BIOCHEMICAL CHARACTERIZATION OF THE OPTIC NERVE IN MICE OVEREXPRESSING THE P53 GEN. OXIDATIVE STRESS ASSAYS

CARACTERIZACIÓN BIOQUÍMICA DEL NERVIO ÓPTICO EN EL RATÓN QUE SOBREEXPRESA EL GEN P53. ANÁLISIS DE ESTRÉS OXIDATIVO

GALLEGO-PINAZO R[1], ZANÓN-MORENO V[2], SANZ S[3], ANDRÉS V[3], SERRANO M[4], GARCÍA-CAO I[4], PINAZO-DURÁN MD[5]

ABSTRACT

Purpose: The tumour inhibitor p53 gene has the ability of triggering proliferation arrest and cellular death by apoptosis subsequent to several factors, among them oxidative stress. The p53 protein is a major regulator of gene expression. Using genetically manipulated mice carrying an extra copy of gene p53 (transgenic mice super p53) versus control mice, we have investigated the generation of reactive oxygen species and antioxidant activity in the optic nerve of mice in relation to p53 availability.

Methods: We studied two groups of 12-month-old mice of the strain C57BL/6: 1) super p53 group (Sp53) and 2) wild-type control group (CG). Mice were anesthetized in ether atmosphere and the eyeball and retrobulbar optic nerves were excised, washed, soaked in PBS, and stored in liquid nitrogen at –85ºC until processing. Three-four optic nerves from the same group were placed in an eppendorf tube, homogenized and enzymatic-colorimetric methods used to determine oxidative and antioxidant activities and the nitric oxide synthesis.

RESUMEN

Objetivos: El gen supresor tumoral p53 detiene la proliferación y la muerte celular por apoptosis subsecuente a la acción de diversos factores, entre ellos el estrés oxidativo. La proteína p53 es fundamentalmente un regulador de la expresión génica. Utilizando ratones genéticamente manipulados para presentar una copia extra del gen p53 (transgénicos super p53) frente a ratones controles, hemos investigado el estado oxidativo y antioxidante en los nervios ópticos, en relación a p53.

Método: Se han utilizado ratones de la cepa C57BL/6 de 12 meses de edad en dos grupos: 1) grupo super p53 (Sp53) y 2) grupo de controles wild-type (GC). Los ratones fueron anestesiados en atmósfera de éter, extrayendo los globos oculares y nervios ópticos que se lavaron en PBS, manteniendo las muestras en nitrógeno líquido y en congelador de –85ºC hasta su procesamiento. Se homogeneizaron 3-4 nervios ópticos por cada eppendorf, clasificando por grupos y determinando mediante métodos enzimático-colorimétricos la actividad

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[1] Graduate in Medicine. 4th Year Resident in ophthalmology. La Fe University Hospital. Valencia. Member of the Santiago Grisolía Ophthalmological Research Unit. Valencia. Spain.
[2] Graduate in Biology. CSIC Biomedicine Institute. Valencia. Santiago Grisolía Ophthalmological Research Unit. Valencia. Spain.
[3] Ph.D. in Biology. CSIC Biomedicine Institute. Valencia. National Biotechnology Center. Madrid. Spain.
[4] Ph.D. in Biology. National Biotechnology Center. Madrid. Punta de Europa Hospital. Gibraltar Health Area. Cádiz. Spain.
[5] Ph.D. in Medicine. Ophthalmologist. Santiago Grisolía Ophthalmological Research Unit. Valencia. Punta de Europa Hospital. Gibraltar Health Area. Cádiz. Spain.

