OXIDATIVE STRESS IN A MODEL OF EXPERIMENTAL DIABETIC RETINOPATHY: THE UTILITY OF PEROXINYTRITE SCAVENGERS

ESTRÉS OXIDATIVO EN UN MODELO DE RETINOPATÍA DIABÉTICA EXPERIMENTAL II: UTILIDAD DE AGENTES SECUESTRANTES DE PEROXINITRITOS

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ABSTRACT

Purpose: The retina is the neurosensorial tissue of the eye and is extremely rich in polyunsaturated lipid membranes. This feature makes it especially sensitive to oxygen and/or nitrogen activated species and lipid peroxidation. Several authors have postulated the importance of superoxide (O2–) and peroxynitrite production in the development of diabetic complications. In the present study, we have used two different antioxidants, ebselen and lutein, that present as a common feature their peroxynitrite scavenging capacity, to ameliorate the oxidative stress that exists in the retina in diabetic patients.

Methods: Hyperglycemia was accomplished by the intraperitoneal injection of Alloxan in a mouse model of diabetic retinopathy. Malondialdehyde (MDA) and glutathione (GSH) concentrations in eye homogenates (without the lens) were determined. We also recorded serial electroretinograms (ERG) and measured latency and implicit times.

Results: The MDA concentration increased and the GSH concentration decreased in the eyes of the dia-

RESUMEN

Propósito: La retina es el tejido neurosensorial del ojo y es extremadamente rica en membranas con lípidos poliinsaturados. Esta característica la hace especialmente sensible a los radicales libres derivados de oxígeno o nitrógeno y a la peroxidación lipídica. Diversos autores postulan la importancia de la producción de superóxido (O2–) y peroxinitrito en el desarrollo de las complicaciones de la diabetes. En este trabajo hemos empleado dos antioxidantes, ebselen y luteína, que presentan la característica común de ser secuestrantes de peroxinitrito, para evitar el estrés oxidativo que la hiperglucemia induce en la retina.

Métodos: La hiperglucemia se consiguió mediante la inyección de Aloxana. Se determinaron la concentración de malondialdehído (MDA) y de glutatión (GSH) en homogenado de ojo. También se realizaron electroretinogramas (ERG) y medida latencia y de culminación.

Resultados: La concentración de MDA aumentó y la de GSH disminuyó en los animales diabéticos.
**INTRODUCTION**

The retina is the neurosensorial tissue of the eyes and it is extremely rich in membranes with polyunsaturated lipids (1). This characteristic makes it particularly sensitive to oxygenated free radicals and lipidic peroxidation. In fact, there are different retinal pathologies which have been related to the excessive production of free radicals such as uveitis, retinopathy in prematures and diabetic retinopathy.

In literature we find several studies indicating that retinal metabolism is altered in diabetes. Kolluru et al (2) studied mice in which diabetes was induced by the injection of alloxane to prove that the retina experienced an increase in the concentration of substances which react to thiobarbituric acid (TBARS), increases in the activity of protein kinase C and an increase in nitric oxide (NO). They also found that the concentration of GSH is reduced in diabetic retinae. Other studies found in literature prove that retinal metabolism in diabetes is also affected (3,4). However, there are very few studies which prove that the administration of antioxidants reduces a diabetic retinopathy.

Several authors postulated the importance of the production of superoxide ($O_2^-$) in the development of diabetic complications (5). Recently it has also been proved that the benevolence of nitrotyrosine in the plasma of diabetic $O_2^-$ patients are increased, which suggests the possible implications of peroxynitrite in the development of diabetic complications (6). The increase in NO and $O_2^-$ is damaging because it causes a reaction that produces peroxynitrite. The increase in NO and $O_2^-$ is damaging because it causes a reaction that produces peroxynitrite, a powerful oxidative agent with a long mean life cycle. The peroxynitrite anion is cytotoxic because it inhibits the mitochondrial electronic transport chain, it oxidizes protein sulphhydryl groups, it initiates lipidic peroxidation without needing transition metals and it nitrosylates aminoacids such as tyrosine, which affects numerous pathways of signal transduction (7).

