die in early stages of the disease, leading to an overload of oxygen in the retina. The cone redox balance is then disturbed and the resulting oxidative stress exceeds the antioxidant capacity of cones, contributing to their death. The *oxidative stress hypothesis* is supported by several

lines of evidence in experimental models of RP [6, 7, 13, 14]. In these models, oxidative markers such as decreased reduced form of gluthatione, increased malondialdehyde, or nitric oxide [7, 13, 15] have been found. Supporting this idea, antioxidant formulations have reduced cone cell death in models of RP [6, 7, 16, 17]. In addition, overexpression of the endogenous antioxidant enzymes, including SOD and glutathione peroxidase, decreased oxidative damage and prolonged cone survival in some RP mouse models [18, 19].

In this study, we evaluated the presence of some markers of the antioxidant-oxidant status in aqueous humor and peripheral blood of RP patients and compared them with those found in healthy controls. We measured total antioxidant capacity, extracellular superoxide dismutase (SOD3) activity, nitric oxide formation and protein concentration in aqueous humor. We also determined total antioxidant capacity, SOD3 activity, SOD3 content, cyclic GMP, nitrotyrosine, nitric oxide and TBARS formation in peripheral blood. In addition, we evaluated the relationship between visual function and ocular antioxidant status. To our knowledge, this is the first time that ocular antioxidant status has been evaluated in RP patients.

MATERIAL AND METHODS

Participants in the study

Fifty-six patients with typical forms of RP characterized by an elevated final dark-adaptation threshold, retinal arteriolar narrowing, and a reduced and delayed electroretinogram were enrolled in the study. Thirty-seven patients donated aqueous humor and blood and only nineteen patients donated blood. Smoking and antioxidant consumption were taken into account in the study. Sixty age-matched subjects with no confounding ocular or systemic disease (blood donors) and thirteen patients suffering from cataracts without any other ocular or systemic

disease (aqueous humor donors) served as controls. Since this was a retrospective non-randomized, observational study it was difficult to know whether there were a hidden non-measured variable (diet, environmental, etc.) affecting our results. However, it is possible to look for these hidden data mentioned above, using the Rosenbaum Sensitivity test [20], that addresses the possibility of hidden bias in observational studies. This analysis may answer the question "how high an unmeasured covariate's effect would have to be to alter the conclusions of the study". The result of the Rosenbaum Sensitivity test showed that the differences found among groups are highly insensitive against hidden bias, and therefore that there are not hidden variable affecting our results.

The characteristics of the patients enrolled in the study, all of them Caucasian, are shown in Table 1. Written informed consent was obtained before extraction of aqueous humor and peripheral blood. The procedure complied with the Declaration of Helsinki and was reviewed and approved by the Ethics Committee of La Fe University Hospital (Valencia, Spain).

Table 1. Characteristics of participants included in the study

	Aqueous humor samples		**Blood samples**	
	Control	**RP**	**Control**	**RP**
Nº of subjects	13	37	60	56
Males	7	26	31	26
Females	6	11	29	30
Age (yr)	60 ± 3	46 ± 2	41 ± 2	44 ± 2
Type of RP	--	3 AR 7 AD 6 USH 21 unknown	--	5 AR 8 AD 15 USH 28 unknown

Note: RP: non syndromic RP, USH: Usher syndrome, AR: autosomic recessive, AD: autosomic dominant

Patients diagnosed with RP were recruited from *Retina Comunidad Valenciana- Asociación Afectados por Retinosis Pigmentaria* and also from the Ophthalmology Service of La Fe University Hospital (Valencia, Spain). Healthy controls were recruited from the Ophthalmology Service of La Fe University Hospital (Valencia, Spain) and also from the Biobank La Fe (Valencia, Spain).

Ophthalmic examination

Patients from which aqueous humor was obtained underwent a full ophthalmic examination including best-corrected visual acuity (BCVA) and automated visual field (VF). The BCVA was measured according to the Early Treatment Diabetic Retinopathy Study (ETDRS) protocol adapted for use in the Age-Related Eye Disease Study [21]. We used the measurement of static perimetric sensitivities (i.e., total point score) (30-2 program with size V target; Humphrey Field Analyzer (HFA); Carl Zeiss Ophthalmic Systems, Inc., Dublin, CA). The size V target was used to minimize the number of locations with floor effects (sensitivity, ≤0 dB). The FASTPAC test strategy was used to test the central 30-2 visual field [22, 23].

Aqueous humor extraction

Aqueous humor samples from 37 RP patients (31 with non-syndromic RP and six with Usher syndrome) were collected under sterile conditions in a cabin for controlled air quality (ARCSterile) (ARCMedical, Barcelona, Spain). We applied one drop of povidone iodine applied before and after the anterior chamber was punctured using a 30-gauge needle. Antibiotic prophylaxis was subsequently administered for several days. At the time of the sample collection, six patients were smokers and 14 took some kind of antioxidant supplements including vitamins, omega-3 fatty acids, and minerals. Aqueous humor samples from 13 patients suffering from cataracts without any other ocular or

systemic disease were collected with a 30-gauge needle just before the beginning of the cataract surgery and served as controls. The aqueous samples of controls were collected under the same conditions before cataract surgery began. Undiluted aqueous samples of at least 100 μL were collected from each patient, placed in sterile tubes, and stored immediately at - 80°C until use. All specimens were assayed to evaluate antioxidant status in a double-blind arrangement with respect to their group. For each patient, aqueous humors were collected from the eye with the more severe retinopathy or alternatively from the eye with the worse visual acuity.

Serum preparation

Whole blood (8 mL) without EDTA from 56 RP patients (41 with non-syndromic RP and 15 with Usher syndrome) and 60 healthy controls was obtained and centrifuged at 2000 g for five min at 20°C. At the time of the sample collection, 10 patients were smoker and 24 took some type of antioxidant supplements. Serum was collected, aliquoted and frozen at -80°C prior to biochemical determinations were performed. To avoid cGMP degradation 7 mM EDTA was added to an aliquot before freezing.

Evaluation of antioxidant-oxidant status

Antioxidant-oxidant status was analysed in aqueous humor and serum from RP patients and healthy controls. Serum samples were assayed for total antioxidant capacity (TAC), TBARS formation as indicator of malonyldialdehyde (MDA) formation, and SOD3 activity.

TAC was measured using a commercial kit (Cayman Chemical, Ann Arbor, MI) according to manufacturer's instructions. Serum and aqueous humor TAC levels were expressed as μmol/mL.

MDA levels were detected by a colorimetric method involving thiobarbituric acid (TBA) adduct formation (Cayman Chemical, Ann

Arbor, MI) according to manufacturer's instructions. Serum TBARS levels were expressed as μmol/L.

SOD3 activity was measured using a commercial kit (Cayman Chemical, Ann Arbor, MI) according to manufacturer's instructions. Serum and aqueous humor SOD3 activity were expressed as U/mL.

Western blot

Fifty μg total serum protein from 28 RP patients and 27 healthy controls were subjected to electrophoresis in a 10 % polyacrylamide gel containing 0.1 % SDS under reducing conditions, transferred to a polyvinylidene difluoride (PVDF) membrane, and probed with rabbit anti-human SOD3 (1:3000, abcam plc, Cambridge, UK) or rabbit anti-human transferrin (1:1000, abcam plc. Cambridge, UK). The images were captured using an EPSON SCAN from EPSON Corporation (EPSON IBERICA, Barcelona, Spain) and quantified using the Alpha Imager 2200 (version 3.1.2) software (AlphaInnotech Corporation, San Francisco, CA, USA). SOD3 content was normalized to transferrin, a loading control for serum samples [24].

Cyclic GMP determination

Serum preserved in 7 mM EDTA was used to determine the amount of cGMP. cGMP was measured by using the BIOTRAK cGMP enzyme immunoassay kit (GE Healthcare Europe GmbH, Barcelona, Spain). cGMP levels were expressed as pmol/mL.

Nitrites and nitrates (NOX) determination

Nitrites (stable end-product of NO) and nitrates (NOX) levels were measured in serum by the spectrophotometric Griess reaction [25] using nitrate reductase. Serum NOX levels were expressed as nmol/mL.

Nitrotyrosine determination

Serum-free nitrotyrosine was measured by the analytical method LC-MS/MS system, which consisted of an Acquity Ultraperformance LC® liquid chromatography system coupled to an Acquity® TQ-S triple quadruple mass Spectrometer (Waters, Manchester, UK).

Nitrotyrosine and phenyl-alanine-D5 (internal standard) stock solutions were separately prepared at 200 μM in deionized water. Two internal standard solutions at 2 μM and 0.1 μM, and a nitrotyrosine solution at 10 μM were prepared separately in deionized water from the respective stock solutions. Subsequent work solutions were prepared by dilution 1:1 (v/v) with deionized water of the nitrotyrosine solution (10 μM) within the concentration range from 625 nM to 0.153 nM. After thawing at room temperature, 50 μL of serum was precipitated (1:2, v/v) with ice-cold acetonitrile (ACN), vortexed, and centrifuged at 13000 g for 15 min at 4 °C. Then, 100 μL of the supernatant was evaporated to dryness using a Savant Speed Vac SPD121P concentrator (Thermo Fisher Scientific SLU, Madrid, Spain) and redissolved in 100 μL of deionized water containing the internal standard (0.1 μM). Finally, the solution was injected into the LC-MS/MS system. Mobile phase consisted of deionized water and ACN, both containing 0.1 % (v/v) of formic acid (FA). The injection volume was 5 μL.

The triple quadrupole MS detector operated in positive electrospray ionization (ESI) mode by selected reaction monitoring (SRM). Calibration curves were obtained by plotting the target analyte/internal standard peak area ratio for each analyte against its added concentration. Free nitrotyrosine levels were expressed as nmol/L.

Protein concentration

Protein concentration was measured in aqueous humor and serum by the bicinchoninic acid (BCA) protein assay (BCA Kit; Pierce Scientific, CA). Protein content was expressed as mg/mL.

Human mononuclear cell isolation

Whole blood (4 mL) with EDTA from 20 RP patients and 20 healthy controls was obtained and diluted with one volume of phosphate-buffered saline (PBS). Peripheral blood mononuclear cells (PBMC) were obtained as described by Kimura *et al.* [26]. In brief, diluted blood was layered onto Lymphoprep solution (density 1.077 g/mL, AXIS-SHIELD, Oslo, Norway) and centrifuged at 800 g for 20 min at 20 °C. The pellet containing mononuclear cells was collected and washed with three volumes of phosphate-buffered saline and centrifuged at 450 g for 10 min. The cellular pellet was frozen at -80 °C until RNA extraction.

RNA extraction and cDNA synthesis

Total RNA was isolated from PBMC using RNeasy mini kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. RNA concentration and purity were determined by UV spectrophotometry at 260 and 280 nm wavelengths. Then, cDNA was synthesized from 1 μg of total RNA by reverse transcription using the GeneAmp Gold RNA PCR Reagent kit (Applied Biosystems, Carlsbad, CA, USA) following manufacturer's instructions.

Quantitative real-time PCR analysis in PBMC

The relative expression of inducible nitric oxide synthase (iNOS) (Hs01075529_m1) and heme oxygenase 1 (HO-1) (Hs01110250_m1) was evaluated by qPCR using the TaqMan® gene expression assay (Applied Biosystems, Life Technologies Corporation, Carlsbad, California,

USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Hs02758991_g1) gene was used as a housekeeping gene control. A positive control of iNOS was obtained from human umbilical vein endothelial cells (HUVEC) treated with 10 µg/mL LPS for 4 hours.

Statistical analyses

All statistical analyses were done using R software (version 2.15.3) (Foundation for statistical computing, Vienna, Austria). Multivariate analysis of covariance (MANCOVA) [27], fuzzy clustering [28, 29], Mann-Whitney test, Rosenbaum's Sensitivity test [20] and multiple linear regression models were used to analyze the data. A 0.05 significance threshold was used.

RESULTS

Antioxidant status in eyes from patients with retinitis pigmentosa

Fifty aqueous humor samples were collected from 37 RP patients (31 with non-syndromic RP and six with Usher syndrome) and from 13 healthy controls. Table 1 shows demographic features of all participants enrolled in this study. Few markers could be explored because of limited availability of aqueous humor including TAC, SOD3 activity, NOX formation and protein concentration. Descriptive statistics of each marker are shown in Table 2.

Table 2. Antioxidant-oxidant markers and protein in aqueous humor from RP patients and healthy controls.

	Control	RP
TAC (µmol/mL)	1.46 ± 0.1	1.06 ± 0.1*
SOD3 (U/mL)	9.8 ± 1.5	3.7 ± 0.4**
Protein (mg/mL)	3.6 ± 0.2	3.1 ± 0.2
NOX (nmol/mL)	198 ± 25	231 ± 22

Note: RP: retinitis pigmentosa; TAC: total antioxidant capacity; SOD: superoxide dismutase; NOX: nitrates and nitrites. Values are expressed

as mean ± SEM. MANCOVA was carried out considering all response variables (TAC, SOD3, Protein and NOX) simultaneously (* $p < 0.05$; ** $p < 0.01$).

Since several markers (response variables) were measured from the same individuals and since these variables were correlated, performing analysis of covariance (ANCOVA) tests for each response variable would yield non- independent results and make the results difficult to interpret. This approach would also increase the family-wise type I error rate. A sensible approach would be to assess group differences on all the response variables considered simultaneously. Following this approach, we performed a multivariate analysis of covariance (MANCOVA) with the disease, age, gender, and antioxidant consumption as predictive variables. This revealed that RP significantly altered ocular antioxidant status in aqueous humor ($p = 0.001$) (Table 3). We found no statistical evidence for gender, age or antioxidant consumption effects. Further analysis of each of the response variables indicated that TAC and SOD3 activity were decreased in patients with RP ($p = 0.017$ and $p < 0.001$, respectively). Stable end-products of nitric oxide, NOX, were not affected by RP ($p = 0.630$). However, protein concentration showed a tendency to decrease in RP patients ($p = 0.057$). No statistical differences ($p = 0.450$) were found between patients with syndromic RP (Usher syndrome) and non-syndromic RP (data not shown).

Table 3. MANCOVA in aqueous humor and blood from patients with RP and healthy controls.

	Aqueous humor		**Blood**	
Predictive Variables	**Pillai´s trace**	**P-value**	**Pillai´s trace**	**P-value**
Age	0.1261	0.2753	0.1832	0.0119*
Gender	0.1003	0.4043	0.1107	0.1307
Disease RP	0.3841	0.0010**	0.2579	0.0025**
Antioxidant consumption	0.2014	0.0737	0.0752	0.3403

Note: Pillai´s trace, multivariate test criteria used in the MANCOVA.

Due to the large number of oxidants contained in cigarette smoke, we analyzed whether smokers (16% of patients) showed an altered antioxidant status compared to non-smokers in aqueous humor. No statistical differences were found between smoker and non-smoker patients ($p = 0.490$).

Antioxidant-oxidant status in the peripheral blood of patients with retinitis pigmentosa

In light of these results, we assessed whether antioxidant-oxidant status was also disturbed in peripheral blood. Blood samples were collected from 41 patients with non-syndromic RP, 15 patients with syndromic RP (Usher syndrome) and 60 healthy controls (Table 1). We analyzed the following markers: TAC, SOD3 activity and content, TBARS formation (as an indicator of lipid peroxidation) and metabolites of the NO/cGMP pathway.

MANCOVA revealed alterations in the antioxidant-oxidant status in serum of RP patients ($p = 0.002$) (Table 3). Descriptive statistics of each response variable are shown in Table 4. This analysis indicated that age seems to play a role in serum antioxidant-oxidant status, with higher levels of oxidative-nitrosative markers in older participants than in younger participants ($p = 0.012$). However, we did not find statistical evidence for gender or antioxidant consumption effects. Our results revealed that while serum SOD3 activity was decreased, serum TBARS formation was significantly increased in RP patients ($p = 0.025$ and $p = 0.004$ respectively) compared to healthy controls. No significant differences were found in serum TAC.

Table 4. Antioxidant-oxidant markers in blood from RP patients and healthy controls.

	Control	RP
TAC (µmol/mL)	1.15 ± 0.04	1.17 ± 0.05
SOD3 (U/mL)	11.60 ± 0.70	8.30 ± 0.40*
TBARS (µmol/L)	5.10 ± 0.20	7.10 ± 0.40**
NOX (nmol/mL)	49 ± 2	57 ± 3*
cGMP (pmol/mL)	0.41 ± 0.03	0.68 ± 0.06*

Note: RP: retinitis pigmentosa; TAC: total antioxidant capacity; SOD: superoxide dismutase; TBARS: thiobarbituric acid reactive substances; NOX: nitrates and nitrites; cGMP: cyclic guanosine monophosphate. Values are expressed as mean ± SEM. MANCOVA was carried out considering all response variables (TAC, SOD3, TBARS, cGMP and NOX) simultaneously (* $p < 0.05$, ** $p < 0.01$).

No statistical differences were found between healthy controls (0.33 ± 0.04 a.u) and RP patients (0.23 ± 0.03 a.u, Mann-Whitney test, $p = 0.21$) in serum SOD3 content (Fig. 1A). We analysed whether SOD3 content correlated with SOD3 activity in serum samples (Fig. 1B). SOD3 activity almost correlated with SOD3 content in healthy subjects (Spearman´s coefficient = 0.37, $p = 0.06$) (Fig. 1C). However, no correlation was found for RP patients (Spearman´s coefficient = -0.07, $p = 0.74$) (Fig. 1D).

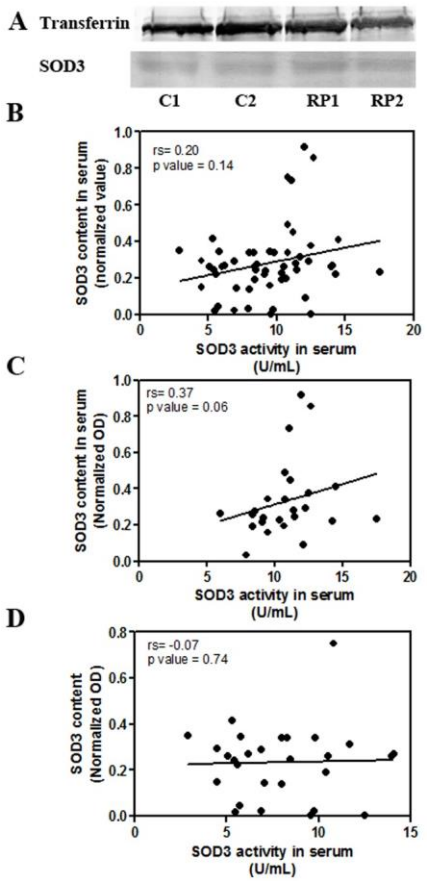

Figure 1. Protein content of SOD3 in serum from RP patients and healthy controls. (A) Fifty µg of total serum protein were subjected to electrophoresis and extracellular SOD3 content was analysed by inmunoblotting, as described in Material and Methods. The immunological signal of SOD3 was normalized to transferrin, a loading control for serum samples; Correlation analysis between SOD3 activity and SOD3 content in serum from patients and healthy controls **(B)**, healthy controls **(C)** or patients **(D)**. Spearman's correlation test was used (rs=correlation coefficient).

The serum NO/cGMP pathway was also analyzed by measuring NOX formation, free nitrotyrosine and cGMP levels and expression of iNOS in PBMC. Serum NOX was significantly higher in RP patients ($p = 0.033$) than in healthy controls. Free nitrotyrosine, another indicator of NO formation *in vivo*, was not included in MANCOVA due to the high number of missing values in this variable. However, results clearly suggested that

free nitrotyrosine levels were higher in RP patients (2.6 ± 0.2 nM, n = 41) than in healthy controls (1.7 ± 0.1 nM, n = 34) (Fig. 2). No iNOS expression was detected by qPCR in PBMC of RP patients or healthy controls. The content of cGMP, a downstream effector of NO, was higher in RP patients than in healthy controls ($p = 0.015$). Elevated serum cGMP could be a consequence of higher soluble guanylate cyclase (sGC) activation by NO or carbon monoxide (CO). CO is generated through heme oxygenase enzymes HO-I and HO-II. qPCR analysis revealed that HO-I was up-regulated (Mann-Whitney test, $p < 0.05$) in PBMC from RP patients (Fig. 3).

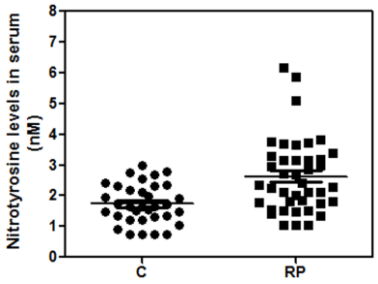

Figure 2. Blood free nitrotyrosine concentration in RP patients and healthy controls. Free nitrotyrosine was measured by LC-MS/MS system as described in Material and Methods. Values are expressed as mean ± SEM.

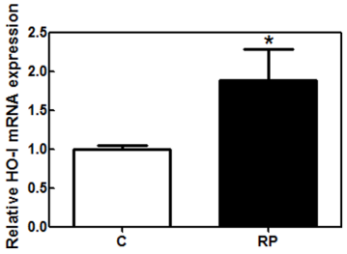

Figure 3. Gene expression of heme oxygenase I in peripheral blood mononuclear cells of RP patients and healthy controls. mRNA expression was quantified by qPCR analysis as described in Material and Methods. qPCR data were normalized to housekeeping gene, *GAPDH*. C; healthy controls, RP: patients with retinitis pigmentosa; PBMC: peripheral blood mononuclear cells. Mann-Whitney test was used (* $p < 0.05$).

Similar to what we found in the aqueous humor samples, there were no statistical differences between patients with syndromic RP (Usher syndrome) and non-syndromic RP ($p = 0.710$), and between smoker and non-smoker patients ($p = 0.550$) in all studied response variables (data not shown).

Taken together, these results allowed us to conclude that RP patients present reduced ocular antioxidant status that could contribute to the progression of the disease, especially to cone death. Moreover, the RP patients show, unexpectedly, imbalances in their peripheral blood antioxidant-oxidant homeostasis.

Ocular antioxidant status and visual function in retinitis pigmentosa

In order to assess the possible relationship between ocular antioxidant status and best-corrected visual acuity or visual field in RP patients, we first assigned to each individual a value between 0 (lowest antioxidant status) and 1 (highest antioxidant status) computed through fuzzy clustering classification according to their NOX, SOD3, protein and TAC values (Fig. 4). The new computed variable was used as a predictor of best-corrected visual acuity and visual field in a multiple linear model including age, sex and antioxidant consumption as covariates. This revealed a strong association between the ocular antioxidant status and the visual field ($p = 0.009$), and suggests that RP patients with better visual field are more likely to belong to cluster 1 (highest antioxidant status). However, no effect was found on best-corrected visual acuity ($p = 0.927$). Analysis showed that 35 % (multiple R-squared (R^2) = 0.352) of the variability in visual field was associated to changes in the antioxidant status.

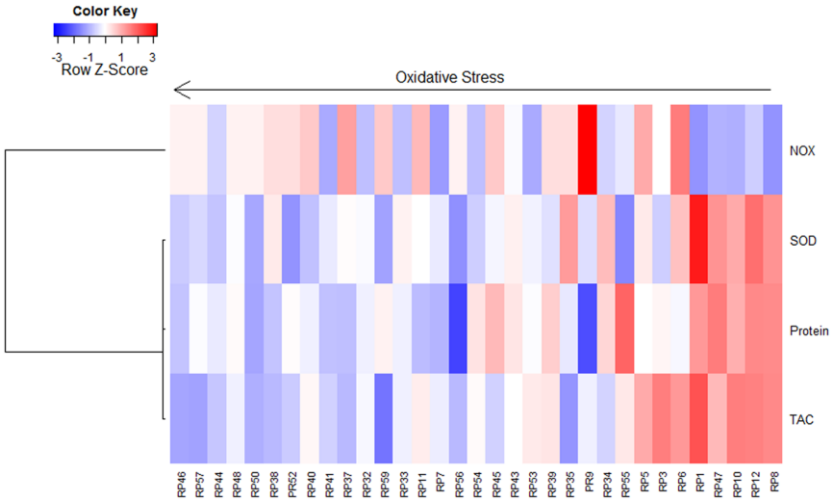

Figure 4. Heatmap representation of the fuzzy clustering results for antioxidant-oxidant markers in aqueous humor of RP patients. The dark red to dark blue colors correspond to high to low values. Individuals classified as with higher oxidant status show low TAC, protein and SOD3 levels and medium to high NOX levels. Individuals classified as with lower oxidant status show low NOX levels and high SOD3, protein and TAC levels.

DISCUSSION

The retina is highly susceptible to oxidative stress because of its high oxygen consumption, its high content of polyunsaturated fatty acids, and direct exposure to light. Increasing evidence suggests that oxidative stress contributes to the pathogenesis of RP [6, 7, 18]. To our knowledge this is the first time that the level of ocular antioxidant status in RP patients has been analyzed. We determined the levels of different markers of the antioxidant-oxidant status in aqueous humor and blood from RP patients, and compared them with those in healthy controls to confirm an alteration of this status.

Recently, it has been shown that the composition of the aqueous humor is affected by the pathophysiology of RP [30] as occurs in other retinal diseases such as glaucoma [31], diabetic retinopathy [32], or age-related macular degeneration [33]. We found that the aqueous humor

levels of TAC and SOD3 activity were lower in RP patients than in healthy controls. TAC and SOD are considered good biological markers of the endogenous antioxidant defence system. Therefore our results suggest that the endogenous antioxidant defence system is reduced in the eye of RP patients, and hence they have less capacity to cope with toxic oxygen intermediates.

The SODs are important antioxidant enzymes that catalyze the dismutation of the free radical superoxide into oxygen and hydrogen peroxide in many tissues, including the retina. The human eye expresses three types of SOD: the extracellular superoxide dismutase or SOD3, the cytosolic copper- and zinc-containing SOD or SOD1 and, the mitochondrial manganese-containing SOD or SOD2 [34]. SOD3 regulates extracellular concentrations of reactive oxygen species (ROS) and reactive nitrogen species (RNS) preventing the production of peroxynitrite, and possibly contributes to tissue protection during inflammatory insults [35, 36]. Therefore the reduced activity of SOD3 detected in the eye of RP patients could result in elevated superoxide [37], and SOD3 down-regulation may contribute to the inflammatory process recently described in RP [30, 38].

Concerning the other two SOD enzymes, it has been shown that retinal overexpression alone of SOD1 protected retinal cells from a severe oxidative stress induced by hyperoxia [39] but had deleterious effects in *rd1* mice [18]. However, the authors showed that combined overexpression of SOD1 or SOD2 with the peroxide-metabolizing enzyme (glutathione peroxidase 4 or catalase) within the same cellular compartment was able to reduce cone cell death in RP models [18, 19].