Correspondence:
Roberto Gallego-Pinazo
Unidad Investigación Oftalmológica Santiago Grisolía
Avda. Gaspar Aguilar, 90
46017 Valencia
Spain
E-mail: robertogallego@comv.es

Results: A significant increase in free radical formation (via lipid peroxidation; p<0.001), antioxidant activity (p<0.001) and nitric oxide synthesis (p<0.001) was found in the optic nerves from transgenic super p53 mice compared to respective controls.
Conclusion: The presence of an extra copy of the p53 gene correlated with redox status in the mouse optic nerve. This transgenic mouse could be useful as an experimental model to study cell resistance to neurodegenerative processes in relation to oxidative stress and to apoptosis induction, such as glaucomatous optic neuropathy or age-related macular degeneration *(Arch Soc Esp Oftalmol 2008; 83: 105-112).*

Key words: P53 gene, super p53 mice, oxidative stress, nitric oxide, optic nerve.

peroxidativa y actividad antioxidante total y la concentración de oxido nítrico.
Resultados: Existe aumento significativo en la formación de radicales libres via peroxidación lipídica (p<0,001), de la actividad antioxidante (p<0,001) y síntesis de óxido nítrico (p<0,05) en los nervios ópticos de los ratones transgénicos super p53, frente a los ratones controles.
Conclusiones: La presencia de una copia extra del gen p53 está ligada a modificaciones de la actividad redox en el nervio óptico del ratón, sugiriendo que p53 otorga una mayor resistencia a la agresión oxidativa. Valoramos la utilización de este modelo de ratón transgénico en procesos neurodegenerativos relacionados con el estrés oxidativo y la inducción de la apoptosis, como la neuropatía óptica glaucomatosa o la degeneración macular asociada a la edad.

Palabras clave: Gen p53, ratón super p53, estrés oxidativo, óxido nítrico, nervio óptico.

INTRODUCTION

Protein p53 has a molecular weight of 53 kD (which accounts for its name) and its main characteristic is that it intervenes directly in controlling the cellular cycle and in the replication and repair of deoxyribonucleic acid (DNA), maintaining genomic stability, activating apoptosis and participating in the cellular response to noxious external agents. Mainly, protein p53 is a regulator of genic expression, acting as transcription factor capable of activating or inhibiting specific genes, notably gene p21, bax and fas, gene IGF-BP3 (insulin-like growth factor-binding protein-3), genE gadd45 (growth arrest and DNA damage-45) and the cycline G gene, all of which are involved in the cellular division cycle and therefore in the proliferation and apoptosis cycle (1,2). Said functions determine that the tumor-suppressing protein p53 is related to cancer prevention due to its ability to regulate the transformation, proliferation and death of cells in the presence of a variety of agents, including attacking nucleic acids and oncogenic stress (1-4). Gene p53 encodes protein p53 and the human genome seems to include about 200 copies of this gene in chromosome 17.

Damage to DNA can come from exogenous natural agents (ultra-violet radiations, micro-organisms) or endogenous (hypoxia, oxidative stress) and also from chemical agents (drugs, pesticides, preservatives, contaminating gases and the like). Therefore, how is p53 stimulated after damages against DNA? It has been proposed that p53 can join the single-chain DNA and thus localize the injury, or that p53 acts in response to anomalies in nucleotide metabolism (1,2,5). In addition, it has been demonstrated that protein p53 becomes inactivated when cellular oxidative activity increases, and for that reason it has been suggested that protein p53 may act as a sensing agent for endocellular oxidative stress (5,6) and that, in biologically favorable situations, there may be mechanisms for inducing or inhibiting apoptosis (1,2,7-9).

The above concept is particularly interesting in ophthalmology because apoptosis, or programmed cellular death, has been related to a number of processes, notably the death of retinal ganglionary cells in the course of glaucoma (10,11).

The apoptosis process responds to the activation of specific mechanisms culminating in the death of cells (1,2,7-11). The activation of the suicide program involves the synthesis of specific messenger RNA and its corresponding translation, which demonstrates that cellular death involves intrinsic intracellular mechanisms (4). In multiple cell types, said apoptosis process depends on gene p53. In fact,

the activation of protein p53 triggers a complex transcriptional program which, according to the cell type and the properties of the environment, leads to an arrest of the cell cycle or, alternatively, to its death by apoptosis (7,9). The search for balance between the mechanisms which promote cell survival and those which initiate self-destruction sequences is the true goal of many researchers because in-depth knowledge of these mechanisms can be the cornerstone for new therapeutic strategies. In this regard, it has been demonstrated that apoptosis-promoting mutations during the development of the ocular globe cause congenital malformations, while those which inhibit apoptosis suppress the appearance of those phenotypes (9,12).

