RESUMEN

La retina es el tejido neural que tapiza la parte posterior del ojo. En los vertebrados está constituida por cinco clases de neuronas: los fotorreceptores (conos y bastones), las amacrinas, las horizontales, las ganglionares, y las bipolares.

Debido a su relativa simplicidad, accesibilidad y a que los distintos tipos celulares están organizados formando un tejido altamente estructurado, la retina ha sido ampliamente utilizada como tejido modelo de estudio del sistema nervioso central, y es la estructura neural más estudiada.

Las neuronas de retina debido a su constante exposición a la luz, su alta tasa metabólica y el alto contenido de ácidos grasos en sus membranas son muy sensibles a sufrir daño oxidativo. Este daño es el responsable de disparar procesos de apoptosis en este tejido. Muchas enfermedades neurodegenerativas de la retina involucran la muerte por apoptosis de las neuronales retinales, y tienen en común que el daño oxidativo es un desencadenante. Este estrés activa vías que involucran a las mitocondrias y conduce a la apoptosis. El conocimiento de los mecanismos por los cuales induce dicha activación es fundamental en el desarrollo de futuras estrategias terapéuticas.

Trabajos previos de nuestro laboratorio demuestran que la carencia de factores tróficos durante el desarrollo neuronal *in vitro* y el estrés oxidativo inducido por PQ inducen la muerte por apoptosis de las neuronas fotorreceptoras.

El ácido docosahexaenoico (DHA, 22:6 n-3) es un ácido graso poliinsaturado esencial de la familia de los omega 3, sintetizado a partir del ácido α-linolénico (18:3 n-3) y se encuentra altamente concentrado en las células del sistema nervioso, principalmente en las membranas sinápticas y en los fotorreceptores. El DHA en el sistema nervioso desarrolla múltiples funciones, juega un papel importante en el proceso de fototransducción, en la formación de la memoria, es neuroprotector y puede regular procesos antiinflamatorios. Tradicionalmente al DHA en la retina se lo relaciona con un importante rol estructural, el de favorecer los cambios de conformación inducidos por la luz en la rodopsina, y es indispensable para el adecuado desarrollo de la visión. En nuestro laboratorio, se ha establecido un rol completamente nuevo para el DHA, el de ser un factor de supervivencia previniendo la apoptosis de los fotorreceptores por ausencia de factores tróficos durante su desarrollo *in vitro* y previniendo la apoptosis inducida por el oxidante Paraquat (PQ). Además hemos establecido que promueve la diferenciación de los fotorreceptores de retina de rata en cultivo.

Hemos demostrado que el PQ dispara la apoptosis de las neuronas de retina en cultivo; el aumento de la apoptosis se encuentra en estrecha relación con la pérdida de integridad mitocondrial en las neuronas y se observó que los fotorreceptores son más sensibles que las neuronas amacrinas a la apoptosis inducida por PQ.

El H_2O_2 es un agente oxidante que, a diferencia del PQ, es en sí mismo una especie oxígeno reactiva (ROS) y un mediador fisiológico del daño oxidativo en retina. En nuestro laboratorio también se demostró que el H_2O_2 induce un aumento de la muerte celular por apoptosis de los fotorreceptores. Es por eso que decidimos investigar en este trabajo de tesis si el DHA también puede prevenir la apoptosis disparada por H_2O_2, que induce la muerte de las células de retina luego de agotar los sistemas de defensa antioxidante.