Numerous experimental and clinical studies indicate altered ocular blood circulation, including retinal flow [40] and choroidal circulation [40-43] in RP patients, possibly as a result of vascular remodelling in

response to reduced metabolic demand [40, 44] and low aqueous humor VEGF-A levels [45]. Reduced aqueous humor protein concentration detected in patients could be, at least in part, a consequence of this blood flow disturbance.

Interestingly, we found that oxidative-nitrosative stress markers are high in blood from RP patients. During oxidative stress, different ROS and RNS such as superoxide, hydrogen peroxide, NO, and peroxynitrite are generated. Increased superoxide radicals resulting from decreased SOD3 activity could, in combination with high NO formation, lead to increase peroxynitrite formation in blood of RP patients. Peroxynitrite can induce lipid and protein oxidation, and nitration of proteins [46]. Nitration is the most extensive post-translational modification of proteins and can strongly affect their biological function [47]. High blood levels of nitrotyrosine could contribute to the nitration of SOD3 impairing its activity [48] without affecting the total protein content in RP patients. This could be a possible explanation for the lack of correlation between SOD3 activity and SOD3 content in RP patients. Furthermore, elevated levels of nitrotyrosine in retina of *rd1* mice correlated with cone cell death, suggesting that NO exacerbates oxidative damage to cones in RP [49].

NO is a diffusible gas synthesized from L-arginine via three different isoforms of NOS (neuronal NOS, endothelial NOS and inducible NOS). Our results demonstrated a strong tendency to up-regulate NO, free nitrotyrosine, and its downstream effector, cGMP, in the blood of RP patients. NO can act through the stimulation of the sGC with subsequent formation of cGMP. Interestingly, up-regulation of HO-I can also contribute to increasing cGMP levels in RP patients. Up-regulating the HO system should generate cytoprotective products such as bilirubin, biliverdin, and carbon monoxide (CO) that in turns should stimulate sGC [50]. On the other hand, HO induction may play an important role in the

antioxidant defense system in oxidative stress-related diseases such as RP, age-related macular degeneration or in retinal damage situations such as intense light damage, retinal detachment or ischemia-reperfusion injury [51-53].

In blood vessels, cGMP is involved in relaxation of vascular smooth muscles leading to vasodilation and increased blood flow and inhibition of adhesion and aggregation of platelets. Systemic vascular impairment has been described in patients with RP [54]. Cellini *et al* [55] and Ohguro *et al* [56] reported that elevated levels of plasma endothelin- 1 (ET-1) which is a potent vasoconstrictive peptide found in RP patients, could lead to vasoconstriction of cutaneous capillaries. However, the increase of ET-1 in blood of RP patients is controversial [57]. The elevated cGMP can be considered as an adaptive response to vasoconstriction, or may simply be a consequence of the high NO levels associated with oxidative stress.

We found that levels of blood lipid peroxidation (measured by TBARS formation) were significantly elevated in RP patients compared with those in healthy controls, supporting previous findings [58, 59]. Retinal photoreceptor outer segment membranes are very sensitive to lipid peroxidation [60]. Lipid peroxidation injures photoreceptor cell membranes resulting in cell damage in different animal models of RP [7, 61]. It is likely, thus, that the retina of RP patients shows lipid peroxidation.

We found statistical evidence supporting an association between visual field and ocular antioxidant status in RP patients. This association seems to corroborate that an imbalance in the antioxidant machinery system is involved in the pathogenesis of RP, similar to other ocular diseases such as age-related macular degeneration, retinopathy of prematurity, retinal light damage, and glaucoma [29-31, 62, 63].

However, further studies are needed to analyze how antioxidant-oxidant status progresses at different stages of RP.

Under our experimental conditions, we found that patients with antioxidant consumption, including vitamins and omega-3 fatty acids, did not present higher antioxidant status than those without antioxidant consumption. Instead, they showed a trend toward diminishing TAC and SOD3 activity. However, we cannot conclude that, in this case, antioxidant consumption really worsened antioxidant status because our study was not designed to test for this. Each patient took a different kind and dose of nutritional supplement and we did not measure antioxidant status before treatment. Antioxidant supplements may have differential effects on oxidative stress responses, depending on the timing, dose and duration of the antioxidant consumption. Therefore, the effectiveness of antioxidant supplementation on antioxidant-oxidant status needs to be explored in detail. At present, the use of vitamin A and omega-3 fatty acids as appears to be an effective treatment for patient with RP, although the interpretation of clinical trials remains controversial [64-67]. Nonetheless several antioxidant treatments in experimental models of RP suggest a positive action of these treatments in slowing down or reducing the progression of the disease [6, 16, 68].

Concerning the effect of smoking, we did not find statistical evidence for a negative effect on the antioxidant-oxidant status in RP patients. Unfortunately there was no data for the control group but the proportion in the RP group (16 % for aqueous samples and 18 % for serum samples) was below average of smokers in Spanish population (35 %) [69]. Therefore, if we assume that our control group represent the Spanish population this imbalance would raise mean oxidative stress in control group compared to RP group, maybe increasing type II error (false negative) but not type I error (false positive).

In conclusion, we found that RP patients present a reduced ocular antioxidant status, and an imbalance of the antioxidant-oxidant status in the peripheral blood. Therefore the eyes of the RP patients could have less capacity to cope with toxic oxygen intermediates. Although RP is a genetic disease, this reduced capacity could contribute to the progression of the degeneration, especially to cone death [7].

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Capítulo IV. Artículo

Phosphodiesterase inhibition induces retinal degeneration, oxidative stress and inflammation in cone-enriched cultures of porcine retina

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PHOSPHODIESTERASE INHIBITION INDUCES RETINAL DEGENERATION, OXIDATIVE STRESS AND INFLAMMATION IN CONE-ENRICHED CULTURES OF PORCINE RETINA

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ABBREVIATIONS: RP, retinitis pigmentosa; cGMP, cyclic guanosine monophosphate; PDE6, phosphodiesterase 6; PAR, poly (ADP-ribose); PARP, poly (ADP-ribose) polymerase.

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ABSTRACT

Inherited retinal degenerations affecting both rod and cone photoreceptors constitute one of the causes of incurable blindness in the developed world. Cyclic guanosine monophosphate (cGMP) is crucial in the phototransduction and, mutations in genes related to its metabolism are responsible for different retinal dystrophies. cGMP-degrading phosphodiesterase 6 (PDE6) mutations cause around 4-5 % of the retinitis pigmentosa, a rare form of retinal degeneration. The aim of this study was to evaluate whether pharmacological PDE6 inhibition induced retinal degeneration in cone-enriched cultures of porcine retina similar to that found in murine models.

PDE6 inhibition was induced in cone-enriched retinal explants from pigs by Zaprinast. PDE6 inhibition induced cGMP accumulation and triggered retinal degeneration, as determined by TUNEL assay. Western blot analysis and immunostaining indicated that degeneration was accompanied by *caspase-3, calpain-2* activation and poly (ADP-ribose) accumulation. Oxidative stress markers, total antioxidant capacity, thiobarbituric acid reactive substances (TBARS) and nitric oxide measurements revealed the presence of oxidative damage. Elevated TNF-alpha and IL-6, as determined by enzyme immunoassay, were also found in cone-enriched retinal explants treated with Zaprinast. Our study suggests that this *ex vivo* model of retinal degeneration in porcine retina could be an alternative model for therapeutic research into the mechanisms of photoreceptor death in cone-related diseases, thus replacing or reducing animal experiments.

KEYWORDS: Retinal degeneration; cGMP; Zaprinast; oxidative stress; TNF-alpha; poly (ADP-ribose); caspase; retinitis pigmentosa; photoreceptor death.

INTRODUCTION

Inherited retinal degeneration affecting both rod and cone photoreceptors constitutes one of the causes of incurable blindness in the developed world. Several genes causing these genetic diseases have been identified including those involved in visual phototransduction.

Visual phototransduction is the process by which light is converted into electrical signals in the rod cells, cone cells and photosensitive ganglion cells of the retina. The phototransduction cascade occurs via a type of G-protein coupled receptors (GPCR) called opsins, which contain the chromophore 11-cis retinal. When struck by a photon, 11-cis retinal undergoes photoisomerization to all-trans retinal, which changes the conformation of the opsin GPCR, leading to trasducin activation. Active trasducin increases 3´, 5´,-cyclic guanosine monophosphate (cGMP) phosphodiesterase activity (PDE6), thereby lowering the concentration of cGMP. Decrease in cGMP concentration leads to the closure of cGMP-regulated Na^+ and Ca^{2+} ion channels, decreased influx of Na^+ and Ca^{2+}, hyperpolarization of membrane potential and decreased glutamate release. Following isomerization and release from the opsin protein, all-trans retinal generated during phototransduction is reduced to all-trans retinol in the retinal pigmented epithelium (RPE) and travels back to the rod outer segment where it can be conjugated again to opsin to form a new functional visual pigment. Rods and cones recover from excitation and recover their light-sensitivity by both inactivating the PDE6 cascade and synthetizing new cGMP by Ca^{2+}-sensitive membrane guanylyl cyclases (RetGCs). RetGCs are controlled by a calcium negative feedback through Ca^{2+}/Mg^{2+} binding proteins, GCAPs (guanylyl cyclase activating proteins) (Burns *et al.*, 2002; Koch and Stryer, 1988). Alterations in any step of the phototransduction cascade could lead to photoreceptor cell death.

Cyclic GMP is essential for the ability of rods and cones to respond to the light stimuli and the control of its level is critical for proper functioning of photoreceptors. Mutations in genes involved in cGMP synthesis (RetGCs and GCAPs) or degradation (PDE6) (Chang *et al.*, 2011; Dizhoor, 2000; Grau *et al.*, 2011; Piri *et al.*, 2005; Hunt *et al.*, 2010) can lead to various forms of retinal dystrophies such as some types of retinitis pigmentosa (Bowes, 1990; McLaughlin, 1993), progressive cone dystrophy (Thiadens *et al.*, 2009), dominant cone degeneration (Behnen *et al.*, 2010; Jiang and Baehr, 2010), cone-rod dystrophy (Buch *et al.* 2011; Sokal *et al.*, 2005; Tucker et la., 1999) and Leber congenital amaurosis (Perrault *et al.*, 2000, 1996).

Retinitis Pigmentosa (RP) is a common form of rod-cone dystrophy, constituting the largest Mendelian genetic cause of blindness in the developed world. Patients with RP typically loose night vision in adolescence, peripheral vision in young adulthood, and central vision later in life due to progressive loss of rod and cone photoreceptor cells. Photoreceptor cell death starts with rod photoreceptor degeneration and eventually cone cell death that is the major problem affecting RP patients, because it leads to loss of central vision (Kalloniatis and Fletcher, 2004).

Mutations in genes encoding α and β-subunit of PDE6 have been reported to cause recessive RP in humans (Corton *et al.* 2010; Dryja *et al.* 1999; Huang *et al.*, 1995; McLaughlin *et al.* 1995). PDE6A $^{nmf363/nmf363}$, PDE6A $^{nmf282/nmf2823}$ (Sakamoto *et al.*, 2009), PDE6B^{rd1} and PDE6B^{rd10} mice are models of human autosomal recessive RP that carry mutations on the α or the β-subunit of PDE6 (Bowes *et al.*, 1990; Chang *et al.*, 2002), that triggers photoreceptor degeneration (Farber and Lolley, 1974; Paquet-Durand *et al.*, 2009). Others than the available murine models have been reported to carry mutations in PDE6 subunits such as the canine models rcd1 and rcd2 (Petersen-Jones *et al.*, 1999; Sargan *et al.*, 1994; Suber *et al.*, 1993; Tuntivanich *et al.*, 2008; Wang *et al.*, 1999).

Although murine models provide invaluable information about photoreceptor cell death, they present different eye size and anatomic differences in retinal structures compared to humans; for example, they lack macula and fovea. This is why the use of large animal models seems to be critical for the development of retinal rescue strategies (Stieger *et al.*, 2009). Anatomically, the pig eye is remarkably similar to the human eye and it is well-endowed with cones (Gerke C. *et al.*, 1995) especially, in a large horizontal band extending across the retina covering the optic disc and horizontal meridian (Hendrickson and Hicks, 2002). This relative abundance of cones makes the porcine eye a good model for therapeutic research into the mechanisms of photoreceptor degeneration in cone-related diseases.

The mechanisms responsible for photoreceptor cell death still remain unclear however, increasing evidence suggests that oxidative stress (Komeima *et al.*, 2006; Shen *et al.*, 2005) and inflammation (de Kozak *et al.*, 1997; Yang *et al.*, 2007, Yoshida *et al.* 2013a, b) contribute to the pathogenesis of RP. Recently, Yoshida *et al.* (2013a, b) have been found elevated inflammatory mediators in the eye of *rd10* mice and of patients with RP including TNF-alpha.

This study investigated whether PDE6 inhibition produced *ex vivo* retinal degeneration in cone-enriched cultures of porcine retina similar to the degeneration found in murine models of RP with non-functional PDE6. Secondly, we assessed whether the damage was accompanied with oxidative stress and induction of inflammatory mediators.

We found that PDE6 inhibition triggered retinal degeneration with *caspase-3, calpain-2* activation and PAR accumulation (indicator of PARP activity) and induced oxidative stress and cytokine induction in cone-enriched cultures of porcine retina. These results suggest that the cone-enriched organotypic culture of porcine retina exposed to PDE inhibitor

could be a complementary model for therapeutic research into the mechanisms of retinal degeneration, thus replacing or reducing animal experiments.

MATERIAL AND METHODS

Porcine retinal explant cultures

Sixty eyes (both left and right eye) from small miniature pigs 3-7 months old were obtained from the local slaughterhouse. Neuroretina explants enriched in cones were carried out as previously described (Fernández-Bueno *et al.*, 2008) with some modifications. Briefly, each eyeball was immersed in 70% ethanol and washed in Dulbecco's Modified Eagle Medium (DMEM). All extraocular tissues were removed and the sclera was punctured with a 22 gauge needle at the ora serrata and bisected, dividing the ocular globe into anterior and posterior eyecups. The vitreous was removed, and the posterior eyecup was placed into a dish with phosphate buffer saline (PBS). A paintbrush was used to mechanically detach the neuroretina from the RPE, and the optic nerve was cut with Westcott scissors. The visual streak with a high cone density (Hendrickson and Hicks, 2002) was cut into 5x5 mm explants. Explants were transferred to Transwell® culture dishes (Corning Inc., Corning, NY) with photoreceptor side down, containing 1.5 mL culture medium composed of Neurobasal A medium supplemented with 2% B-27 (Invitrogen, Life Technologies, Madrid, Spain), 2 mM L-glutamine (Invitrogen, Life Technologies, Madrid, Spain), 100 U/mL penicillin, and 100 ng/mL streptomycin (Invitrogen, Life Technologies, Madrid, Spain). Explants were cultured at 37 °C with 5% CO_2 in a humidified atmosphere. The culture medium level was maintained in contact with the support membrane beneath the explant. Treatments were added the day of the culture and maintained them for 24 h or 48 h.

To evaluate the effect of PDE6 inhibition we used Zaprinast (Zhang *et al.*, 2005) (100, 200 and 500 nmol/L). Zaprinast (Sigma-Aldrich, Madrid, Spain)

was prepared in dimethyl sulfoxide (DMSO) (AppliChem, Darmstadt, Germany). For controls, the same amount of DMSO was added to the culture medium. Some retinal explants were also exposed to 2 μmol/L A231187, a calcium ionophore, as apoptotic inducer (Sigma-Aldrich, Madrid, Spain).

Freshly detached neuroretinas were also obtained for normal morphologic and biochemical evaluation.

Tissue Processing and Histology

For morphological characterization retinal explants were fixed in 4% filtered paraformaldehyde (Sigma-Aldrich, Madrid, Spain) and 2.5% glutaraldehyde (Electron Microscopy Sciences, Hatfield, UK) in 0.1 M PBS (pH 7.4) for 2h. Afterwards explants were post-fixed with 2% osmium tetroxide, rinsed, dehydrated and embedded in Durcupan resin (Fluka, Sigma-Aldrich, Madrid, Spain). Semithin sections were cut at 1.5 μm, mounted on gelatin-coated slides and stained with 1% toluidine blue. These sections were examined under an Eclipse 80i microscope (NIKON Instruments, Badhoevedorp, The Netherlands) and images were captured with a DS-Qi1 digital camera (NIKON Instruments, Badhoevedorp, The Netherlands). ImageJ software was used to quantify the thickness of the retinal explants. After calibration, total area of at least three visual fields per retinal explant was calculated. The total area was expressed as μm^2. Data are expressed as mean ± SEM.

To evaluate apoptosis with the terminal deoxynucleotidil transferase dUTP nick and labeling (TUNEL) assay, retinal explants were fixed in 4% filtered paraformaldehyde (Sigma-Aldrich, Madrid, Spain) in 0.1 M PBS (pH 7.4) and cryoprotected in a saccharose gradient (15-20-30%) (Panreac Química, Barcelona, Spain). Samples were frozen embedded in Tissue-Tek® O.C.T.™ Compound (Sakura Finetek Europe B.V., Zoeterwoude, The Netherlands). Next, 10 μm sections were cut with a cryostat (Leica CM1900,

Nussloch, Germany) and placed on Super Frost Ultra Plus treated slides (Thermo Scientific, Barcelona, Spain).

TUNEL Assay

The TUNEL assay was performed on 10 µm cryosections by means of an *in situ* cell death detection kit conjugated with rhodamine fluorochrome (ApopTag Red *In Situ*) (Millipore, Schwalbach, Germany) according to the manufacturer´s instructions. The specimens were examined under a Eclipse 80i microscope (NIKON Instruments, Badhoevedorp, The Netherlands) and images were captured with a DS-Qi1 digital camera (NIKON Instruments, Badhoevedorp, The Netherlands). The apoptotic (TUNEL-positive) nuclei per visual field were counted in at least three visual fields per each retinal explant using NIS-Elements imaging software (NIKON Instruments, Badhoevedorp, The Netherlands). The data were analyzed quantitatively and, only cells with red intensity were considered TUNEL-positive. The number of apoptotic nuclei was normalised to the SYTOX Green-labelled cell nuclei. Results are given as percentage of apoptotic nuclei/total nuclei. Data are expressed as mean ± SEM.

Immunofluorescence of Caspase-3 and PAR

Immunofluorescence was carried out on 10 µm cryosections. Sections were post-fixed for 15 min at room temperature in 4% filtered paraformaldehyde (Sigma-Aldrich, Madrid, Spain) in 0.1 M PBS (pH 7.4). Sections were incubated for 1 h in blocking solution containing 5% normal goat serum, 1% bovine serum albumin and 0.25% Triton X-100. They were then incubated with primary antibody against cleaved *Caspase-3* (1:200, Cell Signaling Technology, Barcelona, Spain) or PAR (poly-(ADP-ribose)) (1:200, Enzo Life Sicence, Madrid, Spain) overnight at 4 °C in blocking solution. After washing with PBS three times, samples were incubated for 1 hour at room temperature with the fluorescence-conjugated secondary antibody Alexa Fluor 647 (Invitrogen, Life Technologies, Madrid, Spain). After

washing with PBS three times, sections observed under a confocal microscope (Leica TCS SP5 Confocal microscope, Leica Microsistemas S.L.U, Barcelona, Spain). SYTOX Green (Molecular Probes, Paisley, UK) were used as a specific nuclear counterstain. Cells were counted 40× magnification, and the number of *caspase-3* positive cells was counted manually in four visual fields per each retinal explant. The number of cells positive for the cleaved *caspase-3* immunolabelling was normalised to the SYTOX Green-labelled cell nuclei. Results are given as percentage of *caspase-3* positive cell/total nuclei. Data are expressed as mean ± SEM.

PAR positive cells were difficult to count in outer nuclear layer (ONL). For the quantification of PAR immunostaining we used the following formula to calculate the corrected fluorescence (CF) for each cell layer: CF = Integrated density of the selected area - (area of selected area x mean fluorescence of background) (Burgess *et al.*, 2010). Data are expressed as mean ± SEM.

For co-localization with cleaved *caspase-3* or PAR, staining was followed by TUNEL staining.

Cyclic GMP determination

cGMP was measured by using the BIOTRAK cGMP enzyme immunoassay kit (GE Healthcare Europe GmbH, Barcelona, Spain). Retinal explants were homogenised in 5% trichloroacetic acid and neutralized with 2M potassium bicarbonate. Neutralized supernatant was used for cGMP determination. Protein content was measured by the bicinchoninic acid (BCA) protein assay (BCA Kit; Pierce Scientific, CA). The tissue cGMP levels were expressed as pmol/mg protein.

Caspase-3 Activity Assay

Caspase-3 activity was measured with a colorimetric tetrapeptide (DEVD-*p*NA) cleavage assay kit following the manufacturer's instructions

(Bio-Vision, Mountain View, CA). Total retinal protein was extracted from retinal explants and measured by the BCA protein assay. *Caspase-3* activity was expressed as arbitrary units (au)/mg of protein.

Nitrites and nitrates (NOX) determination

Intracellular and extracellular nitrites (stable end-product of NO) and nitrates (NOX) were measured in retinal explants by spectrophotometric GRIESS reaction (El-Mlili *et al.*, 2008) using nitrate reductase. The tissue NOX levels were expressed as nmol/mg protein (intracellular) or nmol/mL (extracellular).

Oxidative stress evaluation

Retinal explants were assayed for total antioxidant capacity (TAC) and thiobarbituric acid reactive substances (TBARS) formation as indicator of malonyldialdehyde (MDA) formation.

Retinal explants were homogenized in 5 mM phosphate buffer pH 7, 0.9% NaCl, 0.1% glucose, centrifuged at 10,000 × g for 15 min at 4°C, and then the supernatants were used to determine TAC and TBARS. Protein concentrations were measured by the BCA protein assay.

TAC was measured using a commercial kit (Cayman Chemical, Ann Arbor, MI) (Kowluru *et al.*, 2006). The tissue TAC levels were expressed as nmol/mg protein.

MDA levels were detected by a colorimetric method involving thiobarbituric acid (TBA) adduct formation (Cayman Chemical, Ann Arbor, MI). Tissue TBARS levels were expressed as nmol/mg protein.

TNF-alpha and IL-6 measurement

For detection of cytokine levels, retinal explants were homogenized in 20 mM Tris-HCl pH 7.4, 10 mM EDTA containing protease inhibitor cocktail (Complete Protease Inhibitor Cocktail; Roche, Basel, Switzerland) and 200

μM phenylmethylsufloxifluoride (PMSF). The TNF-α and IL-6 protein levels were estimated with the corresponding ELISA kit (Diaclone, Besancon, France), according to the manufacturers' instructions. Tissue cytokine levels were expressed as pg/mg protein.

Values for cGMP, *caspase-3* activity, NOX, oxidative markers and cytokines are given as the mean ± SEM of at least ten different cultures. For each experiment samples were measured in duplicate.

Western blot

Retinal explants were homogenized in lysis buffer (50 mM Tris-HCl buffer pH 7.4 containing 50 mM NaCl, 5 mM EDTA, 1% SDS, protease inhibitor cocktail (Complete Protease Inhibitor Cocktail; Roche, Basel, Switzerland) and 1 mM PMSF). Samples (30 μg) were subjected to electrophoresis and immunoblotting as described in Corbalán *et al.* (2002). The following primary antibodies were used: cleaved *caspase-3* (Asp175) rabbit polyclonal antibody (1:1000, Cell Signaling Technology, Danvers, MA, USA), *calpain-2* rabbit polyclonal antibody (1:1000; Sigma-Aldrich, Madrid, Spain); and β-actin mouse monoclonal antibody (1:2000; Sigma-Aldrich, Madrid, Spain). The images were captured using an EPSON SCAN from EPSON Corporation (EPSON IBERICA, Barcelona, Spain) and quantified using the Alpha Imager 2200 (version 3.1.2) software (AlphaInnotech Corporation, San Francisco, CA, USA).

Statistical analyses

For parametric data, ANOVA followed by Newman–Keul's post hoc test was used. When only two samples were compared the Student's t-test was used.

For non-parametric data, Mann-Whitney test was used. Significance levels were set at $\alpha = 0.05$.

RESULTS

PDE inhibition triggers cGMP accumulation in cultured porcine retina

Retinal explants were cultured for 48 h in the presence or absence of Zaprinast, a selective PDE5/6 inhibitor which raises intracellular cGMP levels in a concentration-dependent manner and causes cGMP-dependent photoreceptor degeneration in small animals closely resembling the *rd1* degeneration (Sahaboglu *et al.*, 2010).

Successful PDE6 inhibition was confirmed by a significant cGMP increase at all time points evaluated (Fig. 1). While untreated retina essentially maintained the same level of cGMP, the Zaprinast-treated retina showed a strong increase of cGMP accumulation at 24 h in a dose-dependent manner (1.72 ± 0.3; 2.04 ± 0.2, 2.78 ± 0.27 pmol/mg protein at 100, 200 and 500 nmol/L respectively, One-way ANOVA posthoc Newman-Keuls $p<0.05$). Under our experimental conditions, the effect of Zaprinast concentration on cGMP accumulation disappeared at 48 h.

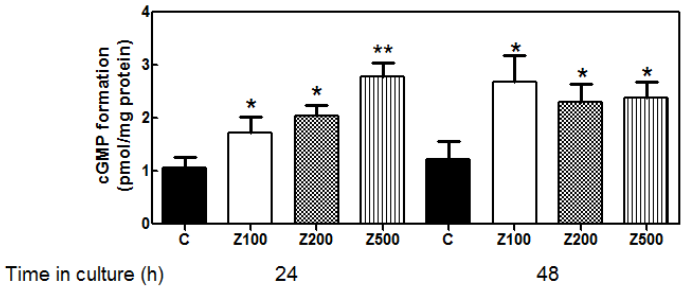

Figure 1. PDE6 inhibition induces cGMP accumulation in cone-enriched cultures of porcine neuroretina. Control retinal explants (C) or explants treated with 100 (Z100), 200 (Z200) or 500 (Z500) nmol/L Zaprinast were prepared as described in Material and Methods. The levels of cGMP at different times in culture are shown. Values are the mean ± SEM of twelve cultures. Values significantly different from own control are indicated by asterisks *$p < 0.05$, **$p < 0.01$ (ANOVA Newman-Keuls post-hoc).

100 nmol/L Zaprinast was the lowest concentration that induced a significant cGMP accumulation at 24 h and 48 h. In view of these results, 100 nmol/L seemed to be the best concentration of Zaprinast for further studies.