We endeavor to analyze the properties of protein p53 in order to understand its function in neurodegenerative eye diseases having unclear etiopathogenic mechanisms, such as glaucomatous optic atrophy which, due to its morbidity, is an important cause of irreversible blindness all over the world. In this study we have utilized an experimental model in mice, genetically manipulated to have supernumerary copies of gene p53 in the form of large transgenes, obtaining super p53 transgenic mice (Sp53) (3).

In addition, as indicated above, as the increase in oxidative activity is capable of inactivating protein p53 and that said protein could be a biological marker of endocellular oxidative stress, regulating at the same time apoptosis-inducting or inhibiting processes in favorable conditions, we aim to make a biochemical characterization of the optic nerves of Sp53 mice to investigate whether these animals exhibit variations as regards the redox activities of mice with non-modified genotypes.

SUBJECTS, MATERIAL AND METHOD

In this study we have utilized mice as experimental models. All experiments complied with European animal manipulation laws (CE, 1986).

Characteristics of the experimental model

C57BL6/J, Charles River mice were selected and genetically manipulated in the National Biotechnology Centre (Madrid) to have two extra copies of the p53 gene (super p53 tg/tg) as per the method of García-Cao et al. (3). In order to ensure the usefulness of the modified genotype it must be taken into account that the transgenic allele p53-tg, when presented in an environment which is genetically neutral for p53, becomes a functional replica of the endogenous gene. This was the case with the mice utilized in this study. In addition, the Sp53 mice having p53-tg alleles in addition to the two endogenous alleles, exhibited a greater response to damages infringed to the DNA and demonstrated greater protection against cancer than normal mice (3).

Therefore, our study was based on the utilization of the transgenic mice model generated by the use of large DNA segments containing the p53 gene in its natural genomic context in order to preserve as far as possible the characteristics of the gene (3). The transgenic animal model which over expresses gene p53 was characterized by polymerase chain reaction genotyping (PCR) and identification of the specific strip which corresponds to the supernumerary gene. Figure 1 shows a specific strip which confirms the presence of the additional transgene p53, containing the p53 promoting region, so that its regulation is identical to that of the endogenous p53 gene.

The functionality of the transgene was demonstrated in vivo and in vitro. The super p53 mice exhibited normal development, were fertile and aged normally in comparison to control animals. This was the case in all the subsequent litters throughout this study and others carried out with the same experimental model (3).

Obtention of samples

The animals came from the National Biotechnology Center of Madrid and were kept in standard laboratory conditions in the CSIC Biomedicine Institute of Valencia. Eighteen mice aged 12 months were selected at random for both groups of the study (GSp53; n=9 vs GC; n=9). These were sacrificed by exposure to ether atmosphere for a few minutes and the ocular globes and optic nerves excised. These were frozen in liquid nitrogen and stored at –85ºC awaiting processing in the labs of the «Santiago Grisolía» Ophthalmological Research Institute of Valencia. The ocular globes were desiccated, separating the optic nerves which were homogenized in Eppendorfs, each containing the

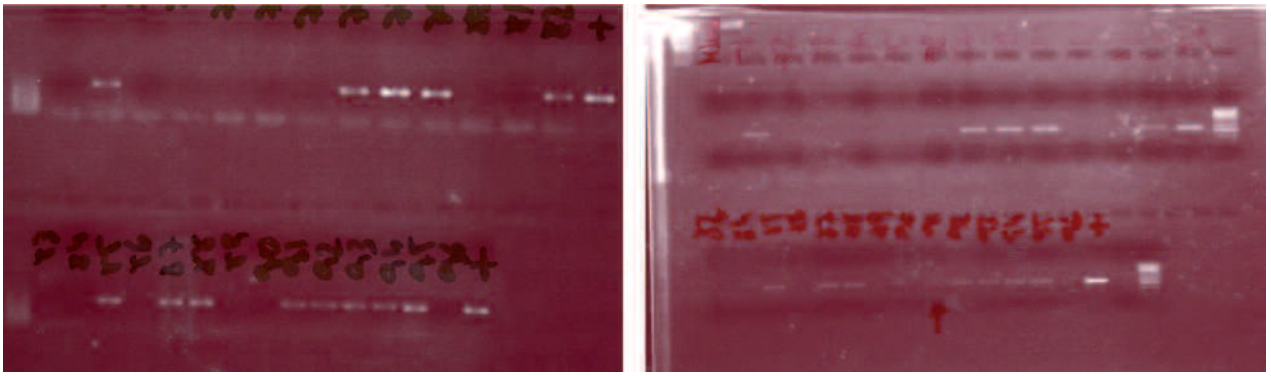
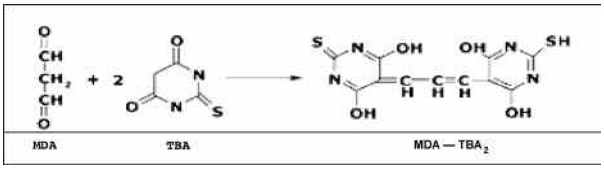