Para ello suplementamos cultivos neuronales de retina con o sin DHA e indujimos daño oxidativo con H_2O_2. Evaluamos viabilidad celular con ioduro de propidio y observamos que, al tratar a los cultivos con H_2O_2, en ausencia de DHA, disminuye la viabilidad celular respecto a los controles. Si previo al tratamiento con H_2O_2 los cultivos fueron suplementados con DHA el porcentaje de células muertas descendió a niveles comparables con los de los controles. Para determinar el efecto del DHA frente a la apoptosis inducida por H_2O_2 realizamos el ensayo de TUNEL y vimos un aumento de los fotorreceptores TUNEL+ en los cultivos tratados con H_2O_2, y una disminución de los mismos en los cultivos preincubados con DHA. Cuantificamos el porcentaje de fotorreceptores con núcleos picnóticos o fragmentados, teñidos con DAPI, para evaluar apoptosis y observamos que el H_2O_2 provocó un aumento de la apoptosis de los fotorreceptores respecto a los cultivos controles y que el DHA agregado previo al tratamiento con H_2O_2 previno la apoptosis de los fotorreceptores.

Estos resultados, junto con el hecho de que el DHA protege a los fotorreceptores del daño oxidativo inducido por PQ, nos permiten concluir que el DHA es un eficaz protector de las neuronas fotorreceptoras de retina expuestas a distintos tipos de agentes oxidantes.

Resultados previos de nuestro laboratorio establecieron que el DHA en cultivos neuronales de retina se incorpora a la membrana de las células, aumenta su concentración en las mismas y tiene la capacidad de activar la vía de la ERK/MAPK y regular la relación de proteínas pro y antiapoptóticas para promover la supervivencia de los fotorreceptores frente al daño oxidativo (Ger)man et al., 2006a;Rotstein et al., 2003. En este trabajo de tesis nos propusimos investigar si el DHA, además de activar las vías de supervivencia citadas, también podría estar ejerciendo su efecto protector actuando como antioxidante. Para determinar si el DHA estaría actuando como un agente antioxidante utilizamos la sonda DCFDA para medir la formación de (ROS en los cultivos y así tener una medición aproximada del daño oxidativo en los cultivos (Halliwell and Whiteman,

2004;Lu et al., 2006). Determinamos el porcentaje de fluorescencia de la sonda DCFDA oxidada respecto al control. Observamos que el porcentaje de fluorescencia aumentó al tratar los cultivos con H_2O_2, indicando un aumento en la formación de ROS. Observamos también que el DHA disminuyó la formación de ROS disparada por H_2O_2 en los cultivos neuronales de retina de rata.

Para investigar si el DHA estimula enzimas del sistema de defensa antioxidante, determinamos la actividad de la GPx, que participa de la reducción de hidroperóxidos al catalizar la oxidación del Glutatión. Vimos que en cultivos suplementados con DHA previo a inducir daño oxidativo, la actividad de la enzima fue mayor que en los cultivos controles, por lo que el DHA estaría estimulando la actividad de esta enzima.

Decidimos medir los niveles de sustancias reactivas con el ácido barbitúrico (TBARS) como indicador de peroxidación lipídica en los cultivos neuronales de retina tratados con H_2O_2. Observamos que el H_2O_2 aumentó los niveles de TBARS y por lo tanto la peroxidación lipídica en los cultivos neuronales de retina. Además al suplementar con DHA, independientemente de si se indujo o no estrés oxidativo con H_2O_2, aumentaron los niveles de TBARS respecto a cultivos sin DHA.

De estos resultados se desprende que el DHA, a pesar de aumentar la peroxidación lipídica en los cultivos neuronales, promueve la supervivencia de los fotorreceptores al activar enzimas de defensa antioxidante, como la GPx, y disminuir la formación de ROS.

La síntesis de DHA a partir de EPA (Acido eicosapentaenoico, 20:5 n-3) requiere de dos elongaciones que forman TPA (ácido tetracosapentaenoico, 24:5 n-3), que es posteriormente desaturado por la Δ6-desaturasa produciendo THA (ácido tetracosahexaenoico, 24:6 n-3). Finalmente este, por acción de una β-oxidasa peroxisomal, da origen al DHA. Este proceso biosintético requiere de la incorporación de los precursores desde la dieta y es principalmente llevado a cabo en el hígado. El DHA así sintetizado es luego distribuido a distintos tejidos por el sistema circulatorio. Si bien la mayor cantidad de DHA del sistema nervioso proviene de la síntesis hepática y su transporte unido a lipoproteínas de la sangre también ocurre la síntesis *in situ*, aunque en menor medida. Las enzimas necesarias para la síntesis de DHA están presentes en el ojo, siendo el epitelio pigmentario el sitio donde es más efectivo este proceso de síntesis. Está comprobado que este camino biosintético puede llevarse a cabo en células gliales, pero no se ha demostrado la síntesis del DHA en neuronas.