Evaluation of Organotypic Retina Cultures

Toluidine blue staining of semi-thin sections demonstrated that the overall architecture of the cultured retina was maintained throughout the culture period (Fig. 2A). However, morphometric analysis revealed that explants treated with 100 nmol/L Zaprinast were significantly thinner (47,915 ± 2938 µm^2, Mann-Whitney test, $p<0.01$ and 33,129 ± 1365 µm^2, Mann-Whitney test, $p<0.05$), compared with controls (58,940 ± 861 µm^2 and 42,000 ± 2220 µm^2) at 24 h and 48 h respectively (Fig. 2B). Zaprinast also decreased photoreceptor outer segments (OS) compared to control explants (Fig. 2A).

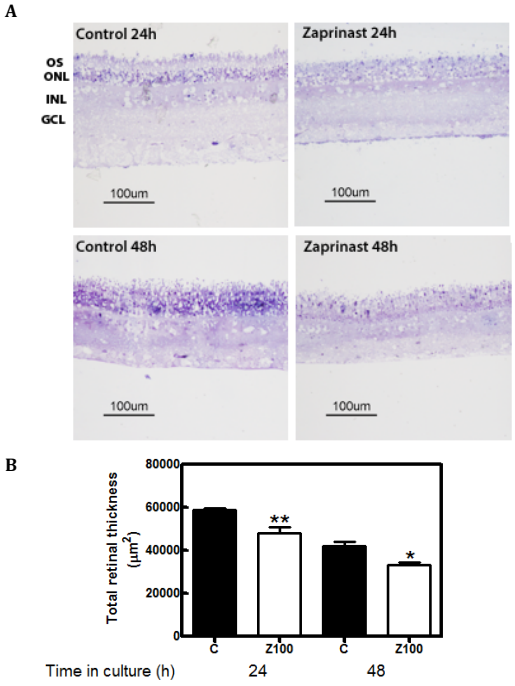

Figure 2. PDE6 inhibition reduces retinal thickness of cone-enriched cultures of porcine neuroretina. Control retinal explants or Zaprinast-treated retinal

explants were prepared as described in Material and Methods. (**A**) Light micrographs from Toluidine blue–stained semi-thin sections of control and 100 nmol/L Zaprinast-treated retinal explants. Abbreviations: OS (outer segment); outer nuclear layer (ONL); inner nuclear layer (INL); ganglion cell layer (GCL). (**B**) Quantitative analysis of retinal thickness. Control (C); 100 nmol/L Zaprinast (Z100). Values are the mean ± SEM of six different cultures. Values that are significantly different from own control are indicated by asterisks *$p < 0.05$ (ANOVA Newman-Keuls post-hoc).

PDE inhibition induces apoptosis in cultured porcine retina

The TUNEL assay was performed to measure the apoptotic cell death in fresh isolated retinas, control explants and explants treated with different Zaprinast concentrations (Fig. 3). While fresh isolated retina did not present any apoptotic nuclei, control explants showed a few apoptotic nuclei (2.4 ± 0.7 % of TUNEL-positive cells) after 24 h in culture.

TUNEL staining demonstrated that inhibition of PDE induced photoreceptor degeneration overall after 48 h of Zaprinast treatment. As shown in Fig. 3A and Table 1, apoptotic nuclei were found in outer nuclear layer (ONL), in inner nuclear layer (INL) and also in the ganglionar layer (GCL) at the lower dose of Zaprinast (100 nmol/L). The major effect was observed in ONL (4.6 ± 1.3 % of apoptotic cells, Mann-Whitney test, $p<0.0001$). Moreover, Zaprinast caused significant increase of TUNEL-positive cells (ANOVA test, post-hoc Newman-Keuls, $p<0.05$) after 24 h in a concentration-dependent manner (Fig. 3B).

Table 1. Effect of Zaprinast on cell death markers in porcine retinal explants.

Layer	TUNEL-positive cells (%)		*Caspase-3* positive cells (%)		PAR content (CF)	
	C	Z100	C	Z100	C	Z100
ONL	0.43 ± 0.13	4.55 ± 1.30***	0.08 ± 0.03	0.28 ± 0.06	7497 ± 722	23,744 ± 5304*
INL	0.92 ± 0.20	2.40 ± 0.40**	0.29 ± 0.09	2.64 ± 0.34***	7019 ± 1163	9640 ± 2380
GCL	0.71 ± 0.15	2.66 ± 0.44**	0.82 ± 0.27	1.72 ± 0.24**	9891 ± 2011	10,413 ± 2301

Note: Mann-Whitney test was used. Values different from control are shown by * $p<0.05$, ** $p<0.01$, *** $p<0.0005$. ONL: outer nuclear layer; INL: inner nuclear layer; GCL: ganglionar nuclear layer; C: control; Z100: 100 nM Zaprinast; CF: corrected fluorescence.

As shown in Fig. 3 control explants showed low numbers of TUNEL-positive cells after 24 h in culture (2.4 ± 0.7 % of apoptotic cells) but, this number increased across the whole retinal explant (ONL, INL and GCL) after 48 h (6.7 ± 1.0 % of apoptotic cells). This cell death was consequence of retinal detachment and culturing time.

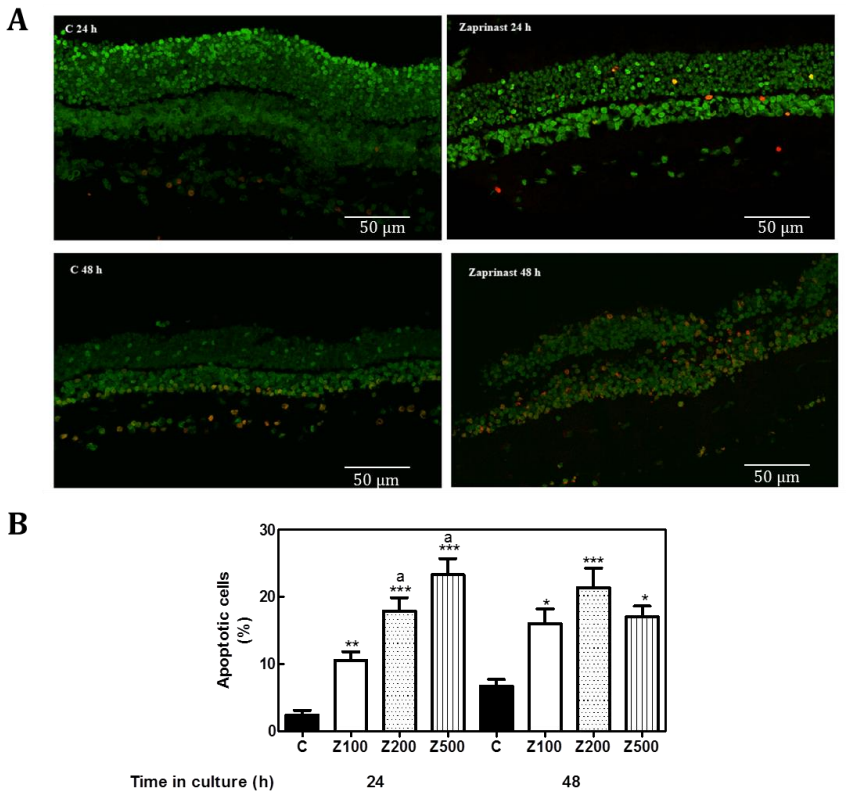

Figure 3. PDE6 inhibition induces apoptosis in cone-enriched cultures of porcine neuroretina. Control retinal explants or Zaprinast-treated retinal explants were prepared as described in Material and Methods. (**A**) Photomicrographs of TUNEL-stained sections visualizing apoptotic photoreceptors (red) and total cell nuclei (green) in control and 100 nmol/L Zaprinast-treated explants. Scale bar: 50 µm. (**B**) Zaprinast induced apoptosis in a *dose-dependent manner* (100, 200, 500 nmol/L) after 24h in culture. Values are the mean ± SEM of eight different cultures. Values significantly different ($p < 0.05$) from Z100 are indicated by 'a'. Values that are significantly different from own control are indicated by asterisks *$p < 0.05$ (ANOVA Newman-Keuls post-hoc).

To further investigate the impact of PDE inhibition on downstream effectors of cell death, we analysed the activity of *caspase-3*, and activation of *calpain-2*.

After 24 h, *caspase-3* activity (Fig. 4A) and protein content of cleaved *caspase-3* (Fig. 4B) were further up regulated in retina treated with 100 nmol/L Zaprinast (2.4 ± 0.1 au/mg protein and 2.1 ± 0.4 fold over control retina respectively, student t-test, $p<0.05$). This up regulation was maintained at 48 h (2.4 ± 0.2 au/mg protein and 2.7 ± 0.8 fold over control retina, student-t test, $p<0.05$). Immunofluorescence of cleaved *caspase-3* revealed that PDE inhibition significantly up regulated *caspase-3* in INL (2.6 ± 0.3 % of *caspase-3* positive cells, $p<0.0005$) and GCL (1.7 ± 0.2 % of caspase-3 positive cells, $p<0.01$) but not in ONL (0.3 ± 0.1 % of *caspase-3* positive cells), after 24 h in culture (Table 1 and Fig. 4C). Control explants also showed up regulation of *caspase-3* mainly in GCL after 48 h.

Western blot analysis showed that PDE6 inhibition also induced *calpain-2* activation (Fig. 4D). A Ca^{2+} ionophore, A231187, was used to confirm *calpain-2* activation.

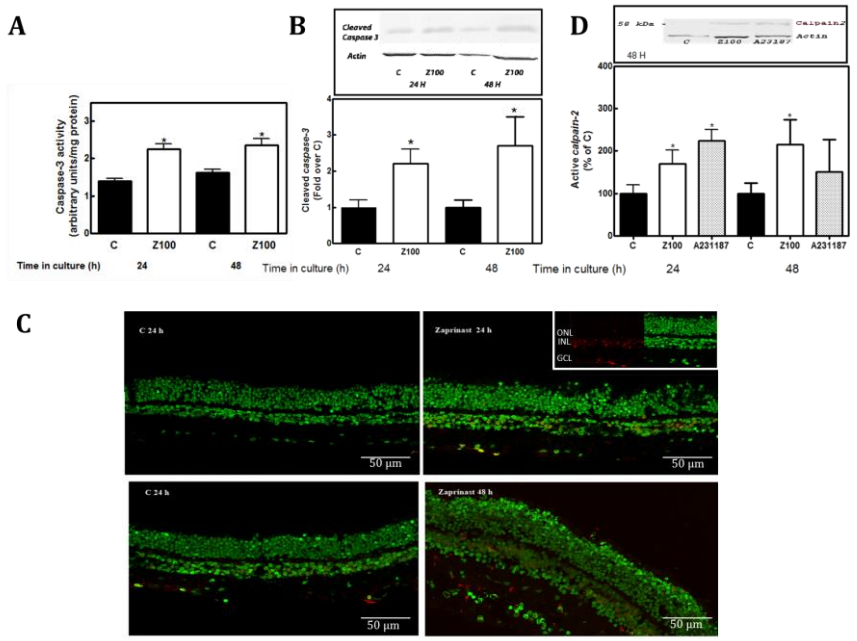

Figure 4. PDE6 inhibition activates *caspase-3* and *calpain-2* in cone-enriched cultures of porcine neuroretina. Control retinal explants or Zaprinast-treated retinal explants were prepared as described in Material and Methods. (**A**) *Caspase-3* activity using the substrate DEVD-pNA in homogenates of control retinal explants or explants treated with Zaprinast (100 nmol/mL). Values are the mean ± SEM of twelve cultures. (**B** and **D**) Retinal homogenates (30 µg of protein) were subjected to electrophoresis and cleaved *caspase-3* (**B**) and *calpain-2* (**D**) activation were analysed by immunoblotting. The intensities of the bands were quantified, normalized respect to internal control (β-actin) and the values are represented as fold over control. Values significantly different from own control are indicated by asterisks *$p < 0.05$ (paired student t-test). (**C**) Evaluation of *caspase-3* activation with cleaved *caspase-3* staining in frozen sections; scale bar, 50 µm.

Strong activation of poly(ADP-ribose) polymerase (PARP) has been found in animal models of retinal degeneration with subsequent accumulation of poly(ADP-ribose) (PAR) polymers (Paquet-Durand *et al.*, 2007; Kaur *et al.*, 2011). Excessive PARP activation may contribute to caspase-independent photoreceptor death (Paquet-Durand *et al.*, 2007; Kaur *et al.*, 2011). To investigate indirectly PARP activity in Zaprinast-treated explants, we performed PAR immunostaining. PDE6 inhibition significantly increased accumulation of poly(ADP-ribosyl)ated proteins in

ONL (23,744 ± 5304 corrected fluorescence, Mann-Whitney test, $p<0.05$) compared to control (7497 ± 722 corrected fluorescence) after 24 h in culture (Table 1).

To determine whether cleaved *caspase-3* or PAR co-localize with TUNEL-positive cells, we performed double labeling (Fig. 5). In Zaprinast-treated explants PAR immunostaining co-localized to a large extent with TUNEL-positive cells in GCL and ONL. A few number of cells in INL also co-localized with PAR. However, *caspase-3* positive cells did not co-localize with TUNEL-positive cells except for a subset of cells in INL. Moreover, double-immunostaining of *caspase-3* and PAR reflected co-localization in a subset of cells in GCL and INL. *Caspase-3* activation occurs mainly in INL and it may partially contribute to cell death in this cell layer. Moreover, *caspase-3* activation also occurs in GCL and could contribute to the future cell death. Taking together, these results suggest that PDE6 inhibition probably induces cell death by caspase-independent mechanisms (PARP activity) in ONL and GCL and by caspase-dependent and caspase-independent mechanisms in INL.

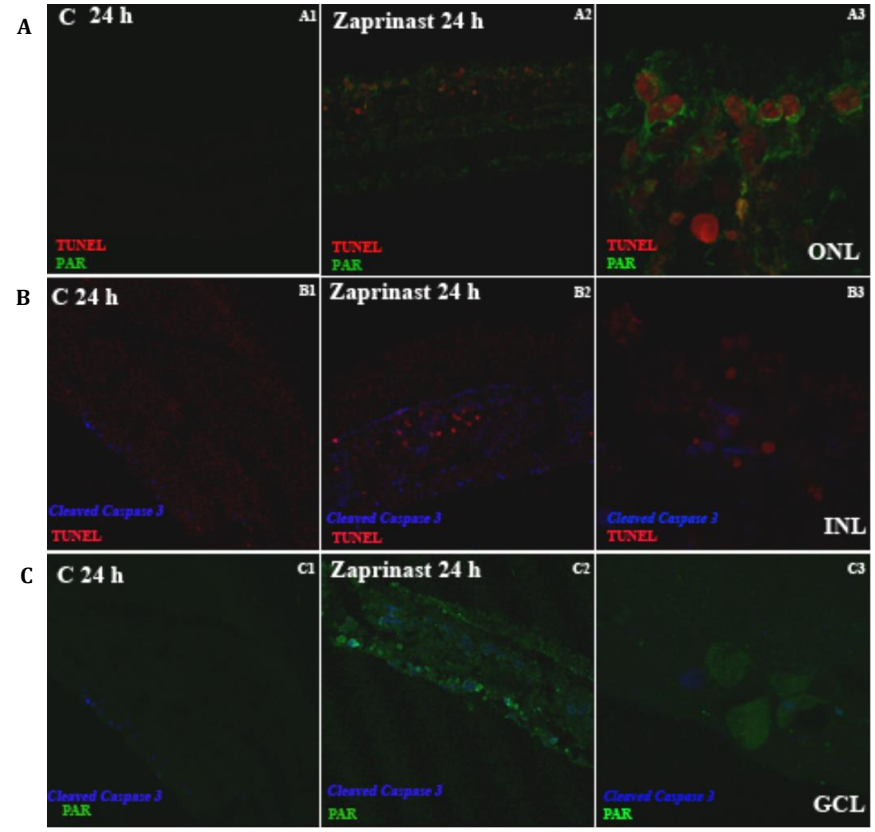

Figure 5. PDE6 inhibition induced different cell markers in cone-enriched cultures of porcine neuroretina. Double- immunostaining of control retinal explants or explants treated with Zaprinast (100 nmol/mL) were prepared as described in Material and Methods. (**A**) Double staining of TUNEL assay and PAR. (**B**) Double staining of TUNEL assay and cleaved *caspase-3*. (**C**) Double staining of PAR and cleaved *caspase-3*; scale bar: 50 µm. Images A3, B3 and C3 were obtained after zooming (factor = 3) with Leica TCS SP5 Confocal microscope.

PDE inhibition induces oxidative stress and cytokine production in cultured porcine retina

It has been described that cGMP accumulation induces oxidative stress in murine models of retinal degeneration (Sharma and Rohrer, 2007). To explore whether PDE inhibition also induced oxidative damage in cultured porcine retina we measured: intracellular and extracellular nitrites formation (iNOX and eNOX) as measurement of nitric oxide, TBARS

formation as indicator of lipid peroxidation and total antioxidant capacity (Fig. 6).

At 24 h, PDE6 inhibition enhanced iNOX levels compared with control (48 ± 4 nmol/mg protein and 30 ± 2 nmol/mg protein, respectively, student t-test, $p<0.05$). As culturing time moved forward eNOX level also increased compared to control (47 ± 3 nmol/mL and 36± 2 nmol/mL, respectively, student t-test, $p<0.05$) (Fig. 6A).

MDA concentration was determined by the thiobarbituric acid (TBA) assay, which measures the amount of TBA reactivity with MDA formed during the acid hydrolysis of lipid peroxide compound. Addition of 100 nmol/L Zaprinast caused a rapid increase of lipid peroxide TBA value compared with control at 24 h (3.9 ± 0.4 nmol/mg protein and 2.0 ± 0.2 nmol/mg protein, student t-test, $p<0.05$), remaining elevated at 48 h (4.8 ± 0.5 nmol/mg protein, student t-test, $p<0.05$) (Fig. 6B).

The overall antioxidant capacity of retinal explants treated with 100 nmol/L Zaprinast, decreased at 24 h and 48 h (174 ± 12 µmol/mg protein and 156 ± 9 µmol/mg protein respectively, student t-test, $p < 0.05$), compared to control retinal explants (225 ± 21 µmol/mg protein and 192 ± 14 µmol/mg protein, respectively) (Fig. 6B).

TNFα and IL-6 are upregulated in several inflammatory ocular diseases, including Adamantiades-Behcet disease (Durrani et al, 2007), retinal vascular tumours (Japiassu *et al.*, 2009), neovascular age-related macular degeneration (Seddon *et al.*, 2005), uveitis (Murray et al, 1990), and retinitis pigmentosa (Yoshida et al, 2013a, 2013b). We assessed whether 100 nmol/L Zaprinast induced these pro-inflammatory mediators in porcine retinal explants (Fig. 6C).

PDE inhibition induced a significant long-lasting upregulation of TNF-alpha at 24 h and 48 h (49.6 ± 10.2 pg/mg protein and 50.8 ± 5.6 pg/mg

protein, respectively, student t-test, $p<0.05$) compared to control (26.6 ± 3.6 pg/mg protein and 24.5 ± 2.9 pg/mg protein, respectively).

At 24h PDE inhibition also produced IL-6 up regulation (6.5 ± 1.8 pg/mg protein, student t-test, $p<0.05$) compared to control (3.1 ± 0.4 pg/mg protein) (Fig. 6C).

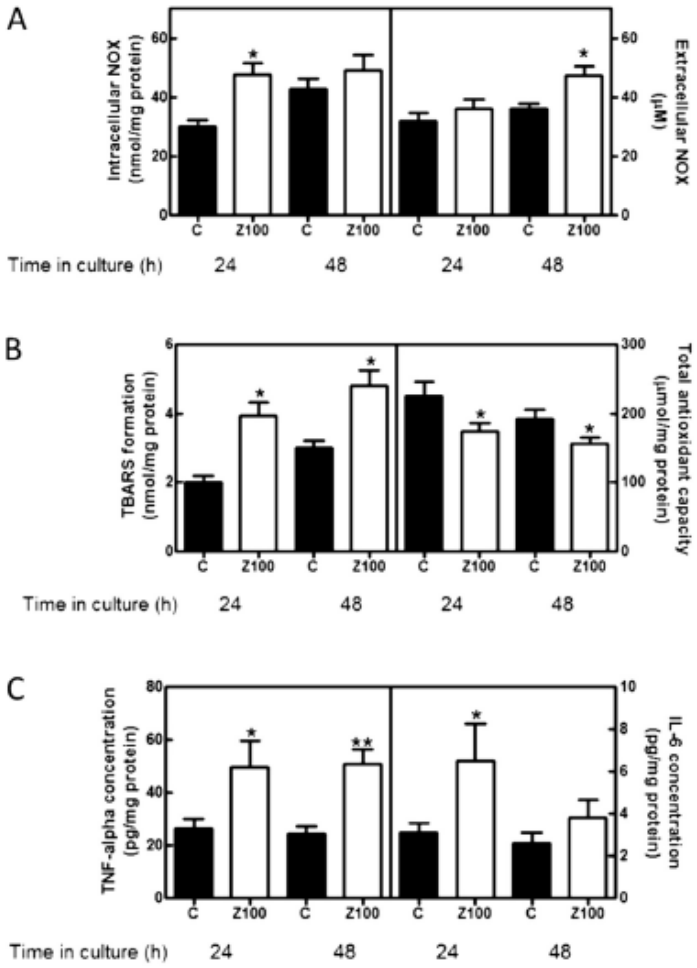

Figure 6. PDE inhibition triggers oxidative stress and cytokine production cone-enriched cultures of porcine neuroretina. Control retinal explants or explants treated with Zaprinast (100 nmol/mL) were prepared as described in Material and Methods. (**A**) Effect of Zaprinast on nitric oxide formation (measurement of nitrites and nitrates (NOX)). Intracellular (iNOX) and extracellular nitrites and nitrates (eNOX) were measured by Griess reaction. (**B**) Effect of

Zaprinast on the total antioxidant capacity and malonyldialdehyde formation (TBARS formation). (**C**) Effect of Zaprinast on TNF-alpha and IL-6 content. Each sample was measured in duplicate, and the values are the mean ± SEM of twelve cultures. Values significantly different from own control are indicated by asterisks *p < 0.05, **p < 0.01 (paired student t-test).

DISCUSSION

Mutations in genes related to cGMP metabolism as RetGC, GCAPs or PDE6 are involved in several retinal dystrophies including retinitis pigmentosa. Between 4 and 5% of patients with retinitis pigmentosa present PDE6 mutations leading to non-functional enzyme.

There is a lot of interest in using the pig as a model of retinal disease and stem cell transplantation therapy due to its resemblance to human retina. However, transgenic pigs are expensive and difficult to manage. Porcine organotypic culture of the retina is a choice that could allow us to evaluate the effect of new therapeutic drugs on some retinal changes faster and cheaper than *in vivo* and has the additional advantage of reducing animal experiments. These organotypic cultures were originally developed to follow cellular and cytoskeletal changes during the culture period (Allamby et al, 1997; Winkler *et al.*, 2002) and more recently to study retinal detachment (Fernández-Bueno, 2008; Fisher and Lewis, 2003). Here we evaluated whether PDE6 inhibition produced retinal degeneration in enriched-cone cultures of porcine retina.

Firstly, we assessed whether this porcine model reproduces the retinal degeneration observed in small animals after PDE inhibition. Our results showed that PDE inhibition induced cGMP accumulation accompanied by retinal degeneration. These results corroborated those found in small animals both *in vivo* and *in vitro* (Sahaboglu et al, 2010; Vallazza-Deschamps *et al.*, 2005). Sahaboglu *et al.* (2010) demonstrated that Zaprinast caused cGMP-dependent photoreceptor degeneration closely resembling the *rd1* degeneration observed in murine retinal explants. In the *rd1* mouse the high levels of cGMP increase the number of the cGMP-gated channels in the open

state, thus allowing intracellular calcium (Ca^{2+}) to rise to toxic levels and leading to rapid photoreceptor degeneration (Fox et al, 1999). Several studies have pursued to reverse the effects of PDE6 loss function by blocking cGMP-gate channel (Vallazza-Deschamps et al, 2005), Ca^{2+} channel (Nakazawa, 2011) or PKG activity (Paquet-Durand *et al.*, 2009). All these treatments reduced photoreceptor cell death.

We reported that sustained elevation of intracellular cGMP in porcine retinal explants triggered different downstream effectors of cell death related to caspase-dependent mechanisms (*caspase-3*) and caspase-independent mechanisms (*calpain-2* and probably PARP activity, measured as PAR accumulation). In 661W cells inhibition of PDE, increases the intracellular Ca^{2+} that in turns activates the cysteine protease calpain, which executes apoptosis via modulation of *caspase-3* activity (Sharma and Rohrer, 2004). Caspase activation occurs mainly through the extrinsic and intrinsic pathways (Bredesen *et al.*, 2006). Although *caspase-3* inhibitors have been transiently effective in delaying retinal degeneration through inhibition of the apoptosis of photoreceptor cells in *rd* gene-carrying mice (Yoshizawa *et al.*, 2002), the role of caspases in inherited retinal degeneration is controversial (Doonan *et al.*, 2003; Sanges et al, 2006; Zeiss *et al.*, 2004). Recent studies have shown that several caspase independent inducers of cell death such as AIF (apoptosis-inducing factor), calpains, PARP are activated during retinal degeneration (Kaur *et al.*, 2011; Paquet-Durand *et al.*, 2010; Sanges *et al.*, 2006). In our model *caspase-3* activation seems to be involved in part of cell death in INL but neither in GCL nor ONL. However, co-localization of PAR accumulation with TUNEL-positive cells suggests that caspase-independent mechanisms are involved in cell death in ONL and GCL. It is important to highlight that although TUNEL is used as an apoptotic marker, it also can detect non-apoptotic DNA fragmentation, e.g. in necrosis (Grasl-Kraupp *et al.*, 1995). Taken together, these findings suggest the involvement of multiple death signalling mechanisms (caspase-dependent

and caspase-independent mechanisms) in retinal cell death after PDE6 inhibition in porcine retina.