Fig. 1: PCR genotyping of the mice over expressing gene p53.

optic nerves and classified according to the study group. By means of Ultraturrax with pauses to avoid overheating and over ground ice the nervous tissue homogenates were obtained. These were utilized in enzymatic-colorimetric techniques to determine the oxidative and antioxidant condition and to determine nitric oxide as per techniques described by other authors in a variety of tissues, albeit with personal modifications applied to the management of ophthalmological samples, as extensively described in previous works of our group (6-11). Basically, the oxidate condition was determined by establishing the end products of lipid peroxidation, malonildialdehyde (MDA) and the thiobarbituric acid technique and the products which react with it (TBARS). The solution containing the homogenate was boiled for 1 hour and the supernatant extracted with butanole. A sample (in triplicate) was deposited in multi-bowl plates for spectral fluorometric reading in cytofluor at 544 nm excitation and 592 nm emission. The formulae of metabolites which took part in the enzymatic-colorimetric reaction are the following:



The determination of the total antioxidant condition (TAXC) was established by means of the total antioxidant capacity of the sample utilizing a specific combination of reactants which measure the TAXC activity of the homogenized tissue, which is marketed by Randox Labs. The end reaction obtains the measure of the TAXC activity by means of a spectrophotometer at a wavelength of 600 nm.

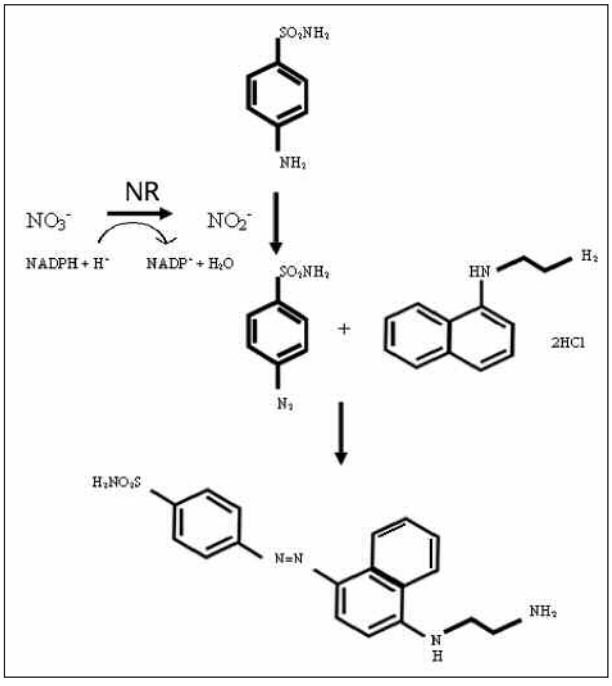
The total nitric acid was determined by means of a commercial preparation by R&D Systems. This essay was based on the enzymatic conversion of nitrate to nitrite by means of nitrate reductase enzyme. After the reaction the colorimetric determination of the nitrite is carried out by Griess' reaction which is based on a two-stage diazotization reaction: 1) acidification of NO2 in order to produce a nitrosating agent and 2) reaction of this agent with sulphanylic acid to produce a diazonium ion which will be joined to N-(1-naphthyl) ethylendiamine to form a chromophore which absorbs light at 540-570 nm, and which is measurable.