En este camino metabólico el ácido eicosapentaenoico (EPA, 20:5n-3) es un intermediario, y al igual que el DHA, el EPA ha demostrado tener efectos importantes en el cerebro y retina. En retinas de rata alimentadas con dietas ricas en ácidos grasos de la serie n-3 aumenta la relación EPA/AA (EPA/Acido Araquidónico) en los segmentos externos de los fotorreceptores y disminuyen

los daños a nivel de dichos segmentos externos cuando se somete a estas ratas a un daño agudo inducido por luz. Por otro lado el aumento de los niveles de EPA plasmático en pacientes humanos sanos se relaciona directamente con el aumento de la densidad del pigmento macular que regula procesos inflamatorios y previene el daño oxidativo previniendo el avance de la degeneración macular. De todos modos, aun se desconocen los mecanismos celulares y moleculares por medio de los cuales el EPA actúa como un agente neuroprotector.

Como el EPA ha demostrado tener efectos muy similares a los que se observan con el DHA en diferentes modelos animales y celulares, en esta tesis investigamos si al igual que ocurre con el DHA, la suplementación de cultivos neuronales de retina con EPA es capaz de prevenir la apoptosis inducida por daño oxidativo en los fotorreceptores y promover su diferenciación. También analizamos si el EPA en neuronas de retina aisladas puede actuar como precursor metabólico del DHA, promoviendo su síntesis y si esta síntesis es indispensable para que el EPA actúe como agente neuroprotector y promotor de la diferenciación de los fotorreceptores.

Para evaluar si el EPA es capaz de proteger a los FR de la apoptosis inducida por PQ, suplementamos los cultivos con EPA en concentraciones crecientes (2, 3, y 6 µM) y los tratamos con PQ. Evaluamos luego viabilidad celular, con IP y apoptosis por el estudio de la morfología nuclear con DAPI. Tanto en los cultivos controles, como en los suplementados con las distintas concentraciones de EPA se observaron porcentajes bajos de muerte celular y de fotorreceptores apoptóticos. La suplementación con EPA, previa al tratamiento con PQ previno el aumento en la mortalidad celular y en la apoptosis inducida por PQ de los fotorreceptores en una forma dosis dependiente. Determinamos que la concentración 3 µM de EPA era la más efectiva. Confirmamos el efecto protector del EPA 3 µM frente a la apoptosis inducida por PQ en los fotorreceptores utilizando el ensayo de TUNEL. El PQ indujo un aumento de los FR TUNEL + y la adición de EPA previa al tratamiento con PQ disminuyó el número de fotorreceptores TUNEL + a niveles comparables con los de los cultivos controles. Por lo tanto, el EPA, al igual que el DHA actúa como un agente protector frente a la muerte inducida por PQ en los fotorreceptores en cultivos.

Esto nos llevó a plantearnos si otros ácidos grasos también podrían actuar de esta manera. Para contestarlo decidimos investigar si los ácidos araquidónico (20:4 n-6, AA), palmítico (16:0, PA) y oleico (18:1 n-9, OA) presentaban efectos antiapoptóticos frente al daño oxidativo inducido con PQ. A diferencia del EPA, ninguno de estos ácidos al ser agregados a los cultivos, evitó los procesos de muerte celular y apoptosis de fotorreceptores desencadenados por el tratamiento con PQ. Podemos afirmar entonces que sólo el EPA y el DHA disminuyen la apoptosis de los fotorreceptores inducida por PQ.