We have described that PDE inhibition induced the inflammatory mediators TNF-alpha and IL-6 in porcine retina. Elevated levels of these two inflammatory mediators have been recently described in the eye of patients with RP and *rd10* mice (Yoshida *et al.*, 2013a, 2013b). TNF-alpha or IL-6 have been also observed in other eye diseases, including uveitis, proliferative vitreoretinopathy, retinal detachment and age-related macular degeneration (El-Ghrably et al, 1997; Klein *et al.*, 2008; Nakazawa *et al.*, 2011). TNF-α is likely secreted from activated macrophages, astrocytes, microglial cells and retinal Müller glial cells. It has been described that TNF-alpha has cytotoxic effects on photoreceptors (Nakazawa *et al.*, 2006, 2011). TNF-alpha can trigger several well-characterized death-promoting (caspase-dependent and caspase-independent cell death) and survival-promoting pathways, depending upon the predominating signalling pathway in the particular cell type (Maianski et al, 2003). TNF-alpha binding to cell surface receptors such as TNF receptor 1 mediates activation of initiator caspases (caspase-8, caspase-10), and finally triggers cleavage of effector caspases (extrinsic pathway of cell death) (Nagata, 1997). TNF-alpha binding to cell surface receptors may also elicit anti-apoptotic responses mediated by the activation of the NF-κB pathway.

Several evidence shows that TNF-alpha is also involved in the intrinsic pathway of cell death that is initiated by cellular and DNA damage and particularly employs mitochondria. TNF-alpha and other stimuli can reduce the mitochondrial transmembrane potential resulting in release of mitochondrial factors such as cytochrome c or AIF. AIF rapidly translocates from mitochondria to the nucleus and induces nuclear fragmentation and cell death by autophagic degeneration (Suo et al, 2010; Munemasa et al, 2010; Tezel et al, 2004; Lorenzo et al, 1999; Daugas et al, 2000). The translocation of AIF has been implicated in several types of neuronal death,

including photoreceptor and ganglion cell death (Hisatomi et al, 2001; Mizukoshi et al, 2012; Thapa et al, 2010; Sanges et al, 2006). Inhibition of nuclear AIF translocation delays retinal degeneration of RCS rats, a model of retinitis pigmentosa (Murakami et al, 2008).

PARP-1 activation in response to excessive DNA damage triggers the release of AIF from mitochondria and promotes PARP-1-dependent cell death or parthanatos. AIF is a high-affinity PAR-binding protein. PAR binding by AIF is required for its release from the mitochondria, translocation to the nucleus, and cell death (Wang *et al.* 2011). PARP activation and PAR accumulation have been linked to mitochondrial death and AIF translocation to the nucleus in photoreceptor cell death in *rd1* mice (Paquet-Durand et al, 2007).

The other inflammatory mediator upregulated in these cultures, IL-6 is a pleiotropic cytokine with a role in inflammation, angiogenesis, cell differentiation and neuronal survival. In the retina, IL-6 is synthesized by Müller cells and the RPE (Benson et al, 1992; Yoshida *et al.*, 2001). A neuroprotective role for IL-6 has been suggested in different animal models of ocular injury, *in vitro* studies, retinal vein occlusion, diabetic macular oedema and experimental glaucoma (Chong *et al.*, 2004; Funatsu *et al.* 2009; Noma et al, 2009) suggesting that IL-6 upregulation after injury may serve to control photoreceptor apoptosis (Chong *et al.*, 2004).

Retinal degeneration induced by PDE inhibition was accompanied by oxidative stress in porcine retina. Retina is normally protected from oxidative damage by the presence of enzymes such as superoxide dismutase and catalase (De la Paz et al, 1996). Photoreceptors, which are the predominant cell type in the retina, are particularly susceptible to free radical damage or lipid peroxidation (Osborne and Wood, 2004), because retinal photoreceptor membranes have an unusually high concentration of docosahexaenoic acid. Oxidative damage is a major factor contributing to

cone cell death after the death of rods has occurred (Komeima *et al.*, 2006; Shen *et al.*, 2005). The increased levels of oxygen (hyperoxia), after death of rods result in progressive oxidative damage to cones in a transgenic pig model of RP and in multiple mouse models, including models of recessive and dominant RP. Antioxidant treatments can scavenger reactive oxygen species and promote cone survival and function (Komeima *et al.*, 2006). In our study, we demonstrated the elevation of NO (NOX), which may increase peroxynitrite via the reaction the superoxide radical (Pryor and Squadrito, 1995). NO is an important regulator of homeostatic processes in the eye and its over-expression could contribute to pathological conditions in RP (Komeima et al, 2008).

We hypothesize that cGMP accumulation induces oxidative stress that probably induces microglial activation, as described in *rd* mice, that in turns upregulates TNF-alpha contributing to the cell death. TNF-alpha can activate different cell death pathways including *caspase-3*, and PARP. On the other hand, cGMP accumulation leads to calcium influx and calpain activation. We believe that PDE6 inhibition activates more than one apoptotic pathway (caspase-dependent and caspase-independent) as occurs in other experimental models of retinal degeneration (Gómez-Vicente et al, 2005; Kaur et al, 2011). Moreover, our findings suggest that different retinal cell types follow different apoptotic pathways.

In summary, PDE6 mutations induce retinal degeneration in small animal models (*rd1*, *rd* and *rd10* mice), but eye size and anatomic differences suggest that should be useful to have alternative models for studying retinal rescue strategies or design new drugs intended for humans. Porcine eyes are closer in size to human eyes and have a rich supply of rod and cones. Our organotypic culture of porcine cone-enriched retina exposed to Zaprinast may provide a helpful model to design and assay some treatments thus replacing or reducing animal experiments.

However, this kind of culture has its own limitations. Organotypic cell culturing involves transaction of the optic nerve and mechanical retinal detachment causing photoreceptor loss and retrograde retinal ganglion cell degeneration. To minimize this problem, use of detached samples as controls is necessary. In the future, we aim at improving the viability of organotypic cell cultures, although it is difficult to culture retinal neurons for long periods perhaps due to the high energy requirements of the retina.

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Capítulo V. Artículo

Infliximab reduces Zaprinast-induced retinal degeneration in cultures of porcine retina

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INFLIXIMAB REDUCES ZAPRINAST-INDUCED RETINAL DEGENERATION IN CULTURES OF PORCINE RETINA

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ABSTRACT

Background

cGMP-degrading phosphodiesterase 6 (PDE6) mutations cause around 4 to 5% of retinitis pigmentosa (RP), a rare form of retinal dystrophy. Growing evidence suggests that inflammation is involved in the progression of RP. The aims of this study were to corroborate the presence of high TNFα concentration in the eyes of RP patients and to evaluate whether the blockade of TNFα with Infliximab, a monoclonal anti-TNFα antibody, prevented retinal degeneration induced by PDE6 inhibition in cultures of porcine retina.

Methods

Aqueous humor from 30 patients with RP and 13 healthy controls were used to quantify the inflammatory mediators IL-6, TNFα, IL-1β, IL-10 by a multiplex enzyme-linked immunosorbent assay (ELISA) system. Retinal explants from pig were exposed to Zaprinast, a PDE6 inhibitor, for 24 hours in the absence or the presence of Infliximab. Cell death was evaluated by TUNEL assay. The number and distribution of *caspase-3* positive cells, indirect poly(ADP)ribose polymerase (PARP) activation and glial fibrillary acidic protein (GFAP) content were visualized by immunolabeling. Antioxidant total capacity, nitrites and thiobarbituric acid reactive substances (TBARS) formation were determined to evaluate antioxidant-oxidant status.

Results

IL-6 and TNFα concentrations were higher in the aqueous humor of RP patients than in controls. Infliximab prevented retinal degeneration, as judging by the reduced presence of TUNEL-positive cells, the reduction of *caspase-3* activation and also reduction of glial activation, in

an *ex vivo* model of porcine retina. Additionally, Infliximab partially reduced oxidative stress in retinal explants exposed to Zaprinast.

Conclusions

Inflammatory mediators IL-6 and TNFα were elevated in the aqueous humor of RP patients corroborating previous studies suggesting sustained chronic inflammation. Our study suggests that TNFα is playing an important role in cell death in an *ex vivo* model of retinal degeneration by activating different cell pathways at different cell layers of the retina that should be further studied.

Keywords: Retinal degeneration, Inflammation, Infliximab, Oxidative stress, TNFα, Poly(ADP-ribose), *caspase-3*, Retinitis pigmentosa, Photoreceptor death

BACKGROUND

Retinitis pigmentosa (RP) is a common form of rod-cone dystrophy, constituting the largest Mendelian genetic cause of blindness in the developed world. Patients with RP typically loose night vision in adolescence, peripheral vision in young adulthood, and central vision later in life due to progressive loss of rod and cone photoreceptor cells. Photoreceptor cell death starts with rod photoreceptor degeneration and eventually cone cell death that is the major problem affecting RP patients, because it leads to loss of central vision [1]. More than 60 genes, including phosphodiesterase 6 (PDE6) subunit genes, have been identified to date that, when mutated, cause different forms of non-syndromic RP [2-7].

Although RP is a genetic disease, increasing evidence in patients and animal models suggests that oxidative stress and inflammation,

especially TNFα, contribute to its pathogenesis, independently of the genes mutated [8-10]. Some reports show the presence of sustained chronic inflammatory reaction including elevated TNFα levels in the eyes of RP patients [11] and *rd10* mice [12]. TNFα is a pleiotropic cytokine essential for the induction and maintenance of the inflammatory immune responses [13] that is also upregulated in inflammatory ocular diseases, including Adamantiades-Behcet disease [14], retinal vascular tumors [15], neovascular age-related macular degeneration [16], uveitis [17], glaucoma [18] and ischemic retinopathy [19].

TNFα mediates a broad range of cellular activities, including proliferation, survival, differentiation, inflammation and cell death. In the retina, TNFα is likely to be secreted from activated macrophages, astrocytes, microglial cells and retinal Müller glial cells. TNFα can trigger several well-characterized death-promoting (*caspase*-dependent and *caspase*-independent cell death) and survival-promoting pathways, depending upon the predominating signaling pathway in the particular cell type [20]. TNFα binding to cell surface receptors such as TNFR1 mediates activation of initiator *caspase*s (*caspase*-8, *caspase*-10) and finally triggers cleavage of effector *caspase*s (extrinsic pathway of cell death) [21]. TNFα is also involved in the intrinsic pathway of cell death that is initiated by cellular and DNA damage which particularly involves mitochondria. Finally, TNFα can also activate a subset of programmed necrosis called necroptosis. The mechanism that leads cells to undergo apoptosis or necroptosis and the mechanism that mediates the execution of necroptosis still remains unclear. The poly(ADP-ribose) polymerase (PARP) pathway can also activate this mode of programmed necrosis. PARP-1 activation in response to excessive DNA damage results in the massive synthesis of poly(ADP-ribose) polymers (PAR), NAD+ depletion and subsequent release of apoptosis inducing factor (AIF) from mitochondria, which translocates to the nucleus where it forms an active

DNA-degrading complex (*caspase*-independent pathway). The PARP pathway has been considered as an integral part of TNF-induced necroptosis; however, it has been recently described that both pathways represent distinct and independent routes to programmed necrosis [22].

The mechanisms responsible for photoreceptor cell death in RP are still unclear. However, increasing evidence suggests that inflammation [11,12,23,24] and especially TNFα could contribute to the pathogenesis of RP. Therefore, inhibition of TNFα and downstream cellular signaling mechanisms, following interaction of TNFα with its receptors, could be a possible target in the treatment of retinal neurodegenerative disorders such as RP.

In the current study we found that IL-6 and TNFα were increased in the aqueous humor of RP patients. We also observed that pharmacological inhibition of TNFα with Infliximab, a specific monoclonal antibody against TNFα, prevented retinal degeneration in cultures of porcine retina exposed to Zaprinast. This model reproduces some events of the degeneration found in murine models of RP caused by non-functional PDE6 [25]. We also observed in our model a reduction of *caspase-3* activation, GFAP reactivity and partially oxidative stress, caused by Infliximab treatment. These results suggest that inflammation, especially TNFα upregulation, is playing an important role in retinal degeneration and, importantly, that strategies that promote its blockade could be promising therapies.

METHODS

Participants in the study

Human samples were obtained, informed consent from all subjects previously having been given. The procedure was in accordance with the tenets of the Declaration of Helsinki and was approved by the IRB of La

Fe University Hospital (Valencia, Spain). Thirty adult patients with typical forms of RP characterized by an elevated final dark-adaptation threshold, retinal arteriolar narrowing, and a reduced and delayed electroretinogram were enrolled in the study. Thirteen Caucasian patients suffering from cataracts without any other ocular disease served as controls. Further details of the patients enrolled in the study are shown in Table 1.

Table 1. Description of the participants included in the study

	Control	RP
Number of subjects	13	30
Males	7	21
Females	6	9
Age (years)	60 ± 3	48 ± 2

Patients diagnosed of RP were recruited from Retina Comunidad Valenciana - Asociación Afectados por Retinosis Pigmentaria and also from the department of Ophthalmology of La Fe University Hospital (Valencia, Spain). Healthy controls were recruited from La Fe University Hospital (Valencia, Spain).

Ophthalmic examination

The best-corrected visual acuity (BCVA) and automated visual field (VF) were measured in RP patients as previously described [8]. Individual data for each patient is shown in Additional file 1: Table S1. Macular edema secondary to RP was only present in one patient.

Aqueous humor extraction

Aqueous humor samples from 30 RP patients and from 13 patients with cataracts without any other ocular disease (controls) were collected as previously described [8]. Undiluted aqueous humor samples were

collected from each patient, placed in sterile tubes, and stored immediately at −80°C until use. All specimens were assayed to evaluate cytokine concentration in a double-blind arrangement with respect to their group. For each patient, aqueous humors were collected from the eye with the more severe retinopathy.

Cytokine levels in aqueous humor

The concentrations of cytokines in aqueous humor were measured using a multiplex enzyme-linked immunosorbent assay (ELISA) system. To measure the concentrations of IL-1β, IL-6, IL-10 and TNFα, the SearchLight Custom Human Cytokine-Inflammation Q-Plex Array (Aushon Biosystems, MA, USA) was used. Array was used according to the manufacturer's instructions. The signal of the cytokine array was determined by a cooled CCD camera (Fujifilm, Tokyo, Japan) using chemiluminescence. SearchLight CCD Imaging and Analysis System were used to quantify cytokine concentrations. The cytokine levels were expressed as pg/mL.

Porcine retinal explant cultures

Seventy eyes (both left and right eyes from each animal) from small miniature pigs aged 3 to 7 months were obtained from the local slaughterhouse. Neuroretinal explants were carried out as recently described [25]. Treatments were added the day of the culture and maintained for 24 hours. To inhibit PDE6 and induce retinal degeneration, we used a final concentration of 100 nmol/L Zaprinast [25,26]. Zaprinast (Sigma-Aldrich, Madrid, Spain) was diluted in dimethyl sulfoxide (DMSO) (AppliChem, Darmstadt, Germany). The equivalent amount of DMSO was added to the culture medium of controls. To evaluate the possible neuroprotective effect of TNFα blockade we used Infliximab (2 μg/mL, alone or combined with

Zaprinast) as TNFα blocker (Remicade®, Schering-Plough, Madrid, Spain). Infliximab is a chimeric human immunoglobulin G1 with a mouse variable fragment having high TNFα affinity and neutralizing capacity.

Tissue processing and histology

Retinal explants were fixed in 4% filtered paraformaldehyde (Sigma-Aldrich, Madrid, Spain) in 0.1 M PBS (pH 7.4) and cryoprotected in a saccharose gradient (15-20-30%) (Panreac Química, Barcelona, Spain). Samples were frozen embedded in Tissue-Tek® OCT™ Compound (Sakura Finetek Europe BV, Zoeterwoude, The Netherlands). After this, 10-µm sections were cut with a cryostat (Leica CM1900, Nussloch, Germany) and placed on Super Frost Ultra Plus treated slides (Thermo Scientific, Barcelona, Spain).

TUNEL assay

To evaluate apoptosis the terminal deoxynucleotidil transferase dUTP nick and labeling (TUNEL) assay was used as previously described [25]. The apoptotic (TUNEL-positive) nuclei per field were counted in at least three fields per retinal explant using NIS-Elements imaging software (NIKON Instruments, Badhoevedorp, The Netherlands). The number of apoptotic nuclei was normalized to the SYTOX Green-labeled cell nuclei. Results are given as percentage of apoptotic nuclei/total nuclei. Data are expressed as mean ± SEM.

Immunofluorescence of caspase-3, GFAP and PAR

Immunofluorescence was carried out on 10-µm cryosections. Sections were post-fixed for 15 minutes at room temperature in 4% filtered paraformaldehyde (Sigma-Aldrich, Madrid, Spain) in 0.1 M PBS (pH 7.4). Sections were incubated for 1 hour in blocking solution containing 5% normal goat serum, 1% BSA and 0.25% Triton X-100.

They were then incubated with primary antibody against cleaved *caspase-3* (1:200, Cell Signaling Technology, Barcelona, Spain), glial fibrillary acidic protein (GFAP, 1:400, Sigma-Aldrich, Madrid, Spain) or PAR (1:200, Enzo Life Science, Madrid, Spain) overnight at 4°C in blocking solution. After this samples were incubated for one hour at room temperature with the fluorescence-conjugated secondary antibody Alexa Fluor 647 (Invitrogen, Life Technologies, Madrid, Spain) and observed under a confocal microscope (Leica TCS SP5 Confocal microscope, Leica Microsistemas SLU, Barcelona, Spain) belonging to the Microscopy Unit of the IIS-La Fe (Valencia, Spain). Cells were counted at 40× magnification, and the number of *caspase-3* positive cells was counted manually in 4 fields per retinal explant. The number of cells positive for the cleaved *caspase-3* immunolabeling was normalized to the SYTOX Green-labeled cell nuclei (Molecular Probes, Paisley, UK). Results are given as percentage of *caspase-3* positive cell/total nuclei. Data are expressed as mean ± SEM.

GFAP and PAR positive cells were difficult to count in different retinal layers. For the quantification we used the following formula to calculate the corrected fluorescence (CF) for each cell layer [27]:

CF = Integrated density of the selected area – (area of selected area x mean fluorescence of background)

Data are expressed as mean ± SEM.

For co-localization of cleaved *caspase-3* (combined with Alexa Fluor 647) and PAR (combined with Alexa Fluor 488 (Invitrogen, Life Technologies, Madrid, Spain)) staining was followed by TUNEL staining.

caspase-3 activity assay

caspase-3 activity was measured with a colorimetric tetrapeptide (DEVD-pNA) cleavage assay kit following the manufacturer's

instructions (Bio-Vision, Mountain View, CA, USA). Total retinal protein was extracted from retinal explants and measured by the bicinchoninic acid (BCA) protein assay. *caspase-3* activity was expressed as arbitrary units (au)/mg of protein.

Nitrites and nitrates (NOX) determination

Intracellular nitrites (stable end-product of nitric oxide (NO)) and nitrates (NOX) were measured in retinal explants by spectrophotometric GRIESS reaction using nitrate reductase [28]. The tissue NOX levels were expressed as nmol/mg protein.

Oxidative stress evaluation

Retinal explants were assayed for total antioxidant capacity (TAC) and thiobarbituric acid reactive substances (TBARS) formation as indicator of malonyldialdehyde (MDA) formation.

Retinal explants were homogenized in 5 mM phosphate buffer pH 7, 0.9% NaCl, 0.1% glucose, centrifuged at 10,000 × g for 15 minutes at 4°C, and then the supernatants were used to determine TAC and TBARS. Protein concentrations were measured by the BCA protein assay.

TAC was measured using a commercial kit (Cayman Chemical, Ann Arbor, MI, USA) [29]. The tissue TAC levels were expressed as nmol/mg protein.

MDA levels were detected by a colorimetric method involving thiobarbituric acid (TBA) adduct formation (Cayman Chemical, Ann Arbor, MI, USA). Tissue TBARS levels were expressed as nmol/mg protein.

Values for *caspase-3* activity, NOX and oxidative markers are given as the mean ± SEM of at least eight different cultures. For each experiment samples were measured in duplicate.

Statistical analyses

All statistical analyses were done using R software (version 2.15.3) (Foundation for Statistical Computing, Vienna, Austria). Multivariate analysis of covariance (MANCOVA) and multiple linear regression models were used to analyze human data. For parametric data, ANOVA followed by Newman-Keuls post hoc test was used. For non-parametric data, Kruskal-Wallis test followed by Dunn's Multiple Comparison test was used. Significance levels were set at $\alpha = 0.05$.

RESULTS

Increased levels of TNFα and IL-6 in aqueous humor of RP patients

We performed a multiplex ELISA to determine the concentration of TNFα, IL-6, IL-1β and IL-10 in aqueous humor of RP patients. IL-1β and IL-10 were below detectable levels. Descriptive statistics of the results of the measurements of IL-6 and TNFα are shown in Table 2. We performed a MANCOVA with the results of TNFα and IL-6 as dependent variables while disease, age and gender were taken as predictive variables.

Table 2. Protein levels of cytokines in aqueous humor from retinitis pigmentosa (RP) patients and healthy controls.

	Control	**RP**
TNF-α (pg/mL)	1.1 ± 0.2	1.7 ± 0.2
95%CI	(0.8 to 1.4)	(1.4 to 2.0)
Detectable samples	13/13	28/30
IL-6 (pg/mL)	10.8 ± 3.4	23.5 ± 3.8
95%CI	(3.2- to 18.5)	(15.8 to 31.3)
Detectable samples	13/13	30/30

Note: values are expressed as mean ± SEM; CI: confidence interval.

This analysis revealed that RP significantly increased inflammatory mediators IL-6 and TNFα in aqueous humor (P = 0.03) (See Additional file 2: Table S2). We found no statistical evidence for gender or age effects. Further analysis of each of the response variables indicated that IL-6 is increased in RP patients (P = 0.018). TNFα showed a tendency to increase in RP patients (P = 0.09). We assessed the possible association between inflammatory status (measured as TNFα and IL-6 levels) and stage of the disease (measured as VF and BCVA values) using a MANOVA with VF, BCVA, sex and age as predictors and TNFα and IL-6 levels as response variables. Our results showed no evidence of association between VF and BCVA and inflammatory status (P = 0.09 for VF and P = 0.94 for visual acuity). Additionally, we also analyzed separately the associations among these predictor variables and each of the two cytokine using linear models. In these analyses we found a statistically significant association between higher VF values and higher levels of TNFα (P = 0.03) (Figure 1).

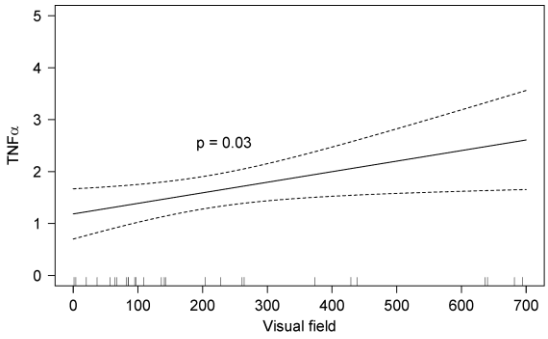

Figure 1. Relation between visual field and TNFα concentration in aqueous humor of retinitis pigmentosa (RP) patients. Statistical analysis revealed a positive relation between visual field and TNFα values controlling the other predictive variables (sex, age and acuity). Ninety-five percent confidence intervals are defined by dotted lines.

Infliximab prevents Zaprinast-induced cell death in cultured porcine retina

We previously described that PDE6 inhibition by Zaprinast triggered retinal degeneration and induced oxidative stress and inflammatory mediators such as TNFα and IL-6 in cultured porcine retina after 24 hours. In particular, TNFα and IL-6 content increased to twice control content [25].

We tested whether incubation with 2 μg/mL Infliximab, a TNFα blocker, for 24 hours prevented Zaprinast-induced retinal degeneration. Firstly, we checked the effect of Infliximab on the TNFα signaling cascade. As we could not measure TNFα, because Infliximab interferes with the ELISA assay as previously described [30], we evaluated its receptor TNF-R1 whose activation is involved in multiple apoptotic pathways. TNF-R1 relative expression increased up to 1.36 ± 0.08 arbitrary units (ANOVA Newman-Keuls post-test, $P < 0.0001$) in Zaprinast-treated explants compared to control explants (1.00 ± 0.08 arbitrary units). However, Infliximab normalized Zaprinast-induced overexpression of TNF-R1 (0.90 ± 0.08 arbitrary units, ANOVA Newman-Keuls post-test, $P < 0.0001$). No significant changes were found in explants treated only with Infliximab (0.91 ± 0.06 arbitrary units).

As shown in Figure 2, Infliximab significantly reduced the number of TUNEL-positive cells in Zaprinast-treated explants from 7.0 ± 0.7% to 2.2 ± 0.3% (Kruskal-Wallis, Dunn's post-test, $P < 0.001$) (Figure 2A). As shown in Table 3, this reduction occurred mainly in the outer nuclear layer (ONL).

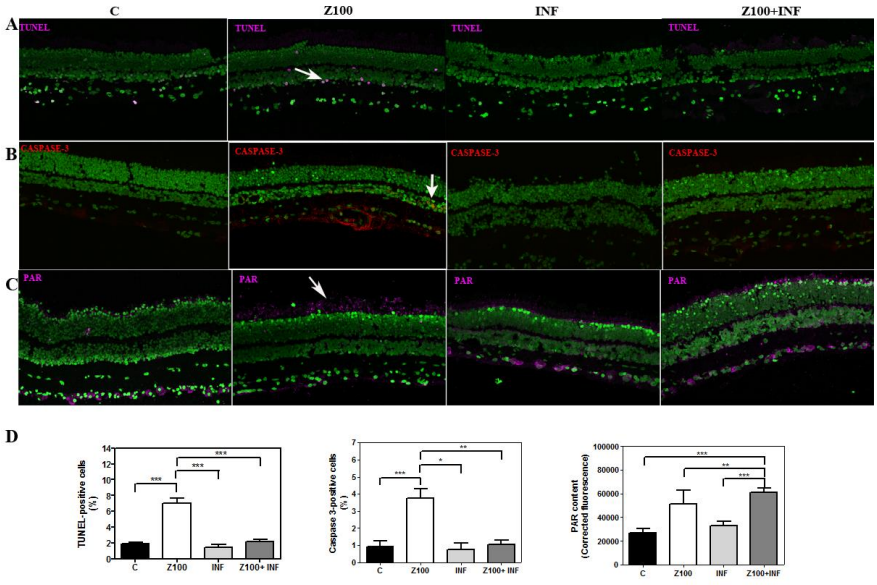

Figure 2. Infliximab prevents Zaprinast-induced cell death in cultured porcine retina. Retinal explants were incubated with dimethyl sulfoxide (DMSO), Zaprinast and Infliximab alone or combined with Zaprinast as described in Methods. Confocal laser scanning micrographs of retinal sections showing TUNEL-stained sections visualizing apoptotic photoreceptors (pink) **(A)**, cleaved *caspase*-3 positive cells (red) **(B)** and PAR accumulation (pink) **(C)** in SYTOX Green-counterstained retinal sections. Scale bar: 50 µm. **(D)** Bar graphs showing the quantification of TUNEL, cleaved *caspase*-3 and PAR accumulation. Values are the mean ± SEM of seven different cultures. Values that are significantly different are indicated by asterisks *P <0.05; **P <0.01; ***P <0.001 (Kruskal-Wallis, Dunn's post-test). C: control; Z100: 100 µM Zaprinast; INF: 2 µg/mL Infliximab; Z100 + INF: 100 µM Zaprinast with 2 µg/mL Infliximab. TUNEL, terminal deoxynucleotidil transferase dUTP nick and labeling.