The statistical processing of data was made with the Excel for Windows v 2003 spreadsheet software by Microsoft corporation (USA) and SPSS v 15.0 (Inc. Redmond WA, USA). The groups were compared by the "t for student" test and the Kolmogorov-Smirnov test. All the date are presented as mean and standard deviation. The confidence interval of the sample was of 95%.

RESULTS

The mean determinations of oxidative activity by means of lipid peroxidation show a significant increase of the MDA-TBARS values ($p<0.001$) in the optic nerves of the Sp53 group vis-à-vis the control mice (2.2 SD 0.6 vs 1.3 SD 0.2) as shown in figure 2.

The mean total antioxidant activity of optic nerve homogenates was significantly greater [TAXC



(p<0.001)] in Sp53 mice than in controls (1.8 SD 0.4 vs 0.7 SD 0.1) as can be seen in figure 3.

The mean determination of nitric oxide synthesis demonstrated a significant increase of values (p<0.001) in the optic nerves of the Sp53 group in relation to the optic nerve values of wild type animals (143 SD 54 vs 105 SD 39), as can be seen in figure 4.

DISCUSSION

By the use of transgenic models a greater knowledge can be obtained about genic transmission

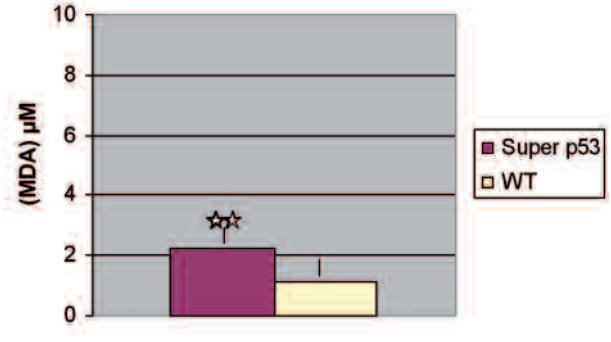


Fig. 2: Determination of MDA TBARS in both groups of mice. The values (µMol/l) are mean ± standard deviation. Significance was set at 95%.

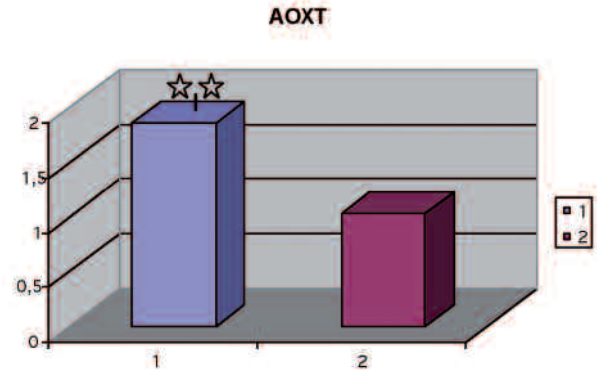


Fig. 3: Determination of total antioxidant activity TAXC in mice. The values (µMol/ml) are mean ± standard deviation. Significance was set at 95%.

mechanisms and cell cycle stages as well as obtaining a fundamental basis for developing therapies (1,19). The model we present in this study which contains an extra copy of the gene which encodes protein p53 allows for an analysis of certain characteristics derived from the functions of this protein and for a description of variations in relation to the supernumerary presence of gene p53 (3).

In the study of the optic nerve of Sp53 mice we have described an increase in the formation of free radicals through the lipid peroxidation pathway (MDA-TBARS) and also the increase of TAXC capacity and nitric oxide synthesis. These results prove that the presence of an extra copy of gene p53 confers redox characteristics in the tissue being studied which are different to those observed in animals with normal genome. In this respect, Lotem et al (8) have demonstrated that apoptosis induced by p53 in myeloid leukemia cells can be inhibited by cytokines IL6, IL3 and interferon g as well as by