Quisimos evaluar si el EPA también podría tener efecto protector frente a otro oxidante como el H_2O_2. El tratamiento con H_2O_2 provocó un marcado aumento de la apoptosis, aumentando significativamente el número de fotorreceptores con núcleos picnóticos y mitocondrias despolarizadas. La suplementación de los cultivos con EPA previo al tratamiento con H_2O_2 previno el aumento del número de fotorreceptores con núcleos picnóticos o fragmentados y mantuvo intacto el potencial de membrana mitocondrial en la mayoría de los fotorreceptores, al igual que en los controles. Estos resultados demuestran el rol protector del EPA frente a la apoptosis inducida por distintos tipo de daño oxidativo inducido a los fotorreceptores en cultivo.

Resultados anteriores de nuestro laboratorio establecieron que *in vitro*, debido a la carencia de factores tróficos, la diferenciación de los fotorreceptores está restringida y en nuestros cultivos, los fotorreceptores, presentan morfología característica de fotorreceptores no diferenciados. El DHA estimula la diferenciación de los fotorreceptores. En este trabajo quisimos ver si el EPA jugaba algún rol en la diferenciación de los fotorreceptores en cultivo. Establecimos que la adición de EPA provocó un aumento de la expresión de opsina en los fotorreceptores y promovió el crecimiento de procesos apicales, rudimentos de los segmentos externos. Por lo que el EPA estimula la diferenciación de los fotorreceptores durante el desarrollo en cultivo.

También nos propusimos evaluar si el EPA es capaz de modificar la composición de ácidos grasos de los fosfolípidos de la retina neural. El análisis por GLC de la composición de ácidos grasos de los cultivos neuronales reveló valores de EPA muy bajos en los cultivos controles. Sorprendentemente, no existió una acumulación significativa de este ácido graso en los fosfolípidos luego de la incubación con EPA pero que sí se duplicaron los niveles de DHA. Esto nos llevó a suponer que los fotorreceptores y/o neuronas amacrinas en nuestros cultivos eran capaces de elongar y desaturar al EPA para producir DHA. Para que este proceso metabólico pueda llevarse a cabo es clave la presencia de la enzima Δ6 desaturasa, responsable de la transformación del TPA al THA. Investigamos por técnicas inmunocitoquimicas y western blot si esta enzima se expresa en los cultivos neuronales de retina. El western blot reveló la expresión de la enzima en los cultivos neuronales de retina. La citoquímica mostró que la enzima estaba presente tanto en el citoplasma como en los núcleos de los fotorreceptores y las células amacrinas. Estos resultados demuestran, por primera vez, que las neuronas de retina son capaces de sintetizar DHA a partir de EPA como precursor.

El hecho de que el EPA en nuestros cultivos esté promoviendo la síntesis de DHA sumado a que los efectos que presenta son los mismos que se vieron con anterioridad por parte del DHA, nos llevó a plantearnos la hipótesis de que este ácido graso no estuviera actuando por sí mismo, sino como consecuencia de su metabolización a DHA. Para respondernos este interrogante, preincubamos a los cultivos con CP24879 (CP), un inhibidor de las Δ5 y Δ6 desaturasas, antes de

suplementarlos con EPA. Como era de esperar, la presencia del inhibidor previno el aumento de los niveles de DHA y condujo a un aumento del contenido de DPA en los cultivos suplementados con EPA.

Evaluamos el efecto del agregado de CP sobre la protección del EPA frente al tratamiento con H_2O_2. El agregado de CP bloqueó por completo el efecto protector del EPA frente a la apoptosis inducida por H_2O_2, y el porcentaje de fotorreceptores apoptóticos fue el mismo que el que observamos en los cultivos tratados con H_2O_2 sin EPA.