Table 3. Effect of Infliximab treatment on cell death markers in Zaprinast-treated retinal explants

	TUNEL-positive cells (%)				*caspase-3* positive cells (%)				PAR content (CF)			
Layer	C	Z100	INF	Z100 + INF	C	Z100	INF	Z100 + INF	C	Z100	INF	Z100 + INF
ONL	0.3 ± 0.1	3.0 ± 1.1[a]	0.4 ± 0.2[b]	0.2 ± 0.1[c]	0.07 ± 0.04	0.2 ± 0.1	0.01 ± 0.01[b]	0.04 ± 0.03[c]	7,647 ± 676	22,925 ± 5111[a]	15,982 ± 2,019	24,970 ± 1,807[d,e]
INL	1.1 ± 0.2	2.5 ± 0.4[a]	0.8 ± 0.3[b]	1.1 ± 0.3	0.2 ± 0.1	2.1 ± 0.3[a]	0.4 ± 0.3[b]	0.4 ± 0.1[c]	7,019 ± 1,163	9,348 ± 2,288	11,156 ± 1,879	21,511 ± 2,251[c,d,e]
GCL	0.7 ± 0.1	2.0 ± 0.4[a]	0.6 ± 0.3[b]	1.0 ± 0.3	0.6 ± 0.3	1.3 ± 0.2[a]	0.3 ± 0.1	0.6 ± 0.2[c]	9,891 ± 2,011	10,019 ± 2,212	10,204 ± 1,496	17,134 ± 2,274[c,e]

Note: Kruskal-Wallis test and Dunn's Multiple Comparisons were used. Values different from control are shown by a($P < 0.05$). Superscripts represent statistical differences ($P < 0.05$) between bZ100 and INF; cZ100 and Z100 + INF; dINF and Z100 + INF; eC and Z100 + INF respectively. ONL: outer nuclear layer; INL: inner nuclear layer; GCL: ganglion nuclear layer; PAR, poly(ADP-ribose) polymers; C: control; Z100: 100 µM Zaprinast; INF: 2 µg/mL Infliximab; Z100 + INF: 100 µM Zaprinast with 2 µg/mL Infliximab; CF: corrected fluorescence.

As mentioned above, TNFα can trigger programmed cell death by activating the extrinsic and intrinsic apoptotic pathways that converges on the execution pathway, which is initiated by the cleavage of *caspase*-3 [31]. The activity of *caspase*-3 in Zaprinast-treated explants was 2.3 ± 0.2 au/mg protein (ANOVA Newman-Keuls post-test, $P < 0.01$) and 1.3 ± 0.2 au/mg protein in control explants. Infliximab almost normalized *caspase*-3 activity (1.7 ± 0.2 au/mg protein) compared to Zaprinast-treated explants (ANOVA Newman-Keuls post-test, $P < 0.05$) and the percentage of cleaved *caspase*-3 positive cells (1.1 ± 0.3%) compared to Zaprinast-treated explants (3.8 ± 0.6%, Kruskal-Wallis, Dunn's post-test, $P < 0.01$). Moreover, immunostaining of cleaved *caspase*-3 revealed that Infliximab treatment reduced the percentage of *caspase*-3 positive cells at all cell layers (outer, inner and ganglion layer (ONL, INL and GCL)) (Kruskal-Wallis, Dunn's post-test, $P < 0.05$) (Table 3 and Figure 2B).

We have previously observed an over activation of poly(ADP-ribose) polymerase (PARP) in our model of porcine retinal degeneration [25]. Moreover, other authors have described similar results in other animal models of retinal degeneration [32,33]. Therefore, we investigated whether TNFα mediated cell death via the PARP pathway. Accumulation of poly(ADP-ribose) polymers (PAR) was used to analyze indirectly PARP activity indirectly. Immunostaining of PAR revealed a significant accumulation of these polymers in ONL and outer segments (OS) in Zaprinast-treated explants (Kruskal-Wallis, Dunn's post-test, $P < 0.05$) that were not prevented by Infliximab treatment (Table 3 and Figure 2C). Infliximab treatment increased PAR accumulation at all cell layers of Zaprinast-treated explants. Thus, the inhibition of TNFα by Infliximab is not causally linked to PARP activation, and therefore does not prevent the secondary PAR accumulation.

To determine whether cleaved *caspase-3* or PAR accumulation co-localize with TUNEL-positive cells, we performed triple labeling (Figure 3). In Zaprinast-treated explants PAR immunostaining co-localized with TUNEL-positive cells in some cells of ONL, in a few cells of the INL and in several cells of GCL. This co-localization disappeared after Infliximab treatment in ONL and GCL but remained in a subset of cells of the INL. PAR accumulation remained high, and even increased, at all cell layers, although the number of TUNEL-positive cells decreased.

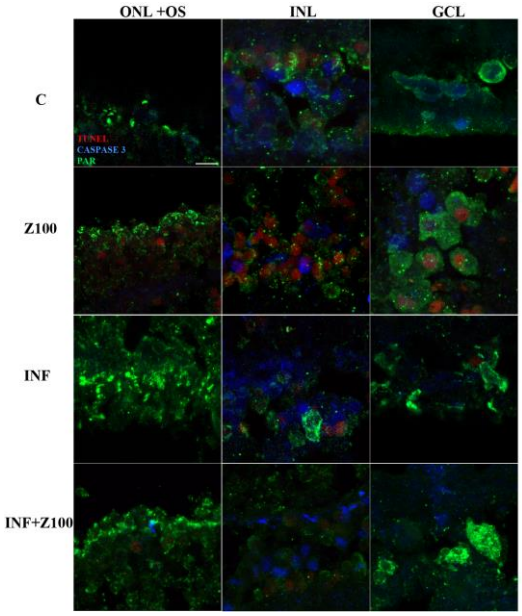

Figure 3. Co-localization of *caspase*-3, PAR and TUNEL at different nuclear layers in culture of porcine retina. Triple-imnunofluorescence labeling of retinal explants treated with dimethyl sulfoxide (DMSO), Zaprinast and Infliximab alone or combined with Zaprinast was carried out as described in Methods. Confocal laser scanning micrographs of retinal sections showing immunolocalization of TUNEL (red), cleaved *caspase*-3 (blue) and PAR (green)-positive cells in the nuclear layers of retina. Scale bar: 10 μm. GCL: ganglion nuclear layer; INL: inner nuclear layer; ONL: outer nuclear layer; OS: outer segments; C: control; Z100: 100 μM Zaprinast; INF: 2 μg/mL Infliximab; Z100 + INF: 100 μM Zaprinast with 2 μg/mL Infliximab. TUNEL, terminal deoxynucleotidil transferase dUTP nick and labeling.

However, *caspase-3* positive cells did not co-localize with TUNEL-positive cells except for a subset of cells in INL in Zaprinast-treated explants. Co-localization of *caspase-3* with TUNEL-positive cells disappeared after Infliximab treatment but increased co-localization of *caspase-3* with PAR in INL.

Infliximab ameliorates Zaprinast-induced glial activation in cultured porcine retina

Gliosis commonly involves upregulation of the intermediate filament protein, GFAP, in Müller glial cells. We studied whether Zaprinast-induced retinal degeneration was accompanied by altered glial reactivity, and if it was the case, whether the blockade of TNFα could prevent it.

In control explants, GFAP were located in the inner half of the retinal Müller cells and their endfeet (GCL layer). However, Zaprinast-treated explants exhibited strong GFAP-positive staining of Müller cells. After PDE6 inhibition, GFAP was massively upregulated throughout the retinal explant. After Infliximab treatment the GFAP-positive labeling was significantly decreased (Figure 4).

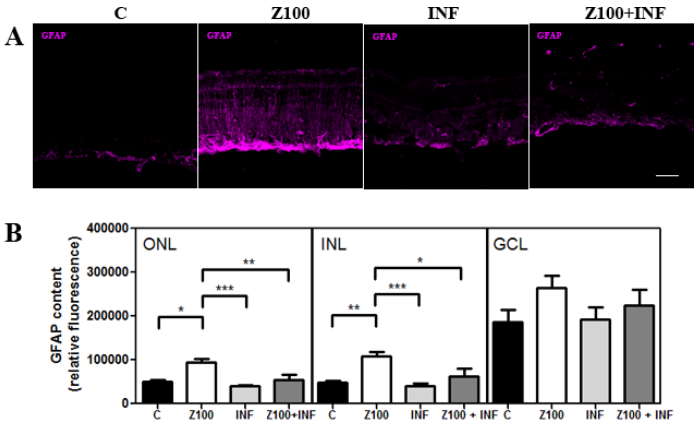

Figure 4. Infliximab prevents Zaprinast-induced glial fibrillary acidic protein (GFAP) overexpression in cultured porcine retina. Retinal explants were incubated with dimethyl sulfoxide (DMSO), Zaprinast and Infliximab alone or combined with Zaprinast as described in Methods. (A) Confocal laser scanning micrographs of retinal sections showing GFAP content. Scale bar: 50 µm. (B) Bar graphs showing the quantification of GFAP content. Values are the mean ±SEM of six different cultures. Values that are significantly different are indicated by asterisks *P <0.05, **P <0.01, ***P <0.001 (Kruskal-Wallis, Dunn's post-test). C: control; Z100: 100 µM Zaprinast; INF: 2 µg/mL Infliximab; Z100 +INF: 100 µM Zaprinast with 2 µg/mL Infliximab.

Infliximab partially prevents Zaprinast-induced oxidative stress in cultured porcine retina

cGMP accumulation induces oxidative stress in murine models of retinal degeneration [34] as it does in our model of porcine retina treated with Zaprinast [25]. To explore whether Infliximab also prevented Zaprinast-induced oxidative damage in cultured porcine retina, we measured intracellular nitrite formation (iNOX), as stable NO metabolite, TBARS content as indicator of MDA and total antioxidant capacity (TAC).

As shown in Figure 5, Infliximab normalized TAC but did not prevent oxidative stress in Zaprinast-treated explants. Total antioxidant capacity returned to control level (230 ± 15 µmol/mg protein, ANOVA Newman-

Keuls post-test, P <0.05) (Figure 5A), but TBARS formation (Figure 5B) and intracellular NOX (Figure 5C) remained high after the blockade of TNFα.

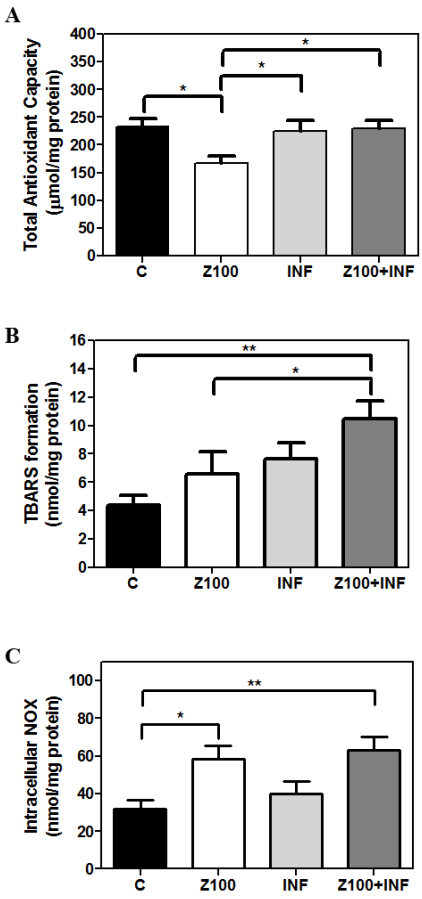

Figure 5. Infliximab partially prevents Zaprinast-induced oxidative stress in cultures of porcine retina. Retinal explants were incubated with dimethyl sulfoxide (DMSO), Zaprinast and Infliximab alone or combined with Zaprinast as described in Methods. Effect of Infliximab on the total antioxidant capacity **(A)**, TBARS formation **(B)** and intracellular NOX **(C)**. Each sample was measured in duplicate, and the values are the mean ±SEM of eight cultures. ANOVA Newman-Keuls post-test was used for TAC analysis. Kruskal-Wallis test and Dunn's post-test was used for TBARS and iNOX analysis. *P <0.05, **P <0.01. C: control; Z100: 100 µM Zaprinast; INF: 2 µg/mL Infliximab; Z100 +INF: 100 µM Zaprinast with 2 µg/mL Infliximab. iNOX, intracellular nitrites and nitrates; TAC, total antioxidant capacity; TBARS, thiobarbituric acid reactive substances.

DISCUSSION

Abnormal pathological pathways such as oxidative stress and inflammation, including upregulation of TNFα, have been described in retinal neurodegenerative diseases both affecting the outer retina, such as RP and age-related macular degeneration (AMD), and the inner retina, such as glaucoma and ischemic retinopathy [35-39]. Low-grade inflammation is present in AMD and glaucoma. For instance, in AMD, many mediators of chronic low-grade inflammation such as C-reactive protein, immunoglobulins, and acute phase molecules, the complement-related proteins, autoantibodies, macrophage infiltration and microglial activation have been found [40]. In glaucoma, microglial activation and an inflammatory response involving Toll-like receptors (TLRs), complement molecules and cytokines, such as TNFα and IL-1β, is associated with secondary phase of the disease [41]. Much less is known about the inflammatory response to retinal ischemic-reperfusion (IR) injury. However, pro-inflammatory gene upregulation, accumulation of leukocytes, and microglial activation is found following IR in rodent retinas [42].

In RP, retinal degeneration is caused by various mutations that result in rod death followed by gradual death of cones [43]. Growing evidence suggests that, regardless of the causative mutation, neuroinflammation contributes to photoreceptor degeneration [44, 45]. For instance, different animal models of RP (rds mice, rd1 mice, P23 rats, RCS rats) carrying mutations in different genes (Prph2, PDE6, Rho, Mertk) show signals of an inflammatory process [23, 46-49]. In early stages of retinal degeneration the photoreceptor cells and surrounding cells, such as microglia, respond to unfavourable conditions with the production of cytokines, chemokines, growth factors, and so on, in an attempt to protect neurons and to preserve retinal function. As disease progresses,

sustained inflammatory mediators and others such as oxidative stress may exacerbate photoreceptor cell death and RP progression.

Early studies suggested the presence of immune reactivity in RP patients, including the presence of retinal autoantibodies in blood and lymphocytes in vitreous humor. However, these results were variable, maybe due to the inherent genetic heterogeneity of this disease [36]. Afterwards, microglial activation, a common hallmark of both inherited and induced retinal degeneration, was described in RP patients and murine models of RP [12, 45, 50-53]. It has been shown that microglial activation leads to proliferation, followed by migration to damaged sites and release of cytokines (TNFα, IL-1α, IL-1β) chemokines, neurotrophins, glutamate, NO, superoxide anions and prostaglandins to repair tissue damage. Although these events are triggered to prevent cell damage, sustained high levels of these molecules, especially cytokines, can cause progressive neurodegeneration. In models of RP, microglial activation coincides, or precedes, the peak of photoreceptor cell death and with high levels of TNFα [12, 44, 45, 50, 54, 55] that seems to be toxic for photoreceptor cells in vitro [23]. Besides, microglial inhibition reduces photoreceptor cell death, TNFα content and improves visual function [46].

In our human study we confirmed (1) the presence of high levels of TNFα and IL-6 in aqueous humor in a larger population of RP patients than previously reported [11]; and we observed that (2) RP patients with higher TNFα values show better visual function (visual field). The conflictive positive correlation, between TNFα and better visual function, may be due to the different stage of the disease of the patients. It has been shown that an increase of proinflammatory markers, including TNFα, in mice models of RP occurs just before photoreceptor cell loss [12]. Therefore, it is tempting to speculate that at early onset of RP, when

proinflammatory markers are elevated, visual function is better in patients, and after these stages patients lose visual function in parallel with TNFα decrease. In any case, these conflicting, and interesting, results strongly suggest that further studies are needed for clarification.

In the last few years, TNFα has been widely recognized as an attractive therapeutic target for the treatment of retinal diseases. Different types of monoclonal antibodies against TNFα, such as Infliximab, Adalimumab, Certolizumab pegol and Golimumab, or circulating receptor fusion protein, such as Etanercept, have been used to treat glaucoma [56, 57], ischemic retinopathy [58] or AMD [59].

The role of TNFα in photoreceptor degeneration and the possible therapeutic use of antibodies against TNFα in the treatment of RP or other retinal degenerations remain quite unexplored. Based on previous studies we decided to evaluate the potential protective effect of the blockade of TNFα in an experimental porcine model of retinal degeneration. In a previous report we demonstrated that this porcine model recapitulated some aspects, especially those related to oxidative stress and inflammation, of the retinal degeneration observed in small animals after PDE inhibition [60,61] and RP patients [8,11]. Sustained elevation of intracellular cGMP in porcine retinal explants triggered different downstream effectors of cell death related to *caspase*-dependent mechanisms (*caspase-3*) and *caspase*-independent mechanisms (calpain-2 and probably PARP activity) [25].

Our current study demonstrated that retinal degeneration accompanied by upregulation of TNFα and IL-6, GFAP and oxidative damage was ameliorated by blocking TNFα with Infliximab. Under our experimental conditions, Infliximab reduced retinal degeneration in all cell layers, mainly in the ONL, by decreasing the number of TUNEL-

positive cells, supporting the idea that inflammation plays an important role in the processes of cell death.

We found that Infliximab reduced *caspase-3* activity and the number of cleaved *caspase-3* positive cells across the different cell layers, especially at the INL. Co-localization studies of *caspase-3* and PAR with TUNEL assay suggested that TNFα is promoting cell death through *caspase*-independent mechanisms in ONL and GCL and *caspase*-dependent mechanisms in INL.

TNF signaling can lead to cell death to two distinct outcomes, each of which is initiated by different signaling complexes: the apoptosis mode and the necrosis mode. The apoptosis mode includes the extrinsic pathway, mainly mediated by *caspase*s, and the intrinsic or mitochondrial pathway, that rely on the balance between the pro-apoptotic and the anti-apoptotic proteins from the Bcl-2 family. Both pathways converge on the same execution pathway. The execution pathway is initiated by the cleavage of *caspase-3* and results in DNA fragmentation and cell death.

We measured indirect activation of PARP through quantification of PAR accumulation. We found an upregulation of PAR due to PDE6 inhibition. However, blockade of TNFα did not prevent PAR accumulation but also increased it. PAR polymers are mainly degraded by poly(ADP-ribose) glycohydrolase (PARG) enzymes, some of them activated by *caspase-3* cleavage [62]. On the other hand, PARP can be inactivated by *caspase-3* cleavage [63]. Therefore, the inhibition of *caspase-3* induced by Infliximab could inhibit PARG activity and prevent PARP inactivation thus exacerbating PAR accumulation at all cell layers of retinal explants. These results support that PARP pathway is independent of TNFα-associated pathways in this experimental model of retinal degeneration (Figure 6).

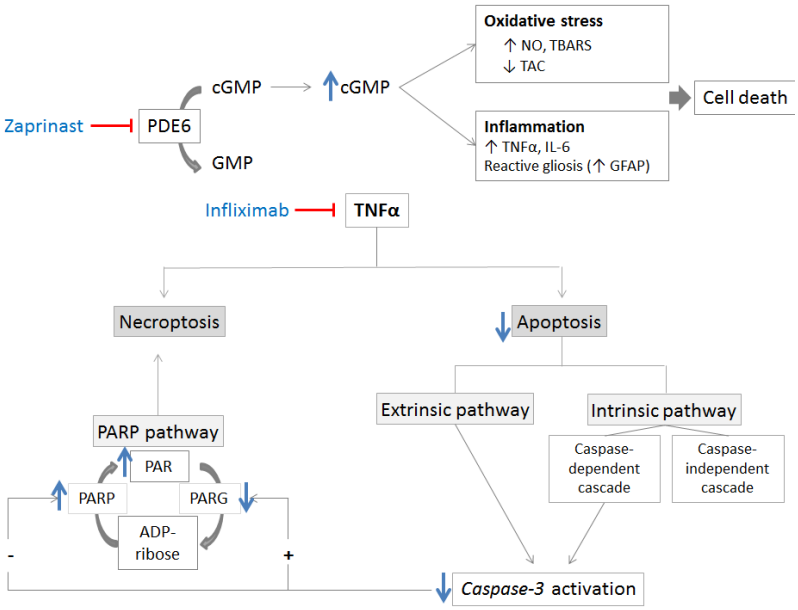

Figure 6. Diagram showing the possible mechanism of Infliximab in the porcine retinal degeneration model. PDE6 inhibition induces cGMP accumulation and triggers retinal degeneration. The degeneration is accompanied by upregulation of inflammatory mediators, PARP pathway, reactive gliosis and oxidative stress markers. According to the current study, TNFα may be involved in the retinal degeneration by increasing *caspase*-3 activation and reactive gliosis. Infliximab may prevent cell death by inhibiting *caspase*-dependent pathways that converge in *caspase*-3 activation in the INL. Infliximab also may prevent cell death by *caspase*-independent pathways that remain unclear in the ONL and GCL. Moreover, Infliximab may exacerbate PARP over activation probably through the *caspase*-3 inhibition. This over activation could contribute to the future cell death. cGMP: cyclic GMP; GCL, ganglion nuclear layer; GFAP: glial fibrillary acidic protein; INL, inner nuclear layer; NO: nitric oxide; ONL, outer nuclear layer; PAR: poly(ADP-ribose) polymers; PARG: poly(ADP-ribose) glycohydrolase; PARP: poly(ADP)ribose polymerase; PDE6: phosphodiesterase 6; TAC: total antioxidant capacity; TBARS: thiobarbituric acid reactive substances; TNFα: tumor necrosis factor alpha.

These results were supported by previous reports in which reactive gliosis (GFAP overexpression) induced by exogenous TNFα was prevented by Adalimumab, other monoclonal anti-TNFα, in a similar model of organotypic culture of porcine neuroretina [64]. Activated Müller cells can release antioxidants, growth factors, and cytokines, including TNFα, contributing to retinal regeneration or to

neurodegeneration. Müller cells are activated in models of RP [49,65-68] resulting in overexpression of GFAP, translocation of Müller cell bodies to the outer retina and thickening of their processes [69].

As previously shown, retinal degeneration induced by PDE inhibition was accompanied by oxidative stress in porcine retinas [25]. This is consistent with the idea that oxidative stress is also contributing to the progression of RP in animal models [70-72] and RP patients [8]. In the current study, we demonstrated that Infliximab partially prevented antioxidant defense depletion but not oxidative stress markers. Infliximab normalized the total antioxidant capacity in Zaprinast-treated explants, but it failed to return TBARS and NOX to control levels. In retinas of *rd10* mice antioxidant treatment reduced inflammatory mediators and photoreceptor cell loss [12]. Based on these data, it is tempting to speculate that the low Infliximab effect could be due to oxidative stress preceding upregulation of inflammatory mediators [12, 73-75]. The other possibility is that Infliximab affects other oxidative stress markers that we did not measure in this study. Anyway, further studies will be needed to explore this issue in more depth.

In summary, our results corroborate that RP patients have ocular inflammation and that TNFα plays an important role in the retinal degeneration induced by PDE6 inhibition in cultured porcine retinas. The mechanisms of cell death vary in the distinct cell layers. TNFα is involved in retinal degeneration through *caspase-3* activation, *caspase*-independent mechanisms and reactive gliosis. Our data suggest that other unknown molecules must be contributing to TNFα-mediated cell death in this model. On the other hand, PARP activation is independent of TNFα signaling and it is probably responsible for a future cell death in ONL. The existence of several distinct pathways that trigger programmed

cell death implies that an efficient protection requires their simultaneous interruption via combined therapies.

The experimental model of organotypic culture has its own limitations because it involves transection of the optic nerve and mechanical retinal detachment causing retrograde retinal ganglion cell degeneration. To minimize this problem, we have used detached retinas as controls. Moreover, the model cannot recapitulate the whole chronic nature of the degeneration, but we believe that it could be useful for studying some aspects related to the retinal degeneration. In our case, we believe that it may provide a helpful model to design and assay some treatments, such as Infliximab, thus replacing or reducing animal experiments. The use of this model allowed us to evaluate the effect of Infliximab faster and more cheaply than using the available *in vivo* models of RP.

The current model of retinal degeneration allowed us to describe an interesting and, in our opinion, neuroprotective effect of Infliximab that strongly encourages further exploration using other experimental models. Due to the importance of the inflammatory process in the pathogenesis of several retinal degenerative conditions such as RP, AMD, ischemic retinopathy, or glaucoma, targeting inflammation could be a promising therapeutic strategy. In particular, TNFα blockers could be a new therapeutic strategy for the treatment of RP and other retinal degenerative conditions.

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Abbreviations

AIF: apoptosis inducing factor, AMD: age related macular degeneration, ANCOVA: analysis of variance, au: arbitrary units, BCVA: best-corrected visual acuity, BCA: bicinchoninic acid, BSA: bovine serum albumin, cGMP: cyclic guanosine monophosphate, DMSO: dimethyl sulfoxide, ELISA: enzyme-linked immunosorbent assay, GCL: ganglion nuclear layer, GFAP: glial fibrillary acidic protein, IL: interleukin, INL: inner nuclear layer, iNOX: intracellular nitrite, IR: ischemic-reperfusion, MANCOVA: multivariate analysis of covariance, MDA: malonyldialdehyde, NO: nitric oxide, NOX: intracellular nitrates and nitrites, ONL: outer nuclear layer, PAR: poly(ADP-ribose), PARP: poly(ADP-ribose) polymerase, PBS: phosphate-buffered saline, PDE6: phosphodiesterase 6, RP: retinitis pigmentosa, TAC: total antioxidant capacity, TBA: thiobarbituric acid, TBARS: thiobarbituric acid reactive substances, TLRs: Toll-like receptors, TNFα: tumor necrosis factor alpha, TUNEL: terminal deoxynucleotidil transferase dUTP nick and labelling, VF: visual field.