Several studies have utilized antioxidants such as vitamin E in an attempt to prevent diabetic complications with contradictory results (8). It could be thought that the treatment with vitamin E would target symptoms rather than causes because it is active only against already made free radicals. An alternative for treatment with antioxidants is to try to interrupt the formation of the superoxide anion or peroxynitrite.

In this study we have utilized two antioxidants, ebselen and lutein, which are very different in their origin but share the characteristic of being peroxynitrite scavengers. The objective of the study was to add new data to the work made in our laboratory (9) which prove that both ebselen and lutein can avoid oxidative stress in diabetic retinopathy and in addition improve functional properties in the retina of diabetic animals.

**SUBJECTS, MATERIAL AND METHODS**

Twenty-five albino male mice were used, in which experimental diabetes was induced with a dose of 200 mg alloxane/kg (66 mg/ml in citrate-phosphate buffer 0.1 M, pH 4.5). Control mice were injected with the same tampon volume. The mice...
having a level of glucemia over 16 mM, 4 days after the treatment with aloxane, were considered to be diabetic. A group of diabetic mice was treated with insulin on days 4, 5 and 6. The treatment with ebse-len (100 mg/kg) was administered orally the last three days of the experiment. The animals were kept in light/darkness conditions (12 hours each) with food and water «ad libitum» and were sacrificed on the seventh day from the beginning of the experiment. Immediately after the sacrifice both eyes were enucleated and the crystalline extracted. Both eyes were homogenized in potassium phosphate tampon 0.2 M pH 7. In order to determine glucemia and glycosilated hemoglobin (HbA1c), two commercially available tests were used manufactured by Boehringer Mannheim Biosystems. The same experimental model was utilized for treatment with lutein.

Determination of proteins. According to the treatment described by Lowry et al with Peterson modifications (10).

MDA measurement. A modification of the Richard et al method was utilized (11), developed in our lab (12), utilizing a high resolution chromatography equipment (Kontron Instruments).

GSH measurement. Determined utilizing the procedure described by Reed (13).

ERG. Practiced on mice anesthesized with ketamine (100 mg/kg weight) and azeproma-zine (2.5 mg/kg weight), adapted to darkness. Anesthetic and mi-driatic liquid was administered. An active electrode of the corneal lens type was used, a reference electrode on the forehead and a mass electrode on the tail of the mice. The stimuli were in flashes with a maximum duration of 5 ms [mean 4; range 100, intensity 1 (0.06 x 22 lumen sec/ft²)]. In front of the white standard flash a 2.5 logarithmic unit optical density filter was placed. A 2-second interval was set between the flash shots. The passing band of the amplifier and preamplifier was set to 350 Hz. Results were recorded on a MacLab computer equipment (Castle Hill, Australia).

Statistical analysis. Data are expressed as mean ± standard deviation. Variance analysis was used (ANOVA) and t-Student for non-paired data.

RESULTS

One week after the induction of diabetes a significant increase of glucose levels in blood was obser-ved. Treatment with ebse-len or lutein did not affect significantly the plasma glucemia levels both in control and in diabetic animals (data not shown).

The MDA values in eye homogenated without lens increase significantly in diabetic mice against the control group. The administration of insulin reduced the DMA concentration in eye homogenated without lens. Treatment with any of both antioxidants (ebse-len or lutein) achieved a reduction of ocular MDA (figs. 1 and 2).

The GSH concentration fell in the retina of diabetic animals. Treatment of diabetic animals with lutein or ebse-len increased the GSH concentration up to the control values (figs. 3 and 4). Treatment with insulin also increased the GSH concentration.