También analizamos si la síntesis de DHA era necesaria para promover la diferenciación de los fotorreceptores. Para ello, investigamos si la incubación con CP afectaba al aumento en la expresión de opsina que provoca el EPA en los cultivos neuronales. El EPA provocó un aumento en la cantidad de fotorreceptores que expresaron opsina, pero la incubación previa con CP bloqueó este efecto y la cantidad de fotorreceptores opsina + en estos cultivos fue comparable con la que observamos en los cultivos controles.

Por lo tanto estos resultados indican que la adición de EPA a los cultivos neuronales de retina previene la apoptosis de los fotorreceptores expuestos a condiciones de estrés oxidativo y estimula el avance de la diferenciación de las neuronas fotorreceptoras. También demuestran que al inhibir la actividad de la ∆6 desaturasa, enzima que cataliza la reacción requerida para la síntesis de DHA a partir de EPA, se bloquean los efectos del EPA sobre los fotorreceptores. Esto implica que no es el EPA en sí mismo el que previene la apoptosis y favorece la diferenciación de los FR en cultivo, sino que es el DHA sintetizado a partir del EPA

En resumen, las conclusiones más destacadas de este trabajo de tesis son las siguientes:

- ✓ El DHA previene la muerte por apoptosis inducida por H_2O_2 de los fotorreceptores en cultivo.

- ✓ El DHA frena la formación de ROS inducida por H_2O_2

- ✓ El agregado de DHA aumenta los niveles de peroxidación lipídica en los cultivos neuronales de retina.

- ✓ El DHA activa enzimas que participan de los mecanismos de defensas antioxidantes para prevenir la formación de ROS que estimula el H_2O_2.

- ✓ El agregado de EPA, y no de otros ácidos grasos, previene la apoptosis inducida por PQ y H_2O_2, efecto que son comparables al observados al suplementar los cultivos con DHA.

- ✓ El agregado de EPA promueve la diferenciación de los fotorreceptores durante el desarrollo *in vitro*, aumentando los niveles de expresión de opsina y la formación de procesos apicales.

- ✓ Los fotorreceptores de rata en cultivo expresan Δ6 desaturasa, esencial para la síntesis de DHA.

- ✓ Las neuronas de retina de rata en cultivo son capaces de sintetizar DHA a partir de EPA.

- ✓ El EPA debe ser metabolizado a DHA para proteger a los fotorreceptores de la apoptosis inducida por estrés oxidativo y para promover la diferenciación de los mismos.

SUMMARY

The neural retina is the tissue in the back of the eye. In vertebrates it consists of five types of neurons: photoreceptor (rods and cones), amacrine, horizontal, ganglion, and bipolar neurons.

The retina is the most studied neural structure because its different cell types are arranged to form a highly structured tissue, and due to its relative simplicity, and its accessibility, and has been widely used as a model for studying the central nervous system.

Due to their constant exposure to light, high metabolic rate and high content of fatty acids in their membranes retinal neurons are very sensitive to oxidative damage. This damage is responsible for triggering apoptosis in this tissue. Many neurodegenerative retinal diseases have in common the apoptotic death of retinal neuronal and that oxidative damage acts as a trigger of this death. This stress-activated pathway involving mitochondria leads to apoptosis. Knowledge of the mechanisms by which this activation is induced is critical in the development of future therapeutical strategies.

Previous work from our laboratory has shown that the absence of trophic factors during neuronal development *in vitro* and induction of oxidative stress by paraquat (PQ) treatment leads to the apoptotic death of photoreceptor neurons.

Docosahexaenoic acid (DHA, 22:6-3) is a polyunsaturated fatty acid of the omega-3 family, synthesized from linolenic acid (18:3 n-3) and is highly concentrated in the cells of the nervous system, especially in synaptic membranes and in photoreceptors. DHA plays multiple functions in the nervous system; it has an important role in the phototransduction process, in the formation of memory, it is neuroprotective and it can regulate inflammatory processes. Traditionally, DHA in the retina has been related to an important structural role, favoring the conformational changes induced by light in rhodopsin, and it is known to be essential for proper development of vision. In our lab, we have established a completely new role for DHA, that of being a survival factor preventing apoptosis of photoreceptors in the absence of trophic factors during *in vitro* development. We have also established that it promotes differentiation of rat retinal photoreceptors.