Additional files

Table S1. Individual data for each patient with retinitis pigmentosa (RP)

Patient	Age (year)	Sex	Acuity of worst eye (logMAR)	Visual Field of worst eye (dB)	Type of RP	Type of inheritance/ Gene
1	43	M	0.3	4	NS	Unknown
2	45	M	0.0	1	NS	ARRP
4	56	M	0.6	---	NS	Unknown
5	57	M	0.05	37	NS	Unknown
6	37	M	0.7	439	NS	Unknown
7	54	F	0.4	261	NS	Unknown
8	35	F	0.5	694	NS	Unknown
9	57	M	0.2	85	NS	SRP
10	39	M	0.5	682	NS	ADRP
11	35	M	0.4	636	NS	ARRP
12	34	F	0.5	640	NS	ADRP
30	47	M	---	---	S	AR/*USH2A*
31	44	F	0.1	65	NS	ADRP
33	48	F	0.05	109	S	AR/*USH2A*
35	57	M	1.0	228	NS	Unknown
37	51	F	0.05	97	NS	Unknown
39	47	M	0.7	204	NS	Unknown
40	45	F	0.05	141	S	AR/*USH2A*
41	63	M	0.1	95	NS	Unknown
44	48	M	0.2	82	NS	Unknown
45	51	M	0.05	57	S	AR/*USH2A*
46	35	F	0.05	20	NS	ARRP
48	46	M	0.8	264	S	AR/*USH2A*
50	39	M	0.4	97	NS	Unknown
53	53	M	0.6	429	NS	ADRP
54	39	F	0.8	85	NS	Unknown
55	68	M	0.05	67	NS	Unknown
56	58	M	0.2	136	NS	Unknown
57	66	M	0.7	373	NS	ADRP
59	39	M	1.0	143	NS	Unknown

Note: RP: retinitis pigmentosa; NS: non-syndromic; S: Syndromic; ARRP, autosomic recessive RP; ADRP, autosomic dominant RP; SRP, sporadic RP; AR: autosomic recessive; *USH2A*: Usher syndrome type 2A.

Table S2. MANCOVA in aqueous humor from retinitis pigmentosa (RP) patients and healthy controls.

Predictive Variables	Pillai´s trace	P-value
Disease RP	0.18	0.033*
Age	0.14	0.081
Gender	0.03	0.547

Note: Pillai´s trace, multivariate test criteria used in the multivariate analysis of covariance (MANCOVA).

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Capítulo VI. Trabajo adicional

Adalimumab delays photoreceptor cell death in a mouse model of retinitis pigmentosa

ADALIMUMAB DELAYS PHOTORECEPTOR CELL DEATH IN A MOUSE MODEL OF RETINITIS PIGMENTOSA

ABSTRACT

Background: Growing evidence suggests that inflammation is involved in the progression of retinitis pigmentosa (RP) both in patients and in animal models. The aim of this study was to investigate the effect of Adalimumab, a monoclonal anti-TNFα antibody, on retinal degeneration in a murine model of human autosomal recessive RP, the *rd10* mice.

Methods: C57Bl6 and *rd10* mice received Adalimumab at 3 mg/kg through intraperitoneal injection twice a week from P9 to P17. Retinas were prepared for biochemical or histological analysis. Retinal sections were nuclei-counterstained with Sytox Green to evaluate nuclei loss. Cell death was determined by TUNEL assay in retinal sections. Indirect poly(ADP)ribose polymerase (PARP) activation, as cell death marker, was visualized by immunolabelling. Reactive gliosis was evaluated by immunolabelling with Iba1 for microglia and with glial fibrillary acidic protein (GFAP) for Müller cells. Gene expression of inflammatory mediators (TNFα, IL-6 and leukemia inhibitory factor (LIF)) were analysed by real time PCR. Antioxidant response including total antioxidant capacity and content of SOD1, SOD2 and catalase, was also evaluated.

Results: We evaluated retinal degeneration from P13 to P20 in *rd10* mice. In our housing conditions, *rd10* retinas were seriously damaged at P18. Adalimumab reduced photoreceptor cell death by decreasing the number of TUNEL-positive cells and attenuating reactive gliosis. We also observed that Adalimumab diminished the poly(ADP) ribose (PAR) content in the outer nuclear layer. Adalimumab prevented TNFα and LIF upregulation and partially improved antioxidant response.

Conclusions: Our study suggests that the TNFα blockade should preserve retinal structure and reduce inflammatory processes involved in the pathogenesis of RP such as microglial activation. Thus, Adalimumab could be used as a promising therapy in patients with RP. However, further studies are needed to characterize its effect along the progression of the disease.

INTRODUCTION

Retinitis pigmentosa (RP) is a group of inherited retinal dystrophies characterized by progressive and irreversible loss of vision that in most models studied, parallels photoreceptor cell death (Portera-Cailliau *et al.*, 1994; Chang *et al.*, 2007; Rivas and Vecino, 2009). RP is the leading cause of genetic blindness in adults with an estimated incidence of 1 in 3,500~4,500 human births (Ayuso and Millan, 2010). Patients with RP typically loose night vision in adolescence, peripheral vision in young adulthood, and central vision later in life, due to progressive, sequential loss of rod and cone photoreceptor cells. Although many therapeutic approaches have been developed to prevent photoreceptor cell death, no effective treatment is still available (Hughes *et al.*, 2004; Mantopoulos *et al.*, 2011; Fernandez-Sanchez *et al.*, 2012). More than 60 genes, including phosphodiesterase 6 (PDE6) subunit genes, have been identified to date whose mutations cause different forms of non-syndromic RP (Huang *et al.*, 1995; McLaughlin *et al.*, 1995; Daiger *et al.*, 1996-2014; Dryja *et al.*, 1999; Corton *et al.*, 2010). Although RP is a genetic disease, evidence suggests that oxidative stress and neuroinflammation could contribute to its progression (Newsome *et al.*, 1988; Shen *et al.*, 2005; Martinez-Fernandez de la Camara *et al.*, 2013a). In particular, inflammatory processes including microglial activation and upregulation of inflammatory cytokines (TNFα, IL-6, IL-1β, etc.) and chemokines (MCP1, RANTES, etc.) have been described in patients as well as in animal

models of RP (Gupta *et al.*, 2003; Zeng *et al.*, 2005; Yoshida *et al.*, 2012; Peng *et al.*, 2014; Zeng *et al.*, 2014).

Tumor necrosis factor alpha (TNFα) is a pleiotropic cytokine essential for the induction and maintenance of the inflammatory immune responses (Vandenabeele *et al.*, 1995). It is a well characterized mediator of cellular activities including proliferation, survival, differentiation, inflammation and cell death. In the retina, it is likely secreted from activated macrophages, astrocytes, microglia and Müller glial cells. TNFα can trigger several well-characterized death-promoting (caspase-dependent and caspase-independent cell death) and survival-promoting pathways, depending upon the predominating signalling pathway in the particular cell type (Li *et al.*, 2013). In the eye, TNFα appears to have a role in the pathogenesis of inflammatory, edematous, neovascular and neurodegenerative disorders (Saurenmann *et al.*, 2006; Al-Gayyar and Elsherbiny, 2013; Genini *et al.*, 2013; Schaap-Fogler *et al.*, 2014).

Several anti-TNFα agents (Infliximab, Adalimumab, Etarnecept, Golimumab, and Certolizumab pegol) have been developed and approved for clinical use in inflammatory diseases such as rheumatoid arthritis, psoriasis and ankylosing spondylitis (Schulz *et al.*, 2014). These anti-TNFα agents are antibodies against TNFα or TNFα receptor. In Ophthalmology, they are widely used as an alternative to traditional immunosuppressive treatments in non-infectious uveitis. More recently anti-TNFα agents have been used for retinal diseases such as neovascular age-related macular degeneration, diabetic macular edema and retinal vein occlusions (Markomichelakis *et al.*, 2005; Abcouwer *et al.*, 2010; Nishida *et al.*, 2011; Mirshahi *et al.*, 2012; Roh *et al.*, 2012). Due to the possible implication of TNFα on RP progression, we previously evaluated the *ex vivo* effect of Infliximab on Zaprinast-induced retinal degeneration in porcine retina. Infliximab reduced retinal degeneration in cultures of porcine retina exposed to Zaprinast that reproduces some

changes found in murine models of RP with non-functional phosphodiesterase 6 (PDE6) (Martínez-Fernández de la Camara *et al.*, 2014). In this study, we suggested that TNFα was playing an important role in cell death by activating different cell pathways at different cell layers of the porcine retina. To further explore the *in vivo* potential benefits of blocking TNFα we adopted the *rd10* mouse, a model of human autosomal recessive RP. This mouse carries a mutation on the β subunit of the cGMP PDE6 gene (*Pde6β*) that produces retinal degeneration (Chang *et al.*, 2007).

In this study, we first analysed the early stages of retinal degeneration and the profile of TNFα expression in *rd10* mice under our housing conditions. We observed that at postnatal day (P) 18 there was a peak of TNFα gene expression and photoreceptor cell death indicated by the loss of nuclei and the increase of TUNEL-positive cells in the outer nuclear layer (ONL). Based on these results we studied the effect of Adalimumab, a monoclonal antibody against TNFα, on the retinal degeneration process at this age. We observed that Adalimumab prevented TNFα upregulation, reduced photoreceptor cell death, slowed microglial and Müller cell activation and partially improved antioxidant response at P18. Based on these results, we suggest that anti-TNFα therapies could be promising treatments to improve photoreceptor cell survival in humans with RP. However, further studies are needed to investigate the molecular mechanisms involved in the protective effect of anti-TNFα agents and their long-term effect.

MATERIAL AND METHODS

Animals and treatment

rd10 mice were used as human model of autosomal recessive retinitis pigmentosa. Wild-type C57Bl6 mice with the same genetic background

as *rd10* mice were used as control. Mice were kept under a 12 hours light/dark cycle, humidity and temperature controlled and with food and water supplied *ad libitum*. All cages were placed on the lower shelf of an IVC rack with light illuminance of 115 ± 7 lux (95% CI: 98-131). Mice were housed in the Animal Facility of *Unitat Central d'Investigació* (UCIM) of Valencia University. The experimental protocol was approved by the Committee of Ethics in Research of the Faculty of Medicine, University of Valencia.

To determine the profile of retinal degeneration at early stages in our housing conditions, untreated *rd10* and C57Bl6 mice were euthanized at P13, P15, P18 and P20. The eyes were rapidly removed and processed as described below.

To evaluate the effect of Adalimumab, each *rd10* mouse received three intraperitoneal injections of Adalimumab (Humira, Abbot Laboratories) saline solution at 3 mg/kg from P9 to P17. Control mice received the same volume of saline at the same time. At P18, treated and untreated *rd10* mice and C57Bl6 mice were sacrificed. The eyes were rapidly removed and processed as described below.

Tissue processing

To obtain retinal sections, the eyes were rapidly removed and fixed in 4 % filtered paraformaldehyde for two hours at room temperature and cryoprotected in a sucrose gradient (15-20-30 %). Eyes were frozen embedded in OCT and 10 μm sections were cut in a cryostat (Leica CM1900, Nussloch, Germany).

For biochemical determinations and gene expression analysis, retinas were isolated, placed immediately into the appropriate buffer and stored at -80°C.

Retinal immunohistochemistry and TUNEL assay

Immunofluorescent staining procedures were developed in 10 µm cryosections. Sections were post-fixed in 4% filtered paraformaldehyde (Sigma-Aldrich, Madrid, Spain) in 0.1 M phosphate buffer pH 7.4 for 15 minutes at room temperature. Sections were pre-treated with citrate buffer pH 6.0 for epitope retrieval and incubated in blocking solution containing 5% normal goat serum, 1% bovine serum albumin and 0.25% Triton X-100 for one hour. Afterward, they were incubated with primary antibody anti-poly(ADP) ribose (PAR) (1:200, Enzo Life Science, Madrid, Spain) as an indirect marker for poly(ADP) ribose-polymerase (PARP) activity, anti-Iba1 (1:300, Wako Pure Chemical Industries Ltd., Osaka, Japan) and anti-GFAP (1:400, Sigma-Aldrich, Madrid, Spain) overnight at 4°C. Sections were incubated with the fluorescence-conjugated secondary antibody Alexa Fluor 647, 555 or 488 (Invitrogen, Life Technologies, Madrid, Spain) for one hour at room temperature. After labelling, the sections were mounted in Fluoromount-G (Southern Biotechnology, Birmingham, AL, USA) and observed under a fluorescence or confocal microscope.

To evaluate cell death the terminal deoxynucleotidyl transferase dUTP nick and labeling (TUNEL) assay was used as previously described (Martinez-Fernandez de la Camara *et al.*, 2013b).

Microscopy and quantification

The retinal sections were examined under an Eclipse 80i microscope (NIKON Instruments, Badhoevedorp, The Netherlands) or under a confocal microscope (Leica TCS SP5 Confocal microscope, Leica Microsistemas SLU, Barcelona, Spain) belonging to the Microscopy Unit of the IIS-La Fe (Valencia, Spain). ImageJ software was used to quantify the thickness and the number of rows of photoreceptor nuclei in the

ONL, the number of TUNEL, PAR and Iba1-positive cells and the corrected fluorescence of GFAP.

We measured the ONL thickness of the entire retina normalized to the thickness of the inner nuclear layer (INL) to avoid the bias derived of the angle of the sectioning plane. The *normalized ONL thickness ratio* is defined as the ONL thickness/INL thickness. At least five entire retinas were analysed per experimental group.

The number of TUNEL or PAR-positive cells was represented as the ratio between the number of TUNEL or PAR-positive cells in the ONL and the normalized ONL thickness ratio of each section.

To evaluate microglial activation we measured the migration index (M.I) which is defined as the number of Iba1-positive cells weighted according to the retinal layer where are located (M.I = ∑(number of Iba1-positive cells in each layer x layer weighted factor)/total number of Iba1-positive cells in the section. The layer weighted factor was 1 for ONL, 0.5 for outer plexiform layer (OPL) and 0.25 for INL.

Corrected fluorescence of GFAP was quantified as previously described (Martínez-Fernández de la Camara *et al.*, 2014). TUNEL and PAR-positive cells, microglial migration index and the corrected fluorescence of GFAP were quantified from four non-adjacent sections of at least five retinas for each experimental group.

Isolation of total RNA and cDNA synthesis

Total RNA was isolated from frozen retina using RNeasy mini kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. RNA concentration and purity were determined by UV spectrophotometry at 260 and 280 nm wavelengths. Then, cDNA was synthesized starting from 1 μg of RNA by reverse transcription using the GeneAmp Gold RNA PCR

Reagent kit (Applied Biosystems, Carlsbad, CA, USA) following manufacturer's instructions.

Quantitative real time PCR

The relative expression of TNFα, IL-6 and LIF was measured by real-time PCR using a commercial thermal-cycler (Applied Biosystems ViiA™ 7 Real-Time PCR System; Life Technologies Corporation, Carlsbad, California, USA), TaqMan® gene expression assay (Mm00443260_g1 (TNFα), Mm00446190_m1 (IL-6) and Mm00434762_g1 (LIF)) and TaqMan® 2X PCR Master Mix (Applied Biosystems, Life Technologies Corporation, Carlsbad, California, USA). β2 microglobulin (β2m) gene (Mm00437762_m1) was used as housekeeping gene.

Real-time PCR was performed with 1 cycle of 2 min at 50°C, followed by 1 cycle of denaturation of 10 min at 95°C, continued by 40 cycles of 15 seconds denaturation at 95°C and 60 seconds annealing at 60°C.

Oxidative stress evaluation

Retinas were homogenized in 5 mM phosphate buffer pH 7, 0.9% NaCl, 0.1% glucose, centrifuged at 1,500 × g for 15 minutes at 4°C. Supernatants were used to determine total antioxidant capacity (TAC) with a commercial kit (Cayman Chemical, Ann Arbor, MI, USA) (Kowluru *et al.*, 2006). Protein concentrations were measured by the bicinchoninic acid (BCA) protein assay. Retinal TAC levels were expressed as nmol/mg protein.

The retinal content of SOD1 (cytosolic), SOD2 (mitochondrial) and catalase were kindly measured by Dr. Jose M. Cuezva and collaborators (Department of Molecular Biology, *Centro de Biología Molecular Severo Ochoa*, Madrid) with a procedure based on reverse phase protein microarray (Aldea *et al.*, 2011).

Statistical analyses

Statistical analysis was done using GraphPad Prism 5.0 program (GraphPad Software, La Jolla, CA). Mann-Whitney U test or Kruskal-Wallis test posthoc Dunns were used to compare different variables between different experimental groups. A p-value < 0.05 was considered statistically significant.

RESULTS

Temporal progression of retinal degeneration in rd10 mice

We first analysed the time course of retinal degeneration in *rd10* mice from P13 to P20 in our Animal Facilities. Between 98 and 131 lux, Sytox Green nuclear staining showed the decline of the number of rows of nuclei in the ONL in *rd10* mice. As expected we did not find a significant difference between control and *rd10* mice at P13. From P15 to P20, the ONL dropped abruptly from eleven to five rows of cell nuclei (Figure 1A).

Then, we assessed photoreceptor cell death by TUNEL staining which detects DNA breaks in apoptotic nuclei but also in necrotic nuclei (Grasl-Kraupp *et al.*, 1995). From P15 to P20 there was a significant increase of TUNEL-positive cells in *rd10* mice, reaching a peak at P18 (12.5 ± 2.4 TUNEL-positive cells/normalized ONL area, Mann-Whitney U test, $p<0.0001$) (Figure 1B).

It has been previously described that TNFα upregulation precedes the peak of photoreceptor cell death (Yoshida *et al.*, 2012; Genini *et al.*, 2013). Therefore we analysed gene expression of TNFα during the time course of retinal degeneration in our housing conditions. As shown in Figure 1C, TNFα was upregulated from P13 to P20 with the highest expression at P18 (6.1 ± 1.2 relative expression, Mann-Whitney U test, $p=0.0002$) concomitantly with the peak of TUNEL-positive cells.

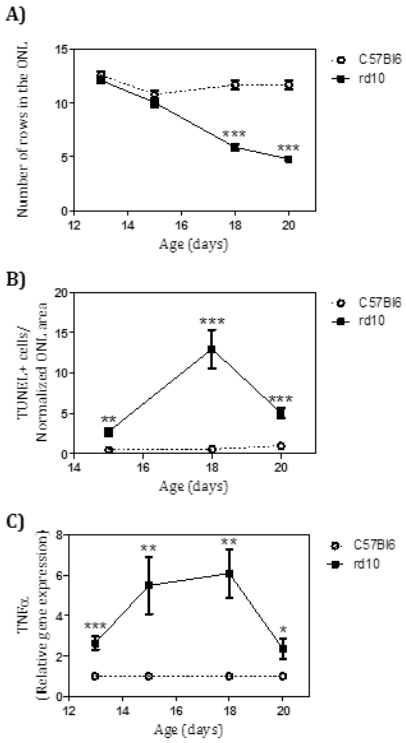

Figure 1. Time course of retinal degeneration in *rd10* mice. (A) Quantitation of the number of rows of photoreceptor nuclei from P13 to P20 in control (C57Bl6) and *rd10* mice. **(B)** Number of TUNEL-positive cells by normalized ONL thickness in control and *rd10* mice from P15 to P20. **(C)** Relative TNFα gene expression from P13 to P20 in *rd10* mice. Values are the mean ± SEM of, at least, five retinas per group. Values that are significantly different are indicated by asterisks *p<0.05, **p<0.001, ***p<0.0001 (Mann-Whitney U test).

In view of these results, P18 was chosen as the reference age for the ensuing analysis of the effect of Adalimumab on the retinal degeneration.

Adalimumab prevented photoreceptor cell death and PARP activation

To determine whether Adalimumab treatment protects against photoreceptor degeneration, we quantified the normalized ONL thickness ratio and the number of rows of nuclei in the ONL at P18. Normalized ONL thickness decreased slower in retinas from *rd10* mice

treated with Adalimumab (1.18 ± 0.05, Kruskal-Wallis, Dunn's post-test, p<0.001) than in retinas from untreated *rd10* mice (0.89 ± 0.04) (Figure 2A). Statistical analysis also revealed that Adalimumab significantly prevented photoreceptor cell loss (7.6 ± 0.4 number of rows of nuclei in the ONL, Kruskal-Wallis, Dunn's post-test, p<0.05) compared to retinas from untreated *rd10* mice (5.9 ±0.3 number of rows of nuclei in the ONL) (Figure 2B).

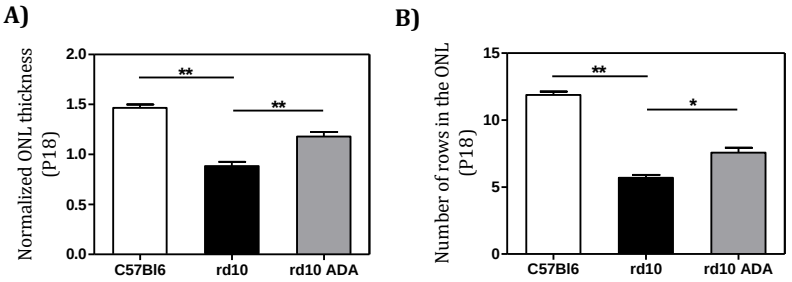

Figure 2. Adalimumab administration decreased photoreceptor cell loss in *rd10* mice at P18. Bar graph illustrates the effect of Adalimumab (ADA) on normalized ONL thickness **(A)** and number of rows of nuclei in the ONL **(B)**. Values are the mean ± SEM of six retinas per group. Values that are significantly different are indicated by asterisks *p<0.05, **p<0.001 (Kruskal-Wallis, Dunn's post-test).

We previously described that Infliximab, other monoclonal anti-TNFα antibody, significantly reduced the number of TUNEL-positive cells in Zaprinast-treated explants of porcine retina (Martínez-Fernández de la Camara *et al.*, 2014). In the current study we observed that intraperitoneal administration of Adalimumab significantly reduced the number of TUNEL-positive cells in the ONL at P18 (2.7 ± 0.4, Kruskal-Wallis, Dunn's post-test, p=0.002) compared to retinas from untreated *rd10* mice (12.5 ± 2.4) (Figure 3A and 3C). Therefore the blockade of TNFα was preventing photoreceptor cell loss in *rd10* mice. However, the mechanisms of TNFα-induced cell death remained unclear.

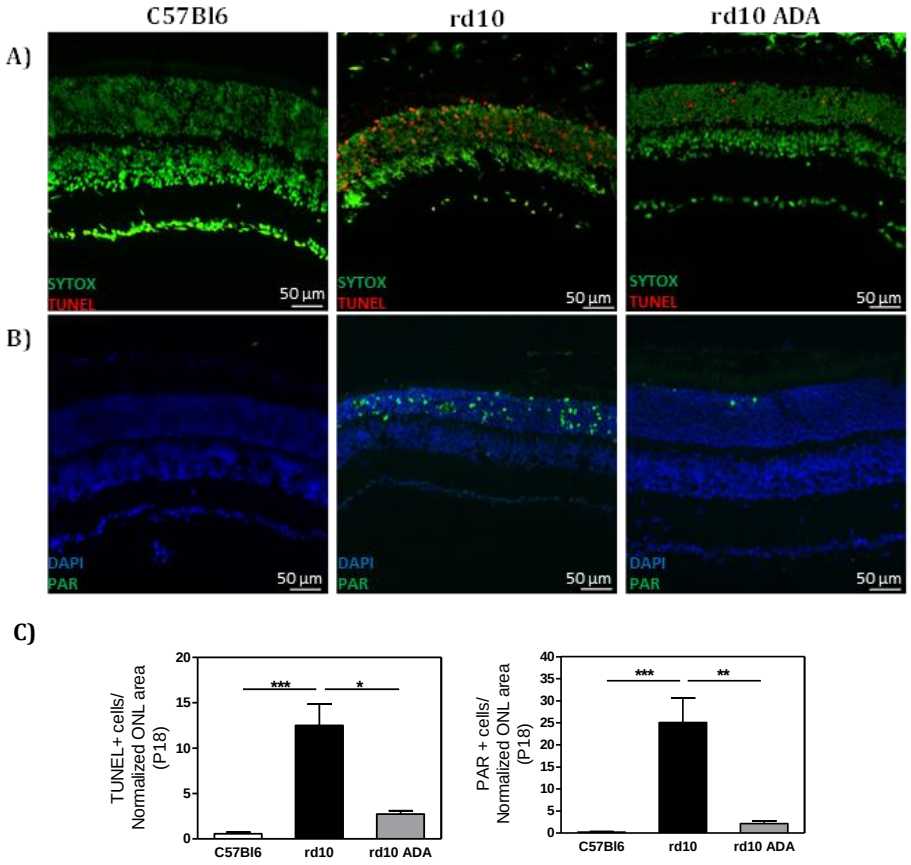

Figure 3. Adalimumab significantly reduced cell death in the ONL at P18. Fluorescence microscopy images of retinal sections showing **(A)** TUNEL-stained sections revealing dead photorreceptors in Sytox Green-counterstained and **(B)** PAR content in DAPI-counterstained retinal sections. **(C)** Bar graph illustrates the effect of Adalimumab (ADA) on the number of TUNEL-positive nuclei and PAR-positive cells. Values are the mean ± SEM of, at least, five retinas in each group. Values that are significantly different are indicated by asterisks *p<0.05, **p<0.01, ***p<0.001 (Kruskal-Wallis, Dunn's post-test).

Paquet-Durand *et al.* (2007) demonstrated non-apoptotic mechanisms involved in photoreceptors cell death such as the activation of PARP which led to necroptosis in a similar mouse model of RP (Paquet-Durand *et al.*, 2007). To assess whether TNFα inhibition prevented PARP activation, we visualized the content of PAR and its

location in retinal cryosections at P18. As shown in Figure 3B, an intense and nuclear PAR staining was found in photoreceptors cells of retinas from untreated *rd10* mice. In contrast, Adalimumab substantially decreased nuclear PAR staining in photoreceptors cells (2.1 ± 0.6 PAR-positive cells, Kruskal-Wallis, Dunn's post-test, $p<0.01$) compared to retinas from untreated *rd10* mice (25.1 ± 5.5 PAR-positive cells) (Figure 3C).

Taking together these results suggested that Adalimumab could slow down photoreceptor cell death through PARP pathway inhibition in *rd10* mice. Further studies are needed to confirm this hypothesis.

