PHOSPHODIESTERASE ACTIVITIES IN THE EYE OF OLD AND YOUNG RATS IN NORMOXIC, HYPOXIC AND HYPEROXIC ATMOSPHERES:

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Phosphodiesterase activity was tested on homogenized eyes of young and old rats kept in hypoxic and hyperoxic conditions, with the aim of correlating any difference in PDE activity with aging and variations in atmospheric oxygen contents. The activities of the two enzymes, cAMP phosphodiesterase (cAMP-PDE) and cGMP phosphodiesterase (cGMP-PDE), were tested. Phosphodiesterases seem to be particularly susceptible to variations in oxygen tension, suggesting an important role of cyclic nucleotides in cellular adaptive processes. Particularly, cAMP-PDE activity increases lightly both in hypoxic and hyperoxic conditions in young and old rats. For cGMP-PDE activity of young rats, a similar behaviour to cAMP-PDE activity is observed with a similar increase in hypoxic and hyperoxic conditions respect to the control rats. Instead old rats seem to be quite insensible to hypoxia, while they show a fair increase in cGMP-PDE activity in the case of hyperoxia.

The second messengers cAMP and cGMP play important roles in mediating the biological effects of a wide variety of first messengers. The intracellular levels of cyclic nucleotides depend upon rates of synthesis and degradation, actuated, respectively, by cyclases and phosphodiesterases (PDEs). Therefore, PDEs seem to play an important role in a wide variety of physiological processes.

At least ten different cyclic nucleotide phosphodiesterase (PDE) isoenzymes have now been identified because of their functional characteristics, such as substrate specificity and susceptibility to selective inhibitors (1). Some of these inhibitors are for specific isoenzymes while others inhibit many different PDEs.

In retinal photoreceptors, the activation of a specific PDE is involved in the enzymatic cascade resulting from light absorption by photo-pigment molecules and cell membrane hyperpolarization (2). Particularly, several studies have shown that the light-sensitive outer segment of rod photoreceptor cells are particularly rich in cyclic GMP and its metabolic enzymes (3-5) and that cyclic GMP phosphodiesterase of rod outer segments is activated by light in the presence of ATP or by addition of bleached rhodopsin preparations (6-9).

The aim of this study was to compare the physiological change in PDE enzymes between young and old rats and the response to chronic hypoxia and hyperoxia. Oxygen tension affects a series of physiological functions, influencing the expression of several enzymes. In some tissues, hypoxia inhibits enzymatic activity, in other tissues it increases it. This apparent dual-effect is related to the oxygen tension and the response is influenced acutely or chronically by the enzymes in the synthesis and the degradation

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(10). Oxygen from free radical production interferes with the aging process (11); it is known that hyperoxia itself has a toxic effect on animals, plants and aerobic bacteria (12). The conflicting data existing in the literature on PDE and oxygen is due first-to the time course of adaptation to hypoxia and hyperoxia, second-to the levels of hypoxia and hyperoxia used and third-on the time of exposure. Normally, hypoxia itself inhibits enzymes. For this reason the body tends to compensate hypoxia with an increase in ventilation (13), an increase in heart rate and haemoglobin stimulation with erythropoietin release from the kidney (14). Accordingly, we tested if PDEs are involved in the process of adaptation to hypoxia and hyperoxia in the eye. The increase in PDEs levels is correlated to the stress response due to variations in the oxygen supply (15). Studies over the past decade have linked retinitis pigmentosa to mutations in genes of the phototrasduction cascade, specifically rhodopsin (16), cGMP-phosphodiesterase, and other photoreceptor -specific proteins. The mechanism by which these genetic or environmental insults lead to PR cell death is unknown, but it is possible that the phosphodiesterases could be involved. The cyclic AMP and cyclic GMP, now known to be the mediator, second messenger, in the action of several different hormones, may regulate not only, the activity of certain target enzymes, as already established, but also by analogy to its role, the catabolite repression and, in some concentration, the apoptosis effect (23) in several cells. As the different cyclic nucleotides metabolism is presumptively regulated by separate mechanisms, we thought that it was important to assay the absolute concentration of cAMP and cGMP, too, and, simultaneously, the activity in its whole of the enzymes which control their metabolism. Therefore, we decided to use a method able to show the real situation in the tissue/cell for both cyclic nucleotide concentration and PDEs expression.

The activity value of cAMP PDE we found is to be assigned to all the PDE families able to metabolise cAMP, as actually we don't have any method to distinguish them. However, considering the high specifity of PDE type IV for this substrate, we can approximate it entirely to that of this family. Following a similar reasoning we ascribed cGMP PDE activity to PDE type V.

MATERIALS AND METHODS

Normoxic treatment

Two groups of Wistar male rats weighting 250-400 g were used, according to the guidelines of the Declaration of Helsinki. Both were kept in room air (21% O_2) as control groups. One group was composed of six young rats (3 months) and another of six aged rats (24 months).

Hypoxic treatment

Two groups of Wistar male rats weighting 250-400 g were used, according to the guidelines of the Declaration of Helsinki. They were kept in a Plexiglas chamber for 12 days in chronic hypoxia (10-12 % inspired oxygen). The temperature was maintained at 25° C. The chamber was recirculated with a pump; CO_2 was removed from the chamber air with baralyme and continuously monitored by a capnograph. Boric acid was mixed with the litter to minimize the emission of urinary ammonia. One group was composed by six young rats (2 months) and the other by six aged rats (24 months). The rats were anaesthetized with Nembutal 40 mg/kg i.p.