We have also shown that the oxidant paraquat (PQ) triggers apoptosis of retina neurons in culture, the increase in apoptosis being closely related to the loss of mitochondrial integrity. We demonstrated that photoreceptors are more sensitive than amacrine neurons to PQ-induced apoptosis.

H_2O_2 is an oxidizing agent that, unlike PQ, is in itself a ROS (Reactive oxygen species) and a physiological mediator of oxidative damage in the retina. In our laboratory we demonstrated that H_2O_2 induces apoptosis of photoreceptors. In this Thesis we investigated if DHA can also prevent H_2O_2-induced apoptosis after exhausting the retinal antioxidant defense systems.

To do this we supplemented retina neuronal cultures with or without DHA and we induced oxidative stress with H_2O_2. We assessed cell viability with propidium iodide and observed that treatment of cultures with H_2O_2 in the absence of DHA decreased cell viability relative to controls. If cultures were supplemented with DHA previous to H_2O_2 treatment, the percentage of dead cells decreased to levels comparable to those found in controls. To determine the efficacy of DHA to prevent apoptosis induced by H_2O_2 we evaluated apoptosis with the TUNEL assay and established that H_2O_2 treatment increased TUNEL + photoreceptors, while they were decreased in cultures preincubated with DHA. To evaluate apoptosis we quantified the percentage of photoreceptors showing fragmented or pyknotic nuclei by staining nuclei with DAPI and observed that H_2O_2 treatment increased apoptosis of photoreceptor compared to controls and pretreatment with DHA prevented H_2O_2-induced apoptosis of photoreceptors.

These results, together with the fact that DHA protects photoreceptors from oxidative damage induced by PQ, allow us to conclude that DHA is an effective protector of retinal neurons exposed to various types of oxidizing agents.

Previous results from our laboratory have established that DHA added to retinal neuronal cultures is incorporated into cell membranes, increasing its levels in neuronal lipids, activating the ERK / MAPK pathway and regulating the ratio of pro to anti-apoptotic proteins, thus promoting photoreceptor survival upon oxidative damage. In this Thesis we investigated whether DHA could also be exerting its protective effect by acting as an antioxidant. To evaluate this we used the DCFDA probe to measure the formation of ROS in culture and get a rough measure of oxidative damage. We determined the percentage of the DCFDA fluorescence probe oxidized over control and established that the percentage of fluorescence increased by treating the cultures with H_2O_2, indicating an increase in ROS formation. We also showed that DHA decreased ROS formation triggered by H_2O_2 in neuronal cultures of rat retina.

To investigate whether DHA stimulates enzymes from the antioxidant defense system, we determined the activity of glutathione peroxidase (GPx), which participates in the reduction of hydroperoxides by catalyzing the oxidation of GSH. We established that in cultures supplemented with DHA previous to the induction of oxidative damage the enzimatic activity was higher than in control cultures, suggesting DHA stimulates the activity of this enzyme .

We measured the levels of TBARS as an indicator of lipid peroxidation in neuronal cultures treated with H_2O_2. We observed that H_2O_2 increased TBARS levels and therefore lipid peroxidation in retinal neuronal cultures. DHA supplementation caused an increase in TBARS levels compared to cultures without DHA, both in the presence or the absence of oxidative damage.

From these results it is apparent that DHA, in spite of the increase it triggers in lipid peroxidation in neuronal cultures, promotes photoreceptor survival by activating antioxidant defense enzymes such as GPx, and reducing the formation of ROS.