Adalimumab reduced reactive gliosis

Chronic microglia activation is associated with various neurodegenerative diseases including RP. We investigated whether Adalimumab treatment prevented microglial activation in retinas from *rd10* mice. Microglial cells were identified by specific labelling with Iba1, a microglia/macrophage-specific calcium-binding protein. As described in Material and Methods section, we analysed the level of migration from the inner to the outer layers of retina as indicator of microglial activation. For this, we did a weighted analysis where values close to 1 indicate a high rate of microglial migration to outer layers. Iba1 immunostaining revealed that microglia was located in all layers, especially in outer layers in retinas from untreated *rd10* mice. But, after intraperitoneal administration of Adalimumab, microglia was preferentially located in the inner layers of the retina. A few microglial cells were observed in the outer plexiform layer but not in the outer nuclear layer in Adalimumab-treated *rd10* retinas (Figure 4A). Statistical analysis confirmed that microglial activation was reduced in retinas from *rd10* mice treated with Adalimumab (0.28 ± 0.03, Kruskal-Wallis, Dunn's post-test, $p<0.05$) compared to retinas from untreated *rd10* mice (0.43 ± 0.02) at P18 (Figure 4C).

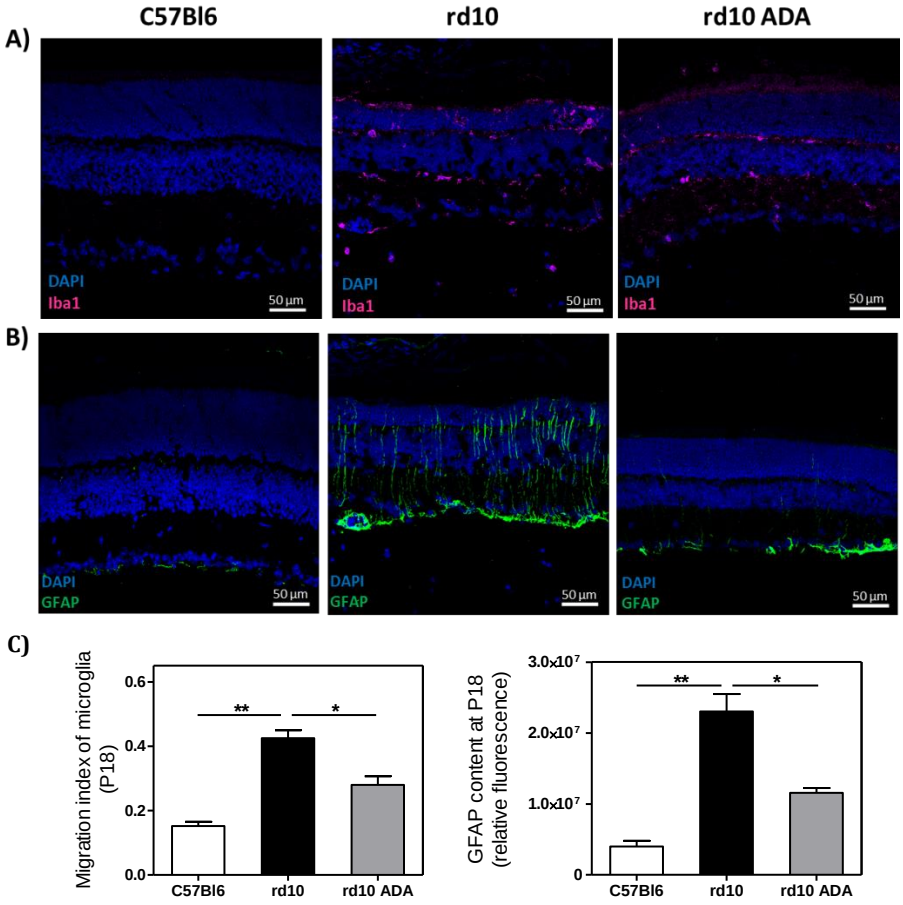

Figure 4. Adalimumab ameliorated reactive gliosis in *rd10* mice at P18. Fluorescence microscopy images of retinal sections showing **(A)** Iba1-labelling to visualize microglial cells and **(B)** GFAP content in DAPI-counterstained retinal sections. **(C)** Bar graph illustrates the effect of Adalimumab (ADA) on migration index of microglia and the corrected fluorescence of GFAP content. Values are the mean ± SEM of, at least, five retinas per group. Values that are significantly different are indicated by asterisks *p<0.05, **p<0.001 (Kruskal-Wallis, Dunn's post-test).

Gliosis commonly involves upregulation of the intermediate filament protein, the glial fibrillary acidic protein (GFAP), in Müller glial cells. We also studied whether the blockade of TNFα with Adalimumab ameliorates glial activation in *rd10* mice. Figure 4B shows that GFAP was upregulated in retinas from untreated *rd10* mice. Adalimumab

significantly decreased GFAP-positive immunolabelling ($1.2x10^7$ ± $0.1x10^7$ corrected fluorescence units, Kruskal-Wallis, Dunn's post-test, $p<0.05$) compared to retinas from untreated *rd10* mice ($2.3x10^7$ ± $0.2x10^7$ corrected fluorescence units) at P18 (Figure 4C).

Adalimumab prevented upregulation of TNFα and LIF and downregulated IL-6 in rd10 retinas at P18.

As previously described by Yoshida *et al.* (2012), TNFα is upregulated in the retina of *rd10* mice previous to photoreceptor cell death peak (Yoshida *et al.*, 2012). We analysed whether Adalimumab affected gene expression of the inflammatory mediators TNFα, IL-6 and LIF by real time PCR at P18. Adalimumab tended toward down-regulating TNFα expression around three-fold in retinas from *rd10* mice (2.4 ± 0.7 relative expression, Kruskal-Wallis, Dunn's post-test) compared to TNFα expression in retinas from untreated *rd10* mice (6.1 ± 1.2 relative expression) (Figure 5A).

Adalimumab also had a tendency to reduce IL-6 expression although there was no difference between retinas from control mice and untreated *rd10* mice (Figure 5B).

LIF is produced by Müller glial cells in response to retinal stress (Burgi *et al.*, 2009). As shown in Figure 5C, LIF was upregulated in retinas from untreated *rd10* mice (0.99 ± 0.28 relative expression, Kruskal-Wallis, Dunn's post-test) and Adalimumab reduced it (0.57 ± 0.08 relative expression). Then, Adalimumab reduced gene expression of all three inflammatory mediators analysed that supports the idea that Adalimumab was preventing from an excessive inflammatory response.

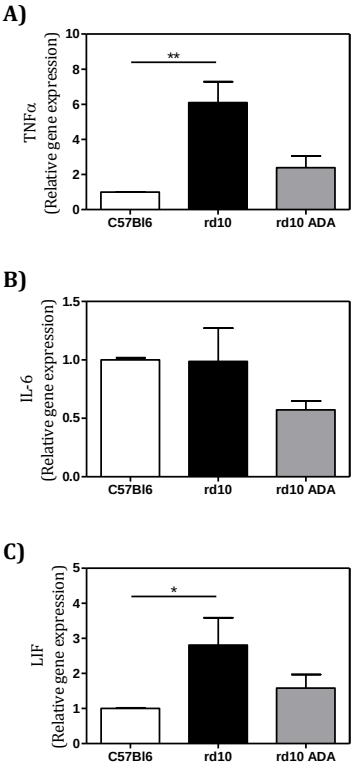

Figure 5. Adalimumab reduced gene expression of inflammatory mediators in the *rd10* mouse retina at P18. (A) TNFα, **(B)** IL-6 and **(C)** LIF gene expression in retinas from control mice, untreated *rd10* mice and *rd10* mice treated with Adalimumab (ADA). Gene expression was analysed as described in Material and Methods section. Values are expressed as mean ± SEM of, at least, nine retinas per group. Values that are significantly different are indicated by asterisks *p<0.01, **p<0.001 (Kruskal-Wallis, Dunn's post-test).

Adalimumab improved antioxidant response

It has been reported that inflammatory processes and oxidative stress are closely linked in several pathologies. The contribution of oxidative damage to cell death in *rd10* mice has been described by several authors (Komeima *et al.*, 2007; Lee *et al.*, 2011; Oveson *et al.*, 2011). Yoshida *et al.* demonstrated that the administration of the antioxidant N-acetylcysteine, reduced the inflammation in *rd10* mice

(Yoshida *et al.*, 2012). On the other hand, we previously described that Infliximab ameliorated antioxidant response in cultured porcine retina exposed to Zaprinast (Martínez-Fernández de la Camara *et al.*, 2014). Therefore we evaluated whether Adalimumab improved the antioxidant response in retinas from *rd10* mice. For this purpose, we measured the total antioxidant capacity (TAC) and the content of three antioxidant enzymes which play a major role in the first line of antioxidant defence: cytosolic and mitochondrial SOD (SOD1 and SOD2, respectively) and catalase.

As shown in Figure 6, the TAC was slightly reduced in retinas from untreated *rd10* mice (92.6 ± 7.8 nmol of Trolox/mg protein) compared to control retinas (119 ± 5.7 nmol of Trolox/mg protein). Adalimumab increased TAC to 204.5 ± 11.6 nmol of Trolox/mg protein (Kruskal-Wallis, Dunn's post-test, $p<0.01$). The content of SOD1 was 4-fold higher in retinas from *rd10* mice treated with Adalimumab (0.80 ± 0.26 arbitrary units (a.u.), Kruskal-Wallis, Dunn's post-test, $p<0.05$) than in retinas from untreated *rd10* mice (0.20 ± 0.04 a.u.). On the other hand, SOD2 content was also increased in retinas from treated *rd10* mice (0.83 ± 0.01 a.u., Kruskal-Wallis, Dunn's post-test, $p<0.05$) compared to retinas from untreated *rd10* mice (0.63 ± 0.04 a.u.). The increase of SOD2 was not as high as for SOD1. We did not find statistical differences for the content of catalase enzyme (Figure 6).

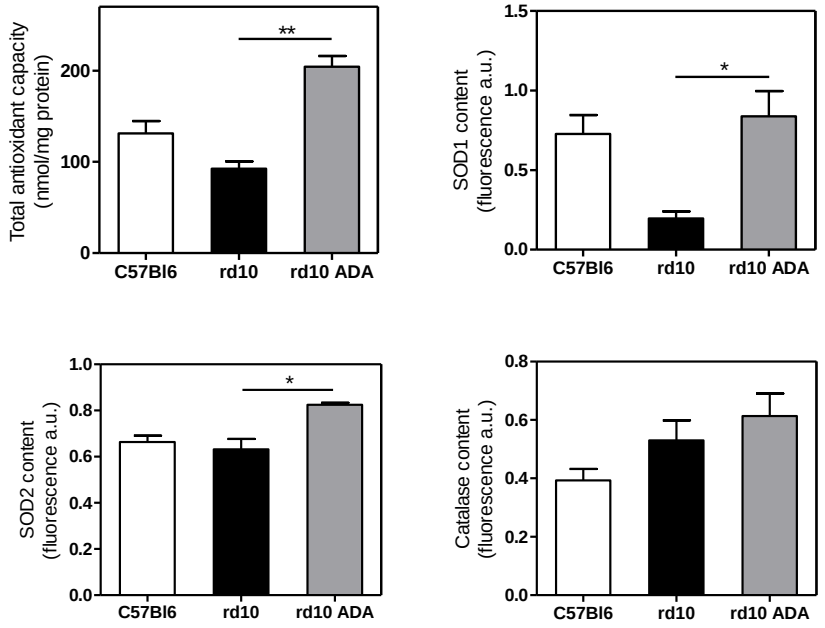

Figure 6. Adalimumab partially improved antioxidant response at P18. Total antioxidant capacity and protein content of SOD1, SOD2 and catalase were measured as described in Material and Methods section. Quantitation of these markers is shown in bar graphs. Values are the mean ± SEM of, at least, six retinas per group. Values that are significantly different are indicated by asterisks *p<0.05, **p<0.01 (Kruskal-Wallis, Dunn's post-test).

DISCUSSION

TNFα inhibitors have been widely used for the treatment of different autoimmune disease, including ocular inflammatory disorders, with promising results (Wu *et al.*, 2012; Interlandi *et al.*, 2014; Schaap-Fogler *et al.*, 2014). Nevertheless, to our knowledge, we present the first study that evaluates the effect of TNFα inhibitors on the progression of the retinal degeneration in a murine model of RP. The results obtained in the present study revealed that intraperitoneal administration of Adalimumab, a recombinant human monoclonal antibody against TNFα,

delayed retinal degeneration decreasing cell death and reactive gliosis at early stages of RP in *rd10* mice.

To evaluate the *in vivo* effect of the blockade of TNFα, we used the model of human autosomal recessive RP, *rd10* mice. This mutant mouse mimics the disease progression seen in humans and offers an appropriate therapeutic window. In general, rod photoreceptors start to degenerate between P16 and P20, with a maximum cell death around P21 and P25. By P60, rods are undetectable and only cones remain (Chang *et al.*, 2007; Gargini *et al.*, 2007; Barhoum *et al.*, 2008; Pennesi *et al.*, 2012). Under our housing conditions, we observed that photoreceptor degeneration started around P15, with a maximum cell death around P18. This time point of maximum cell death agreed with a peak of TUNEL-positive cells recently described by Arango-Gonzalez *et al.* (2014) for *rd10* mice (Arango-Gonzalez *et al.*, 2014). In any case, we performed a genotypic analysis by detecting the c.232C>T mutation in the 13 exon of *PDE6* confirming that our colony was *rd10* mice (data not shown). Therefore we suggest that the discrepancy on the onset of photoreceptor degeneration could be explained by housing conditions. In our study light intensity was higher (98-131 lux) than light intensity described by other authors (around 60 lux) (Doonan *et al.*, 2005; Gargini *et al.*, 2007).

In the present study, we demonstrated that intraperitoneal injections of Adalimumab displayed a neuroprotective effect on photoreceptors survival at the onset of rod death in *rd10* mice. The administration of 3 mg/kg Adalimumab from P9 to P17 contributed to preserve retinal structure reducing the loss of photoreceptors at P18. Adalimumab reduced the number of TUNEL-positive cells by about 80% in the outer nuclear layer. To explore the mechanisms through TNFα inhibition reduced cell death, we studied indirect activation of PARP through quantification of PAR accumulation. We confirmed that there was an

excessive PAR content in retinas from untreated *rd10* mice that was effectively prevented by Adalimumab.

In *rd10* mice, upregulation of TNFα and activation of microglial cells and their migration to the outer retina to eliminate cellular debris are early events involved in photoreceptor death in murine models of RP (Yoshida *et al.*, 2012; Cuenca *et al.*, 2014; Peng *et al.*, 2014). We corroborated microglial activation and migration to outer retina in *rd10* mice. However, we did not visualize Iba1-positive cell in the outer nuclear layer in retinas from *rd10* mice treated with Adalimumab. Thus, inhibition of TNFα prevented excessive microglial activation and migration of microglial cells to the outer layers.

Recent studies showed that Adalimumab decreased glial cell activation (overexpression of GFAP) and preserved retinal organization in organotypic cultures of porcine neuroretina exposed to TNFα (Fernandez-Bueno *et al.*, 2013). Activated Müller cells can release antioxidants, growth factors and cytokines, including TNFα, contributing to retinal regeneration or degeneration. Müller cells are activated in models of RP resulting in overexpression of GFAP, activation of ERK (extracellular signal-regulated kinase), translocation of Müller cell bodies to the outer retina and thickening of their processes (Zhao *et al.*, 2010; Martínez-Fernández de la Camara *et al.*, 2014). In the present study, Adalimumab ameliorated GFAP overexpression in *rd10* retinas.

Adalimumab specifically blocks the interaction of TNFα with its receptors, inhibiting downstream cellular signalling mechanisms (Mirshahi *et al.*, 2012). We found that blocking these TNFα signals we normalized the altered TNFα production, as described in other pathological situations (Zamora-Atenza *et al.*, 2014). TNFα blockade diminished the expression of Th17/Th1 cytokines including TNFα in patients with psoriasis and inflammatory bowel disease (Bose *et al.*,

2011) and prevented the activation of signalling pathways involving the activation of p38 MAPK or NF-κβ (Johansen *et al.*, 2010; Fischer *et al.*, 2013). MAPKs (*mitogen-activated protein kinases*), which comprise a family of protein-serine/threonine kinases, such as ERK, JNK (*c-Jun N-terminal kinase*) and p38, are involved in many inflammatory and degenerative processes including retinal cell death. p38 MAPK plays an important role on microglial activation in different situations and its inhibition suppress the expression of proinflammatory mediators such as TNFα (Ibrahim *et al.*, 2011; Dong *et al.*, 2014). On the other hand, increased ERK activation correlated with an increase in GFAP protein expression and retinal cell death in *RCS* rats and in a model of Leber congenital amaurosis (Zhao *et al.*, 2010; Metrailler *et al.*, 2013). Thus, we speculate that Adalimumab could attenuate the inflammatory process by interfering TNFα signalling as p38 MAPK or ERK activation. Further studies are needed to confirm this hypothesis.

In *rd10* mice, the early stages of the degeneration are also accompanied by upregulation of several signalling molecules implicated in survival pathways as LIF, among others. LIF expression is induced in a subset of Müller glia cells during photoreceptor death (Samardzija *et al.*, 2012). Our results show that LIF expression is increased concurrently with photoreceptor cell death in retinas from untreated *rd10* mice. Treatment with Adalimumab decreased the expression of LIF compared to untreated *rd10* mice possibly due to a lower activation of inflammatory processes derived of TNFα blockade. It is possible that there was an association between the low level of reactive gliosis and the reduced LIF expression in retinas from treated *rd10* mice.

Inflammation and oxidative stress are closely linked processes in several pathological situations. It has been widely suggested that oxidative stress contributes to the pathogenesis of RP in animal models and patients (Komeima *et al.*, 2007; Lee *et al.*, 2011; Oveson *et al.*, 2011).

For instance, we previously found that RP patients present a reduced antioxidant response (Martinez-Fernandez de la Camara *et al.*, 2013a). In the current study we assessed whether downregulation of inflammation (cytokine mediators and reactive gliosis) induced by Adalimumab affected antioxidant response. Adalimumab partially improved antioxidant defence machinery in the retina of *rd10* mice. This treatment normalized the total antioxidant capacity and increased the content of SOD1 and SOD2 in retinas from *rd10* mice. It seems that SOD1 is a key defender against oxidative damage in retina as it has been shown that SOD1 deficient mice are more sensitive to the damaging effects derived of oxidative insults (Usui *et al.*, 2009; Usui *et al.*, 2011). In the present study we have observed that *rd10* mice had a negligible content of SOD1 and Adalimumab restored it.

Although Adalimumab could be a promising therapy for retinitis pigmentosa and other retinal degenerations, further studies are needed. It is important to investigate in detail the molecular mechanisms involved in the neuroprotective effect of Adalimumab and its long-term effect.

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Capítulo VII. Discusión general

DISCUSIÓN GENERAL

En la presente tesis se investigó el papel del estrés oxidativo y la inflamación en pacientes con retinosis pigmentaria (RP) en el modelo murino *rd10* y en el modelo *ex vivo* de retina porcina expuesta a Zaprinast. Para ello, el primer objetivo fue la determinación de factores solubles relacionados con la respuesta antioxidante, el estrés oxidativo/nitrosativo y la inflamación en humor acuoso y sangre periférica, que pudieran estar implicados en la progresión de la degeneración retiniana en pacientes con RP. Esto nos proporcionaría un conocimiento más detallado del microambiente que rodea a las células de la retina y nos permitiría identificar posibles biomarcadores periféricos de la enfermedad. Además, la evaluación de la relación entre el grado de afectación visual y el microambiente nos permitiría conocer en qué medida estarían afectando dichos factores solubles a la progresión de la enfermedad y, por lo tanto, establecer posibles dianas terapéuticas para el diseño de nuevos tratamientos que retrasasen la degeneración retiniana en estos pacientes.

Los resultados de este primer estudio mostraron que la respuesta antioxidante, principalmente la capacidad antioxidante total y la actividad de la enzima antioxidante SOD3, estaba disminuida en humor acuoso de pacientes con RP. Además, se observó una asociación positiva entre una mayor respuesta antioxidante y un mejor campo visual. Aquellos pacientes que tenían peor capacidad para hacer frente a los radicales libres, tenían peor campo visual (Capítulo III) (Martinez-Fernandez de la Camara *et al.*, 2013a). Los resultados de este estudio también revelaron que los pacientes con RP presentaban alteraciones a nivel sistémico de distintos marcadores de estrés oxidativo/nitrosativo y de respuesta antioxidante. Se observó una menor actividad de SOD3, un incremento de TBARS (indicador de peroxidación lipídica) y una

activación de la vía NO/GMPc determinada por valores elevados de nitritos (medida indirecta del contenido de NO), nitrotirosina y GMPc en suero de pacientes con RP. Resulta difícil comprender cómo alteraciones en la retina pueden ser visibles a nivel sistémico, especialmente en lo que se refiere a marcadores de estrés oxidativo que pueden derivar de otras situaciones patológicas o fisiológicas. Una estrategia adecuada para confirmar la presencia de biomarcadores periféricos sería realizar un estudio observacional desde el inicio de los síntomas o antes de su aparición hasta fases más avanzadas de la enfermedad en una amplia cohorte considerando diversas variables que pudiesen influir en los resultados como hábitos alimenticios, consumo de tabaco, otras patologías, etc. Esto nos permitiría saber si los cambios metabólicos observados en sangre periférica son consecuencia de las alteraciones oculares. En este sentido, se han realizado estudios de metaboloma en pacientes con degeneración macular asociada a la edad (Osborn *et al.*, 2013) aunque por el momento, no se ha hecho nada similar en pacientes con RP.

Hasta donde sabemos, este estudio fue el primero que se realizó evaluando el daño oxidativo y la respuesta antioxidante en pacientes con RP. Actualmente se ha iniciado un estudio prospectivo observacional dirigido por el Dr. Campochiaro (Universidad Johns Hopkins en Baltimore, Maryland) en el que se analizarán marcadores de estrés oxidativo en humor acuoso y en sangre periférica en pacientes con RP (ClinicalTrials.gov. Identificador: NCT01949623).

Los trabajos previos en modelos animales han aportado información importante sobre la implicación del daño oxidativo en la muerte de los fotorreceptores. En estos modelos se han ensayado diversas estrategias terapéuticas dirigidas a neutralizar los radicales libres a través de la administración de diferentes antioxidantes (ácido lipoico, vitamina C, tocoferol, etc.) o mediante ingeniería genética, aumentando la expresión

de las enzimas antioxidantes (Komeima *et al.*, 2006; Lu *et al.*, 2009; Usui *et al.*, 2009; Oveson *et al.*, 2011). En pacientes con RP se han llevado a cabo ensayos clínicos con compuestos como la vitamina A, vitamina E, luteína y ácido docosahexanoico que han mostrado algún tipo de beneficio (Aleman *et al.*, 2001; Berson *et al.*, 2004; Bahrami *et al.*, 2006; Berson *et al.*, 2010; Kiang *et al.*, 2014). Por ejemplo, se ha descrito que una dieta rica en ácidos grasos omega-3 suplementada con vitamina A reduce la pérdida de la agudeza visual de cuatro a seis años (Berson *et al.*, 2012) o que la combinación de luteína con vitamina A disminuye la pérdida de campo visual periférico en pacientes con RP (Berson *et al.*, 2010). Actualmente existen, al menos, seis estudios clínicos en distintas fases que evalúan el efecto de diversos compuestos (luteína, vitamina A, vitamina E y compuestos derivados de algas) sobre la fisiopatología de la RP en pacientes (ClinicalTrials.gov). Debido a la importancia que podría tener una terapia antioxidante apropiada se sigue trabajando en nuevos antioxidantes, dosis y combinaciones que retrasen la progresión de la RP.

Entre otros factores, la oxidación de proteínas, lípidos y ADN derivados del daño oxidativo generado tras la muerte de los bastones, puede activar la microglía y la producción de citoquinas y quimioquinas contribuyendo al proceso inflamatorio que, si no es controlado, puede volverse crónico y deletéreo para las células (Roy *et al.*, 2008). En los últimos años, numerosos estudios han puesto de relevancia la importancia de la inflamación en la patogénesis de la RP. En este trabajo hemos confirmado el aumento de TNFα e IL-6 en humor acuoso de pacientes con RP obtenidos por otros autores (Capítulo V) (Martínez-Fernández de la Camara *et al.*, 2014). En 2012, Yoshida y colaboradores detectaron niveles elevados de IL-6 en el humor vítreo y en el humor acuoso de pacientes con RP, mientras que solo detectaron un aumento de TNFα en el humor vítreo (Yoshida *et al.*, 2012a). En este estudio

observaron que los pacientes con mayor número de células inflamatorias en la cavidad vítrea tenían peor función visual, no obstante no evaluaron la relación entre el contenido de citoquinas y la función visual. En nuestro estudio observamos una correlación positiva entre el contenido de TNFα en humor acuoso y el campo visual, es decir, aquellos pacientes con niveles más elevados de TNFα mostraron mejor función visual. Varios autores han demostrado que la activación microglial y el incremento de la expresión de quimioquinas y de TNFα coincide o precede al pico de muerte de los fotorreceptores (Zeng *et al.*, 2008; Yoshida *et al.*, 2012b; Al-Gayyar and Elsherbiny, 2013). Las quimioquinas reclutan células inflamatorias al lugar del daño, por lo que el aumento del contenido de quimioquinas y citoquinas se produciría previamente al infiltrado celular. Esto podría explicar la discrepancia entre los resultados mostrados en esta tesis y el trabajo de Yoshida y colaboradores (2012a). En su caso, la asociación entre un mayor infiltrado celular y una peor función visual estaría relacionada con un proceso inflamatorio más avanzado. En nuestro estudio, sin embargo, la correlación positiva entre TNFα y la función visual podría ser debida a que el incremento de TNFα sería previo a la muerte de los fotorreceptores (inflamación inicial) y después descendería conforme va progresando la degeneración (Yoshida *et al.*, 2012b; Genini *et al.*, 2013). Por tanto, niveles elevados de TNFα estarían presentes en pacientes en los que todavía no está afectada la función visual de forma severa. Sin embargo, es necesario profundizar en el perfil de los niveles de TNFα y otras citoquinas en las distintas etapas de la RP para determinar el papel del TNFα sobre la evolución de la RP.