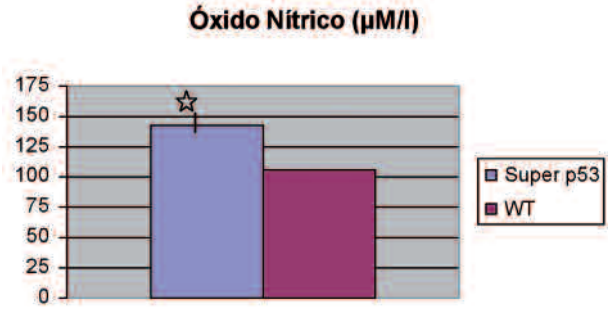


Fig. 4: NO determination in both groups of mice. The values (µMol/ml) are mean ± standard deviation. Significance was set at 95%.

antioxidants. In Proceedings of the National Academy of Sciences, USA, said authors stated that antioxidants and cytokines exhibit a protective effect in the induction of apoptosis. It seems that these cells, which have a high intrinsic level of production peroxides and greater sensitivity to apoptosis, require a greater concentration of cytokines to inhibit it. Accordingly, by reducing the oxidative stress in cells by administering antioxidants, apoptosis is inhibited whereas by increasing intrinsic stress with the addition of hydrogen peroxide, apoptosis is stimulated. This would demonstrate that the intrinsic degree of oxidative stress can regulate cellular susceptibility to the induction of p53 gene-dependent apoptosis as suggested previously (3,7-9). Likewise, from the instant results it can be deduced that the optic nerves of Sp53 mice there is a greater oxidative capacity but also a greater antioxidant activity when compared with the biochemical enzymatic-colorimetric results obtained from the optic nerves of control mice with a normal p53 gene. This suggests that, in the nerve tissue of the study, the supernumerary presence of the gene is translated in an increase of activity of the p53 protein and, biochemically, in a significant increase of antioxidant defenses of cells against oxidative attack. Therefore, the action of said protein on the optic nerve is similar to that described in other tissues, regulating the cellular susceptibility to oxidative stress and possibly to the induction of apoptosis which depends on this process. Protein p53 is crucial for understanding tissue resistance to the formation of oxygen-reactive species in optic nerves and their study will shed further light on the ways to counteract redox-dependent apoptosis.

On the other hand, the significant increase of nitric oxide synthesis in the optic nerves of the Sp53 group matches the results obtained in the aqueous humor of patients with open angle primary glaucoma (20). As glaucoma progresses, the retina ganglionary cells receive less neurotrophines. This mechanism is part of a series of events associated to high IOP which include ischemia, vascular dysfunction and release of cytokines and molecules with cytotoxic effects such as glutamate. The main purpose of neuroprotective therapy is to interrupt said sequence at one or more points. In fact, Neufeld et al of Washington University (20) have demonstrated that an excess of NO is associated to increased IOP and other signs of glaucoma, while an interruption in the synthesis of NO prevents or delays the evolution of the glaucomatous disease. The question is: is NO the agent which causes damage to ganglionary cells or does it appear as a metabolic product of said damage? It seems that the mechanism is that NO induces the injury and death of ganglionary cells independently of the IOP increase or the control thereof. This is a point to be taken into account when analyzing the action of existing anti-glaucoma drugs and also to address anti-glaucoma therapies in the future.

NO is a powerful activator of protein p53. However, the mechanisms of this action are not entirely clear. It seems that NO induces the phosphorylation of p53 in serine 15, which does not require ATP. NO induces the nuclear retention of p53 and the apoptosis of neuroblastoma cells exposed to ionizing radiation (21). This means that, by increasing NO, the efficacy of radiotherapy for treating certain types of cancer would be enhanced. In addition, as it has been demonstrated that p53 plays a crucial role in the response to mutagenic endogenous molecules such as NO and that human cells exposed thereto exhibit an accumulation of p53, it is quite possible that in order to safeguard the integrity of DNA, the possibility of NO-induced damage is reduced by the repression of the p53-mediated syntase-2 nitric oxide enzyme (22).

The Sp53 experimental model mice exhibited a greater antioxidant activity in the optic nerves and therefore it is assumed to have a greater cellular resistance or potentially different behavior against oxidative aggression, particularly in conditions of ischemia-reperfusion or inflammation, and induction of redox-dependent apoptosis.

Protein p53 is essential for understanding tissue resistance to the formation of oxygen reactive species and the action of nitric oxide on optic nerves, in relation to neurodegenerative processes such as glaucomatous optic neuropathy or age-related macular degeneration.

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