DHA synthesis from EPA (eicosapentaenoic acid, 20::5 n-3) requires two elongations that form TPA (tetracosapentaenoic acid, 24:5 n-3), which is subsequently desaturated by the Δ6-desaturase producing THA (24:6 n-3, tetracosahexaenoic acid). Finally THA is decarboxylated to DHA by a peroxisomal β-oxidase. This biosynthetic pathway requires the provision of precursors in the diet and is mainly carried out in the liver. DHA is then distributed to various tissues through the circulatory system. While most of the DHA in the nervous system comes from the hepatic synthesis and lipoprotein transport of blood, *in situ* synthesis also occurs though to a lesser extent. The enzymes necessary for the synthesis of DHA are present in the eye and its synthesis is more effective in retinal pigment epithelium than in the retina. It has been shown that this biosynthetic pathway can be carried out in glial cells, but it is still unknown whether this synthesis occurs in neurons.

In this metabolic pathway EPA is an intermediate, and like DHA, it has been shown to have significant effects on the brain and retina. In rats fed diets rich in fatty acids of the n- 3 series, the EPA/AA (EPA /arachidonic acid) ratio increases in the outer segments of photoreceptors and the damage to these outer segments is reduced when rats are subjected to acute light-induced damage. Moreover, increased levels of EPA in plasma of healthy human patients are directly related to increased macular pigment density, which regulates inflammatory response, and prevents oxidative damage and the progression of macular degeneration. However, the molecular and cellular mechanisms by which EPA acts as a neuroprotective agent are still unknown.

As EPA has demonstrated effects very similar to those observed with DHA in different animal and cellular models, here we investigated whether EPA was able to prevent apoptosis of photoreceptors induced by oxidative damage and promote their differentiation. We also investigated if EPA acted as a metabolic precursor of DHA in retina neurons, promoting its synthesis and if this synthesis was essential for EPA effects in photoreceptors.

To assess whether EPA is able to protect photoreceptors from PQ-induced apoptosis, we supplemented cultures with increasing concentrations of EPA (2, 3, and 6 μM) and treated them

with PQ. Then we evaluated cell viability with PI, and studied apoptosis by DAPI staining. Low percentages of cell death and apoptotic photoreceptors were observed both in control cultures and in those supplemented with different concentrations of EPA. Supplementation with EPA prevented the increased in cell death and apoptosis induced by PQ in a dose dependent manner. We determined that the 3 uM EPA concentration is the most effective. We confirmed the protective effect of 3 µM EPA from apoptosis induced by PQ in photoreceptors by TUNEL assay. PQ induced an increase in TUNEL + photoreceptors and EPA addition prior to treatment with PQ decreased the number of TUNEL + photoreceptors to levels comparable with control cultures. Therefore, ~~the~~ EPA, like DHA acts as a protective agent against PQ-induced death in photoreceptors in culture. This led us to ask whether other fatty acids might also act in this way.

To answer this we decided to investigate whether arachidonic ~~acid~~ (20:4 n-6, AA), palmitic (16:0 PA) and oleic (18:1 n- 9) acids had antiapoptotic effects against oxidative damage induced by PQ. Unlike EPA or DHA, none of these fatty acids when added to the cultures prevented cell death and photoreceptor apoptosis triggered by PQ. We can propose that only EPA and DHA decreased photoreceptor apoptosis induced by PQ.

We wanted to assess whether EPA was also protective against other oxidants such as H_2O_2.Treatment with H_2O_2 caused a marked increase in apoptosis, significantly increasing the number of photoreceptors with pyknotic nuclei and depolarized mitochondria. Supplementation of the cultures with EPA prior to treatment with H_2O_2 prevented the increase in the amount of photoreceptors with fragmented or pyknotic nuclei and retained mitochondrial membrane potential in most photoreceptors, with values similar to control cultures. These results demonstrate the protective role of EPA from apoptosis induced by different types of induced oxidative damage to photoreceptors in culture.