Tras observar alteraciones en la respuesta antioxidante y la presencia de estrés oxidativo e inflamación en pacientes con RP, decidimos caracterizar un modelo *ex vivo* de degeneración retiniana que reprodujese algunos de los cambios observados en pacientes y en

modelos murinos con el fin de estudiar los mecanismos moleculares implicados y ensayar posibles tratamientos. El segundo objetivo del presente trabajo fue la caracterización de un modelo experimental de degeneración retiniana *ex vivo* utilizando cultivos organotípicos de retina porcina en los que se inhibía la PDE6 cuyo gen está mutado en el 4-5% de los casos de RP autosómica recesiva (Capítulo IV) (Martinez-Fernandez de la Camara *et al.*, 2013b). Para inhibir la enzima PDE6 se utilizó Zaprinast (Sigma-Aldrich, Madrid, España). El Zaprinast es un inhibidor de las enzimas PDE5, PDE6 y, en menor medida, de la PDE1. Mientras que la PDE6 es exclusiva de fotorreceptores (Zhang *et al.*, 2005), la PDE5 y la PDE1 tienen una distribución más homogénea a lo largo de la retina (Santone *et al.*, 2006; Foresta *et al.*, 2008).

Tras la puesta a punto de los cultivos, se caracterizó el proceso de degeneración de la retina expuesta a Zaprinast durante 24 y 48 horas. En primer lugar, observamos que la inhibición provocaba la acumulación del segundo mensajero GMPc que, como se ha descrito previamente en otros estudios, provoca la muerte de los fotorreceptores (Vallazza-Deschamps *et al.*, 2005; Sharma and Rohrer, 2007). En segundo lugar, comprobamos que este modelo reproducía algunas de las alteraciones observadas en pacientes como menor respuesta antioxidante (disminución de la capacidad antioxidante total), estrés oxidativo (incremento de nitritos y TBARS) e inflamación (elevado contenido de TNFα e IL-6). No obstante, somos conscientes de que este modelo presenta algunos inconvenientes ya que la realización del cultivo implica la sección del nervio óptico y esto puede desencadenar alteraciones en el modelo que no ocurren en casos de RP, como degeneración de las células ganglionares y muerte acelerada de las células de la retina, no solo de los fotorreceptores, sino también de las células bipolares, amacrinas, etc. A pesar de estas limitaciones creemos que el empleo de la neuroretina porcina es un buen modelo por su mayor similitud con la retina humana,

en cuanto a tamaño, densidad y distribución de los fotorreceptores, etc. Además, el cultivo organotípico de retina porcina sería un buen modelo para ensayar fármacos y profundizar en el conocimiento de los posibles mecanismos protectores de estos fármacos como se muestra en el Capítulo V de esta tesis y en otros trabajos (Saikia *et al.*, 2006; Fernandez-Bueno *et al.*, 2013).

En vista de los resultados obtenidos y los trabajos realizados por otros autores, pensamos que el TNFα sería una buena diana terapéutica sobre la que actuar para enlentecer la degeneración retiniana. Por ello, el tercer objetivo de esta tesis fue estudiar el efecto de la inhibición de TNFα con anticuerpos comerciales sobre la progresión de la degeneración retiniana en el modelo *ex vivo* de retina porcina y en un modelo *in vivo* de RP, el ratón *rd10*. En la primera parte, se evaluó el efecto de la inhibición de TNFα con el anticuerpo monoclonal Infliximab (Remicade®) en el modelo experimental de retina porcina. El Infliximab ya se había empleado previamente como tratamiento de diversas patologías oculares como uveítis (Saurenmann *et al.*, 2006), edema macular (Wu *et al.*, 2012), etc. El Infliximab, anticuerpo quimérico humano con los fragmentos variables de origen murino, tiene efecto antiinflamatorio al inhibir el TNFα y el TNFβ y lisar las células productoras de TNFα (Olson *et al.*, 2007).

En el modelo *ex vivo* de retina porcina el tratamiento con Infliximab redujo la muerte de los fotorreceptores y la gliosis reactiva inducida por la inhibición de la PDE6. No obstante, no observamos un efecto muy beneficioso sobre el daño oxidativo en este modelo ya que, aunque normalizaba la capacidad antioxidante total, no disminuía la formación de marcadores de estrés oxidativo como los nitritos o la formación de TBARS en los explantes tratados con Zaprinast (Capítulo V) (Martínez-Fernández de la Camara *et al.*, 2014). Estos resultados nos sugirieron que el TNFα estaba desempeñando un papel en la degeneración retiniana en

este modelo *ex vivo* y su inhibición podría enlentecer dicha degeneración.

En la segunda parte del estudio evaluamos el efecto de la inhibición de TNFα en un modelo *in vivo* de RP, el ratón *rd10*, en este caso con el anticuerpo monoclonal humanizado, Adalimumab (Humira®). El Adalimumab se une al TNFα, ya sea circulante o unido a la superficie celular, impidiendo su interacción con los receptores TNFR1 y TNFR2. Tiene elevada especificidad por la isoforma alfa del TNF, no teniendo la capacidad de unirse a TNFβ (Neri *et al.*, 2010). En los ratones *rd10*, el bloqueo de TNFα disminuyó la progresión de la degeneración a día postnatal 18, edad a la que se producía un pico de muerte en nuestras condiciones de estabulación. El tratamiento con Adalimumab disminuyó la degeneración de los fotorreceptores (menor número de células TUNEL positivas), la activación de PARP (menor contenido de PAR) y la gliosis reactiva de la microglía (menor tasa de migración de la microglía a las capas más externas de la retina) y de las células de Müller (menor expresión de GFAP) en la retina de los ratones *rd10* (Capítulo VI).

El bloqueo del TNFα con Adalimumab mejoró la respuesta antioxidante de forma similar a lo observado en el modelo porcino tratado con Infliximab. En otras patologías se ha descrito que los anti-TNFα son capaces de mejorar el estado redox. Por ejemplo, en pacientes con enfermedades inflamatorias crónicas como la artritis reumatoide o la espondilitis anquilosante (Kageyama *et al.*, 2008a; Kageyama *et al.*, 2008b; Karkucak *et al.*, 2010) o en modelos experimentales de uveítis, colitis, daño isquémico intestinal o miocárdico (Johnsen-Soriano *et al.*, 2010; Akdogan *et al.*, 2014; Cano-Martinez *et al.*, 2014; Yang *et al.*, 2014) el tratamiento con anticuerpos anti-TNFα (Infliximab, Adalimumab o Etanercept) reduce algunos marcadores de estrés oxidativo y aumenta la respuesta antioxidante. Sin embargo, en nuestro modelo *ex vivo* porcino no vemos un efecto del Infliximab en los marcadores de estrés oxidativo

analizados (nitritos y TBARS). Es posible que el Infliximab reduzca otros marcadores de estrés oxidativo no analizados o que tenga algún tipo de efecto sobre la respuesta antioxidante a más largo plazo. En el caso del estudio en los ratones *rd10* todavía no se han evaluado marcadores de estrés oxidativo-nitrosativo pero, esperamos poder realizarlo en breve.

En los modelos experimentales empleados en este trabajo se produce un aumento de GMPc tras la inhibición de la enzima PDE6 con Zaprinast (modelo *ex vivo* de retina porcina) o por una mutación genética presente en dicho gen (ratón *rd10*) que en último lugar provocaría la degeneración de los fotorreceptores. Para éstos y otros modelos de RP en los que se observa una acumulación de GMPc se han propuesto vías alternativas no apoptóticas responsables de la muerte de los fotorreceptores, como se muestra en la Figura 11 del Capítulo I. Los niveles elevados de GMPc activarían canales iónicos de nucleótidos cíclicos (CNG), aumentando el flujo de calcio al interior celular, y también proteína-quinasas G (PKG) que fosforilarían proteínas (Paquet-Durand *et al.*, 2009; Arango-Gonzalez *et al.*, 2014). Posteriormente, las PKG podrían disparar la activación de las histona deacetilasas (HDAC) (Hao *et al.*, 2011) y éstas, a su vez, podrían activar la poli(ADP) ribosa polimerasa (PARP) (Sancho-Pelluz *et al.*, 2010). Por otro lado, el aumento de calcio podría activar calpaínas que mediarían la vía intrínseca de la apoptosis (Sanges *et al.*, 2006; Paquet-Durand *et al.*, 2010; Kaur *et al.*, 2011). Ambas rutas convergerían provocando la muerte de los fotorreceptores.

Hasta hace unos años, la explicación más aceptada para la muerte inicial de los fotorreceptores en los modelos animales en los que se acumulaba GMPc era la excesiva activación de los canales CNG de sodio y calcio, con la consecuente liberación de glutamato y acumulación de grandes cantidades de calcio intracelular (Sharma and Rohrer, 2004, 2007). Sin embargo, en 2009, Paquet-Durand y colaboradores realizaron importantes hallazgos que revelaron que, más que la activación de los

canales CNG, era una excesiva actividad de las PKG la que contribuiría en mayor medida a la muerte de los fotorreceptores (Paquet-Durand *et al.*, 2009; Sahaboglu *et al.*, 2013). En favor de esta idea habían varios hechos: (1) la mayor sensibilidad de las PKG por el GMPc (100 veces más elevada) que los canales CNG; (2) el aumento de calcio inhibe la guanilato ciclasa, reduciendo el contenido de GMPc y eso no es lo que se observaba en los modelos experimentales con la PDE6 no funcional; y (3) el efecto protector de los antagonistas de canales de calcio es controvertido. Por ejemplo, la prevención de la muerte de los fotorreceptores tras la administración de D-*cis*-Diltiazem, un inhibidor de los canales CNG y dependientes de voltaje (VGC), depende del modelo animal y las condiciones experimentales (Pearce-Kelling *et al.*, 2001; Pawlyk *et al.*, 2002; Nakazawa, 2011). En cambio, la administración de nilvadipina, antagonista de canales de calcio, parece retardar la progresión de la pérdida de campo visual en pacientes con RP (Nakazawa *et al.*, 2013). A esta droga se han atribuido efectos antioxidantes que, posiblemente, podrían estar contribuyendo a la mejora de estos pacientes (Nakazawa *et al.*, 2011). En cambio, la inhibición de la actividad de PKG reduce la muerte de los fotorreceptores en el ratón *rd1* (Paquet-Durand *et al.*, 2009).

La muerte inicial de los bastones activaría diversas rutas de señalización, en principio neuroprotectoras, pero que mantenidas de forma crónica producirían efectos deletéreos. Es el caso de la liberación de citoquinas proinflamatorias como TNFα, formación de radicales libres (ROS o RNS), activación de la microglía, etc. que con el tiempo podrían activar vías de muerte celular.

El TNFα es esencial para la inducción y el mantenimiento de la respuesta inflamatoria. El TNFα activa vías de supervivencia, muerte y diferenciación celular a través de su unión a dos tipos diferentes de receptores, TNFR1 y TNFR2. Como se muestra en la Figura 1 del

presente capítulo, la unión de TNFα o miembros de su familia a TNFR1 induce la trimerización del receptor y el reclutamiento de proteínas adaptadoras con el dominio de muerte (DD) TRADD que sirven como plataforma para reclutar, al menos, tres proteínas adaptadoras más: RIP1 (*receptor-interacting protein 1*), FADD (*Fas-associated death domain protein*) y TRAF2 (*TNF-receptor-associated factor 2*) que activan múltiples vías de transducción de señal: (1) cascada de caspasas y subsiguiente apoptosis; (2) cascada de MAPK (*mitogen-activated protein kinases*) como p38, JNK (*Jun N-terminal kinase*) y ERK (*extracelular signal-regulated kinase*) cuyo equilibrio regula la supervivencia o la muerte celular; (3) activación del factor de transcripción nuclear kappa β (NF-kB) implicado en la supervivencia o muerte celular y (4) necroptosis, vía de muerte celular mediada por las proteínas RIP1 y RIP3 (Wajant *et al.*, 2003; Schneider-Brachert *et al.*, 2013; Sosna *et al.*, 2014).

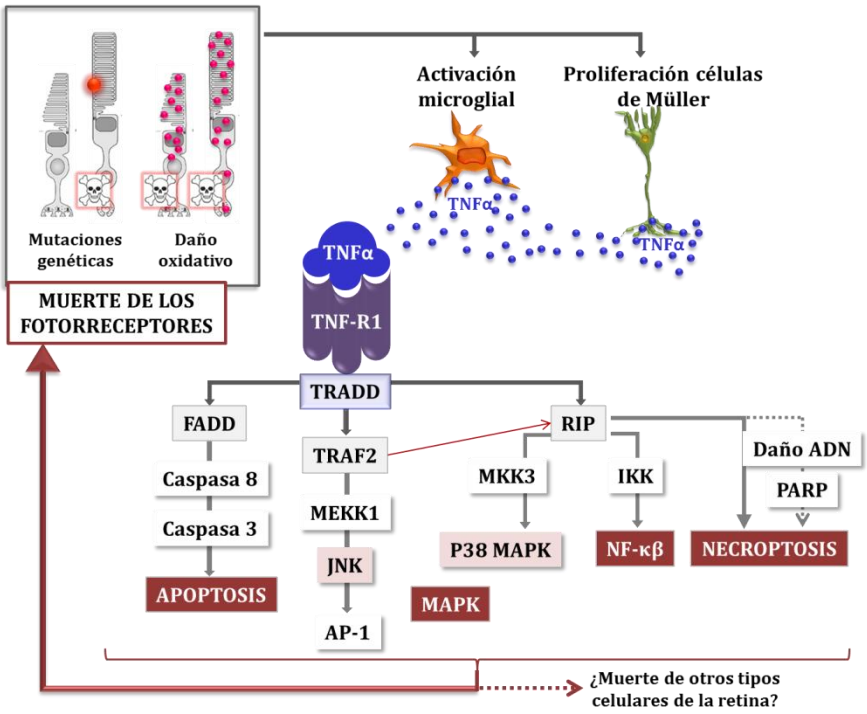

Figura 1. Vías de señalización activadas por la unión del TNFα al receptor TNFR1. Como hemos visto en el Capítulo I, las mutaciones genéticas provocan la muerte de los bastones y, como consecuencia, se genera daño oxidativo e inflamación. Uno de los hitos relevantes del proceso inflamatorio en RP es la activación de la microglía y de las células de Müller que liberan TNFα en respuesta al daño. El TNFα puede activar diversas vías de supervivencia y de muerte celular. Estas últimas prevalecen cuando el daño es severo, provocando la exacerbación de la muerte de los fotorreceptores y, posiblemente, de otras células de la retina. Mientras que la apoptosis y la necroptosis conducen exclusivamente a la muerte celular, la vía mediada por MAPK y NF-κβ pueden ejercer funciones duales, bien regulando la supervivencia celular, o bien regulando la muerte celular.

El reclutamiento de FADD y RIPK (*receptor-interacting protein kinases*) por TRADD activa la cascada de caspasas. Las proteínas FADD y RAIDD (*RIP-associated Ich-1/CED homologous protein with death domain*) participan en la activación de las caspasas iniciadoras 2, 8 y 10 que finalmente activan las caspasas efectoras 3, 6 y 7. Estas caspasas efectoras son las responsables de la degradación proteolítica de muchas dianas celulares que finalmente provoca la muerte celular. El TNFα

también activa la cascada de las MAPK a través de su interacción con TRAF2 que interacciona con MEKK1 activando JNK, y con RIP activando p38 MAPK (Wajant *et al.*, 2003). La activación de JNK activa el factor de transcripción AP-1 y la consiguiente proliferación celular. La activación de JNK puede inhibir la apoptosis interfiriendo con la vía intrínseca de la apoptosis. Por su parte, la activación de p38 MAPK está implicada en el proceso inflamatorio (Metrailler *et al.*, 2013).

El reclutamiento de TRAF2 y RIPK por TRADD activa el factor de transcripción NF-kB que regula la expresión de genes implicados en inflamación, estrés oxidativo y regula un amplio rango de genes que participan en la muerte y supervivencia celular (Yang *et al.*, 2007; Siomek, 2012). RIPK y TRAF2 reclutan el complejo IKK (*inhibitor of* NF-kB *kinase*). NF-κβ está compuesto de homodímeros y heterodímeros, siendo el más estudiado y abundante en las células de mamífero el constituido por las subunidades p50 y p65. La activación de NF-κβ implica la fosforilación del dominio IκB por el complejo IκB quinasa, promoviendo su degradación vía proteosoma. La degradación de IκB libera las subunidades p65 y p50 que se dirigen al núcleo para unirse al ADN y activar la transcripción de múltiples genes como Bcl-2 o inhibidores de la caspasas como c-IAP1, XIAP (Christman *et al.*, 2000; Kern, 2007). NF-κβ es considerado un *master regulator* de las respuestas inmunológicas, de la proliferación celular y de la apoptosis (dell'Omo *et al.*, 2013).

El TNFα también puede activar la necroptosis. La activación de esta vía de muerte celular depende de la actividad de las proteínas RIP1 y RIP3. RIP1 interviene también en la vía de señalización NF-κβ y en la apoptosis, sin embargo, es su actividad serina/treonina quinasa la que la hace esencial en la vía necrótica. Para activar esta vía de muerte debe interaccionar con la proteína RIP3, miembro de la familia RIP1, cuya actividad quinasa es vital para desencadenar el proceso necrótico (Zhang

et al., 2009; Christofferson and Yuan, 2010). RIP3 provoca la activación de las proteínas MLKL y PGAM5 que transmiten la señal necrótica induciendo la fragmentación mitocondrial (Sosna *et al.*, 2014). La necroptosis también puede activarse a través de la vía PARP. La relación entre el TNFα y PARP como mediadores de la necroptosis es controvertida. Hasta hace unos años, el modelo sugerido se basaba en que las funciones de ambas moléculas estaban integradas para llevar a cabo el proceso necrótico. Sin embargo, recientemente Sosna y colaboradores han propuesto un nuevo modelo en el que la vía PARP es activada en respuesta a daños en el ADN y media le necrosis independientemente de la necroptosis inducida por TNFα. RIP1 y RIP3 participarían en ambas vías pero no constituirían una conexión entre ambas (Sosna *et al.*, 2014).

La unión de TNFα a sus receptores puede activar la vía extrínseca e intrínseca de la apoptosis provocando la muerte de los fotorreceptores en diversos modelos animales de RP (Bode and Wolfrum, 2003; Cottet and Schorderet, 2009). El papel de la activación de las MAPK en las degeneraciones retinianas y en concreto en RP no está claro. JNK se activa en el epitelio retiniano, en las células ganglionares y en otros tipos celulares de la capa nuclear interna de la retina en condiciones de isquemia, sección del nervio óptico o glaucoma. Sin embargo, la función de JNK en los fotorreceptores, tanto en condiciones normales como patológicas, no se conoce en detalle. Se ha observado que la fosforilación de miembros clave de la vía de señalización JNK (JNK1, JNK2, c-Jun,…) está reducida en retinas de ratones *knock out* RP1, mientras que la fosforilación de ERK y p38 MAPK permanece invariable. La disminución de la activación de JNK podría desencadenar la muerte de los fotorreceptores a través de la actividad de c-Jun en estos ratones (Liu *et al.*, 2005). Por su parte, la señalización mediada por ERK y p38 MAPK modulan la actividad de las células de Müller: la fosforilación de ERK es

uno de los hitos de la activación de las células de Müller en respuesta a daños en la retina mientras que p38 MAPK parece ser indispensable en la regulación de la expresión de LIF, que ejerce una función neuroprotectora en la retina (Zhao *et al.*, 2010; Cai *et al.*, 2011; Agca *et al.*, 2013).

Las especies reactivas de oxígeno y otros tipos de estrés como la radiación UV, pueden inducir la activación de NF-κβ (Christman *et al.*, 2000; Siomek, 2012). En este sentido, diversos compuestos que se han empleado en modelos de RP por sus propiedades antioxidantes, como la curcumina, el resveratrol, la N-acetilcisteína (NAC) o la vitamina E ejercen funciones neuroprotectoras a través de la inhibición de NF-κβ y la consecuente reducción de la expresión de genes inflamatorios (Vasireddy *et al.*, 2011; Siomek, 2012; Cuenca *et al.*, 2014). La activación de NF-κβ en las células gliales, especialmente en la microglía, tiene un papel normalmente neurotóxico ya que promueve la producción de citoquinas proinflamatorias y sustancias proapoptóticas como TNFα, IL-1β, iNOS y moléculas de adhesión celular (ICAM-1) (Zeng *et al.*, 2008). Algunas proteínas inducidas por NF-κβ, entre ellas el TNFα, parecen ser responsables de la activación persistente de NF-κβ lo que contribuye al mantenimiento de procesos inflamatorios crónicos en la retina de ratones *rd*, aun cuando los fotorreceptores ya han degenerado (Zeng *et al.*, 2008).

El bloqueo de la vía necrótica a través de la inhibición farmacológica y de la inactivación genética de RIPK reduce la muerte de los conos y preserva su función en el ratón *rd10* (Murakami *et al.*, 2012). Por otro lado, se ha observado un incremento de la actividad de PARP, y por consiguiente del contenido de PAR, asociado a la degeneración de los fotorreceptores en dos modelos de rata, P23H y S334ter, y en ratones *rd1* (Paquet-Durand *et al.*, 2007; Kaur *et al.*, 2011) confirmando que la

necroptosis es una vía alternativa a la apoptosis implicada en la degeneración de los fotorreceptores.

Según los resultados mostrados en esta tesis y trabajos previos, el TNFα parece tener un papel importante en la degeneración de los fotorreceptores en RP, al menos en un momento determinado de la degeneración. No obstante, existen otros tipos de alteraciones que podrían estar contribuyendo al avance de la enfermedad como el estrés oxidativo (*hipótesis oxidativa* propuesta en el Capítulo I). En nuestro caso, el bloqueo de TNFα mejora la respuesta antioxidante pero no parece disminuir el daño oxidativo en el modelo experimental de retina porcina y no sabemos su efecto en el ratón *rd10*. Es necesario profundizar en el efecto de los anti-TNFα solos o combinados con agentes antioxidantes sobre el estrés oxidativo con el fin de lograr una terapia más eficaz que retrase la degeneración retiniana.

En vista de los resultados obtenidos en la presente tesis parece que la inhibición de TNFα con anticuerpos monoclonales podría ser una buena estrategia terapéutica en RP. El bloqueo del TNFα estaría inhibiendo los mecanismos de señalización celulares derivados de su unión al receptor TNFR1 que desencadenan, en último término, la muerte celular. Sería necesario profundizar en los mecanismos a través de los cuales los inhibidores de TNFα previenen la muerte celular y reducen los procesos inflamatorios en los modelos empleados en el presente trabajo. Asimismo, ajustar el tratamiento en cuanto a dosis, vía de administración y, posiblemente, combinarlo con otras sustancias con propiedades antioxidantes, como se comenta en el párrafo anterior, podría ser lo más adecuado para conseguir una mayor efectividad.

Como hemos comentado anteriormente, se han empleado anticuerpos anti-TNFα en diversas patologías a través de la administración subcutánea. Esta vía de administración puede tener

efectos adversos que afecten a otros órganos. Para el tratamiento de enfermedades oculares se ha propuesto la administración intravítrea de estos compuestos disminuyendo así, la posible aparición de efectos adversos sistémicos. En este sentido, se ha evaluado la toxicidad de la inyección intravítrea de Adalimumab en conejos, concluyendo que no induce cambios patológicos en la retina ni a nivel morfológico ni funcional (Tsilimbaris *et al.*, 2009; Myers *et al.*, 2014). Estos resultados sugieren que la inyección intravítrea de Adalimumab podría ser segura en ensayos clínicos permitiéndonos evaluar su efecto en enfermedades oculares en las que está implicado el TNFα, como es el caso de la retinosis pigmentaria.

Teniendo en cuenta los resultados obtenidos en la presente tesis, sugerimos que la inflamación y el estrés oxidativo están directamente implicados en la muerte de los fotorreceptores en RP. En los pacientes, hay una respuesta deficiente del sistema de defensa antioxidante endógeno y un alto contenido de mediadores inflamatorios que repercutirían en la capacidad visual. El modelo *ex vivo* de retina porcina sería un modelo útil para ensayar diversos compuestos y estudiar los mecanismos moleculares a través de los cuales estos compuestos prevendrían la muerte de los fotorreceptores, ya que reproduce algunas de las alteraciones encontradas en los pacientes. El ratón *rd10,* ampliamente utilizado como modelo de RP, es útil para ensayar compuestos como el Adalimumab y evaluar *in vivo* su efecto sobre la degeneración retiniana.

Capítulo VIII. Conclusiones

CONCLUSIONES

1. La retinosis pigmentaria altera la maquinaria antioxidante endógena en humanos. Los pacientes con retinosis pigmentaria tienen una menor respuesta antioxidante que se demuestra con una reducción en la capacidad antioxidante total y de la actividad superóxido dismutasa extracelular en humor acuoso. En estos pacientes, además, hay un incremento de marcadores de estrés oxidativo y nitrosativo en sangre.

2. La deficiente respuesta antioxidante ocular afecta de forma negativa a la función visual de los pacientes con retinosis pigmentaria.

3. La inflamación está implicada en retinosis pigmentaria en humanos. Los pacientes con retinosis pigmentaria presentan niveles elevados de las citoquinas TNFα e IL-6 en humor acuoso, confirmando la presencia de inflamación descrita por otros autores. Los niveles de IL-10 e IL-1β no se han podido detectar en humor acuoso.

4. Niveles elevados de TNFα se correlacionan con una mejor función visual sugiriendo que esta citoquina estaría implicada, posiblemente, en las etapas iniciales de la enfermedad.

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5. La inhibición de la PDE6 con Zaprinast en cultivos organotípicos de retina porcina induce la acumulación de GMP cíclico y desencadena la degeneración de la retina que va acompañada de estrés oxidativo e inflamación. Este modelo reproduce algunas de las alteraciones

observadas en otros modelos animales y en pacientes con retinosis pigmentaria.

6. El cultivo organotípico de retina porcina expuesto a Zaprinast podría ser un modelo *ex vivo* útil para diseñar y ensayar estrategias terapéuticas que evitasen o retrasasen la muerte de los fotorreceptores así como para profundizar en los mecanismos responsables de esta muerte.

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7. El tratamiento con Infliximab, anticuerpo monoclonal anti-TNFα, previene la degeneración retiniana inducida por la inhibición de la PDE6 en el modelo *ex vivo* de retina porcina. El bloqueo de TNFα reduce la muerte celular, la gliosis reactiva y mejora la respuesta antioxidante en este modelo.

8. El tratamiento con Adalimumab, anticuerpo monoclonal humanizado anti-TNFα, preserva la estructura de la retina en ratones *rd10*. La administración de Adalimumab reduce la muerte celular, la gliosis reactiva y mejora la respuesta antioxidante en la retina de ratones *rd10*.

9. El TNFα es una molécula clave implicada en la muerte de los fotorreceptores en el modelo *ex vivo* de retina porcina y el ratón *rd10* empleados en este estudio. Por tanto, estrategias dirigidas a bloquear sus vías de señalización podrían ser apropiadas para enlentecer el avance de la retinosis pigmentaria.

Capítulo IX. Bibliografía general

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