All electroretinograms are characterized by their morphology, amplitude, latency and culmination times and duration. The latency time is the time...
elapsed from the moment of stimulation up to the appearance of the wave. The culmination time (implicit time) also begins when stimulation starts and it ends when the wave reaches its maximum amplitude. The latency and culmination times was observed in the different groups of animals of the study without finding significant differences (data not shown).

### DISCUSSION

The research carried out by our lab (9) had proved the importance of oxidative stress in this model of experimental diabetic retinopathy. This is again confirmed with the results presented in this work, as proved by the increase of MDA concentration or the reduction of GSH concentration in eye homogenate without lens of diabetic mice. Many documents in bibliography have demonstrated that 97% of the eye homogenate without lens corresponds to the retina (14). The high concentrations of MDA confirm the importance of lipidic peroxidation in diabetes. there is a general consensus about the fact that MDA measurements made with liquid high-resolution chromatography (HPLC) are effective markers of the involvement of oxygen stress in a determined pathological condition, and these measurements are also useful to evaluate the effects of antioxidant treatments (15). Literature about GSH levels in the retina of diabetic mice are contradictory. Our results match those presented by Kern, Kowluru and Engerman (16), who observed a reduction of GSH levels in retina of mice with two-month old diabetes. However, other researchers (17) were not able to prove these variations. GSH levels are also reduced in other tissue affected by the complications of diabetes such as the lens (16), the peripheral nerve (18) and the kidneys (19).

Treatment with both antioxidants returns said parameters to normal values. This effect can be explained by the common characteristics of ebselen and lutein as peroxynitrite scavengers.

The importance of NO and peroxynitrite in diabetes complications and particularly in diabetic retinopathy is described in many studies. Perhaps the most dramatic change which takes place early in the neurosensory retina of diabetic mice is the tenfold increase in the frequency of apoptosis. This change is observed only one month after the induction of diabetes and continues with the same frequency at least 12 months. The vast majority of apoptotic cells was not found in the vessel cells but in ganglion cells (20). Apoptosis is also found in the retina in other degenerative diseases such as pigmentary retinitis, interior ische-mical optical neuropathy and glaucoma.

The apoptosis of nerve cells has been related to the formation of peroxynitrite (21). NO is synthesised in cells through the conversion of L-arginine into L-citruline by means of the action of the synthetase nitric oxide enzyme (ONS). There are three isoforms of ONS: neuronal, endothelial and inducible. The first two have constitutive expressions and generate a small amount of NO when activated by the calcium/calmoduline complex. In contrast, the inducible isoform has no constitutive expressions; instead, it expresses in many types of cells after being activated by immunological or inflammatory
stimuli and it acts independently of calcium, generating large amounts of NO for long periods of time.

In the eyes, it is believed that neuronal ONS is responsible for producing NO in light receptors and bipolar cells, whereas endothelial ONS is present in the endothelial vascular cells. However, inducible ONS, which is present in the Müller cells and in the pigmented epithelium of the retina, can be involved in the phagocytosis of the external segment of the light receptor, as well as in infectious, inflammatory and ischemic processes and the pathogenicity of diabetic retinopathy (22).

A number of studies have proved an increase of ONS activity in the retina of diabetic mice when compared with control groups (23,24) in the first stages of diabetes. This increase could be related to the clinical alterations typically found in diabetic retinopathy. On the contrary, Roufail et al (25) found that the number of cells containing neuronal ONS had decreased 32 percent one week after the induction of diabetes in mice and remained low up to eight months later. This research did not confirm whether the law number of nONS+ cells was due to the decreased protein synthesis or to the death of said cells.

Our group had already proved that the amplitude of b wave of the ERG was smaller in diabetic animals (9). In this research we studied the latency and culmination time in the b waves of ERGs in the different groups of animals without finding significant differences in spite of the fact that some authors have found a significant increase of b wave latency in diabetes mice (26).

New research is required to confirm the mechanism of action of ebseilen and lutein in this model of experimental diabetic retinopathy.

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