Previous results from our laboratory have established that *in vitro*, due to lack of trophic factors, differentiation of photoreceptors is restricted and in our cultures, photoreceptors exhibit an undifferentiated morphology. DHA stimulates photoreceptors differentiation. In this study we investigated whether EPA played a role in the differentiation of photoreceptors in culture. We established that EPA addition promoted an increase in opsin expression in photoreceptors and promoted the development of apical processes. EPA therefore stimulates the differentiation of photoreceptors during development in culture.

We also set out to assess whether EPA was able to modify the fatty acid composition of phospholipids of cultured neurons. GLC analysis of fatty acid composition of the cultured neurons revealed very low levels of EPA in control cultures. Surprisingly, there was no significant accumulation of this fatty acid in phospholipids after incubation with EPA but DHA levels were

doubled. This led us to presume that photoreceptors or amacrine neurons in our cultures were able to elongate and desaturate EPA to produce DHA. Δ6-desaturase enzyme is a key enzyme in this process. It is responsible for the transformation of TPA to THA. We investigated by Western blot and immunocytochemical techniques if this enzyme was expressed in the retinal neuronal cultures. Western blot analysis revealed the expression of the enzyme in retinal neurons. Cytochemistry showed that the enzyme was present in both the cytoplasm and nuclei of photoreceptors and amacrine neurons. These results demonstrate, for the first time, that retinal neurons are able to synthesize DHA using EPA as a precursor.

The fact that EPA addition leads to DHA synthesis together with the evidence that its effects were the same as those shown for DHA, led us to consider the hypothesis that this fatty acid was not acting by itself, but rather due to its metabolization to DHA. To answer this question, cultures were preincubated with CP24879 (CP), a Δ5 and Δ6-desaturase inhibitor before supplementation with EPA. As expected, the presence of the inhibitor prevented the increase in DHA levels in spite of EPA addition and showed a tendency to increase the levels of DPA in cultures supplemented with EPA.

We evaluated the effect of the addition of CP on EPA neuroprotection. The addition of CP completely blocked the protective effect of EPA against H_2O_2-induced apoptosis, and the percentage of apoptotic photoreceptors was the same as that observed in cultures treated with H_2O_2 without EPA.

We also analyzed whether DHA synthesis is required to promote photoreceptors differentiation. For this, we investigated whether incubation with CP affected EPA-induced increase in opsin expression in neuronal cultures. EPA caused an increase in the amount of opsin expression, but preincubation with CP blocked this effect and the amount of opsin + photoreceptors in these cultures was comparable to that observed in controls.

Therefore these results indicated that EPA addition to retina neuronal cultures prevented apoptosis of photoreceptors exposed to conditions of oxidative stress and stimulated the differentiation of photoreceptors. EPA effects on photoreceptors were blocked when ∆6-desaturase was inhibited. This implies that it is not EPA by itself that prevents apoptosis and promotes differentiation of cultured photoreceptor but it is DHA synthesized from EPA the fatty acid responsible for these effects.

In summary, the main conclusions of this thesis are:

- ✓ DHA prevents H_2O_2- induced apoptosis of photoreceptors

- ✓ DHA decreases the formation of ROS induced by H_2O_2

- ✓ DHA increases the levels of lipid peroxidation in neural retina cultures

- ✓ DHA activates enzymes involved in the antioxidant defense mechanisms to prevent ROS formation stimulating H_2O_2

- ✓ EPA, like DHA, prevents oxidative stress-induced apoptosis

- ✓ EPA and not other fatty acids, prevents PQ-induced apoptosis

- ✓ EPA promotes differentiation of photoreceptors during development in vitro, increasing opsin expression and the formation of apical processes

- ✓ Cultured rat photoreceptors express Δ6-desaturase

- ✓ Rat retinal neurons in culture are able to synthesize DHA from EPA

- ✓ EPA must be metabolized to DHA to protect photoreceptors from apoptosis induced by oxidative stress and for promoting their differentiation.

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