Hyperoxic treatment

Experiments were performed on two groups of Wistar male rats. One group was composed by six young rats (3 months), and the other by six aged rats (24 months). Rats (250-400 g) were exposed to 98-100 % O_2 (760 Torr) for 60 hrs in a large Plexiglas chamber. The temperature was maintained at 25° C. The chamber was recirculated with a pump; CO_2 was removed from the chamber air with baralyme and continuously monitored by a capnograph. Boric acid was mixed with the litter to minimize the emission of urinary ammonia. The rats were anaesthetized with Nembutal 40 mg/kg i.p.

Partial purification of phosphodiesterases

Four rat eyes were weighed and homogenized with K_1K_2 phosphate buffer, 10 mM pH 7,00, in a ratio 1:10 (w/v) utilizing a potter Model T25 basic (Ikalabortechnik, Germany). The homogenate was treated in ultrasonic bath model Sonic 18-35 (Simply, Italy) for one minute at 25°C. The homogenate was centrifuged for 30 sec at 1000 rpm in a Mikro 22R

(Hettich Zentrifugen-Germany). Supernatant was used for PDE assay and protein determination.

cAMP PDE assay

The enzymatic reaction was carried out using the method of Spoto et al. (18) with minor modifications: 0.1 M Tris-HCl buffer, pH 8.3, 10 mM $MgCl_2$, 0.1 M KCl at 37°C. The reaction was initiated by the addition of 44 μM of cAMP. Control experiments were performed using a commercial preparation (Sigma) where the enzyme concentration was 0.4 μM. The time course of reaction was 60 minutes. The reaction was terminated by transferring the tubes with the reaction mixture in a boiling water bath for 3 min. The sample was then centrifuged and filtered through a nylon-66 filter, 0.2 μm (Rainin corporation). The clear filtrate obtained was used directly for HPLC assay or stored at -80°C.

cGMP PDE assay

The enzymatic reaction was carried out using the method of Spoto et al. (18) with minor modifications: 0.1 M Tris-HCl buffer, pH 8.3, 10 mM $MgCl_2$, 0.1 M KCl at 37°C. The reaction was initiated by the addition of 44 μM of cGMP. Control experiments were performed using a commercial preparation (Sigma) where the enzyme concentration was 0.4 μM. The time course of reaction was 60 minutes. The reaction was terminated by transferring the tubes with the reaction mixture in a boiling water bath for 3 min. The sample was then centrifuged and filtered through a nylon-66 filter, 0.2 μm (Rainin corporation). The clear filtrate obtained was used directly for HPLC assay or stored at -80°C.,

Analysis of cyclic nucleotides by reverse-phase HPLC

The HPLC system was from Beckman and consisted of two 110A pumps, a variable wavelength spectrophotometer Spectroflow 783 (Kratos Analytical, Manchester, United Kingdom) measuring at 254 nm and an autosampler Promis (Spark Holland, Emmen, The Netherlands). The column used was a 5-μm Li-Chrospher 100 CH 18/2 Merck (250x4 mm). The mobile phase employed for the separation of nucleotides consisted in 200 mM ammonium acetate (pH 6.0) with 2% acetonitrile (v/v). The flow rate was 1 ml/min; the detection was performed at 254 nm. Peak identities were confirmed by co-elution with standards. Quantitative measurements were carried out by comparison using standard solutions of known concentrations. Analysis was confirmed with the cyclic GMP EIA Kit and the cyclic AMP EIA Kit (Biomol, Plymouth Meeting, PA, U.S.A.)

Analysis of phosphodiesterase activity by reverse-phase HPLC:

The analysis was done as described by Spoto (17-18). In some experiments the presence in the homogenate of oxidase or/and phosphatase produced products of degradation of the nucleotide-monophosphates. From cGMP PDE were obtained guanosine-monophosphate and xanthine with a quantity equivalent to the decrease of cyclic GMP, but this is known in literature (19-20). From cAMP PDE were obtained adenosine-monophosphate and inosine with a quantity equivalent to the decrease of cyclic AMP, but this too is known in literature (21).

Data processing

Fisher's PLSD, Scheffe, Bonferroni/Dunn tests were used to evaluate the presence of statistically significant differences.

Protein content

Protein content was determined using a bicinchoninic acid protein determination kit from Sigma with bovine serum albumin as a standard.

RESULTS

The phosphodiesterase activity was tested on homogenized rat eyes, obtained as described in materials and methods, with the aim of correlating any difference in PDE activity with aging and variations in atmospheric oxygen contents.

The enzymatic analysis on homogenized rat eyes shows a different behaviour between cAMP PDE activity and cGMP PDE activity. In the first case, in normoxic controls the activity is quite the same as in the old (2.3+1.29 nmole/mg proteins) and young rat eyes (2.24+1.38 nmole/mg proteins), while cGMP PDE activity is higher in old rats. Besides, it is immediately evident how the cGMP PDE activity is predominant on cAMP PDE. Data processing with Fisher's PLSD showed statistically significant differences, in cGMP PDE activity, between the control group and the hyperox

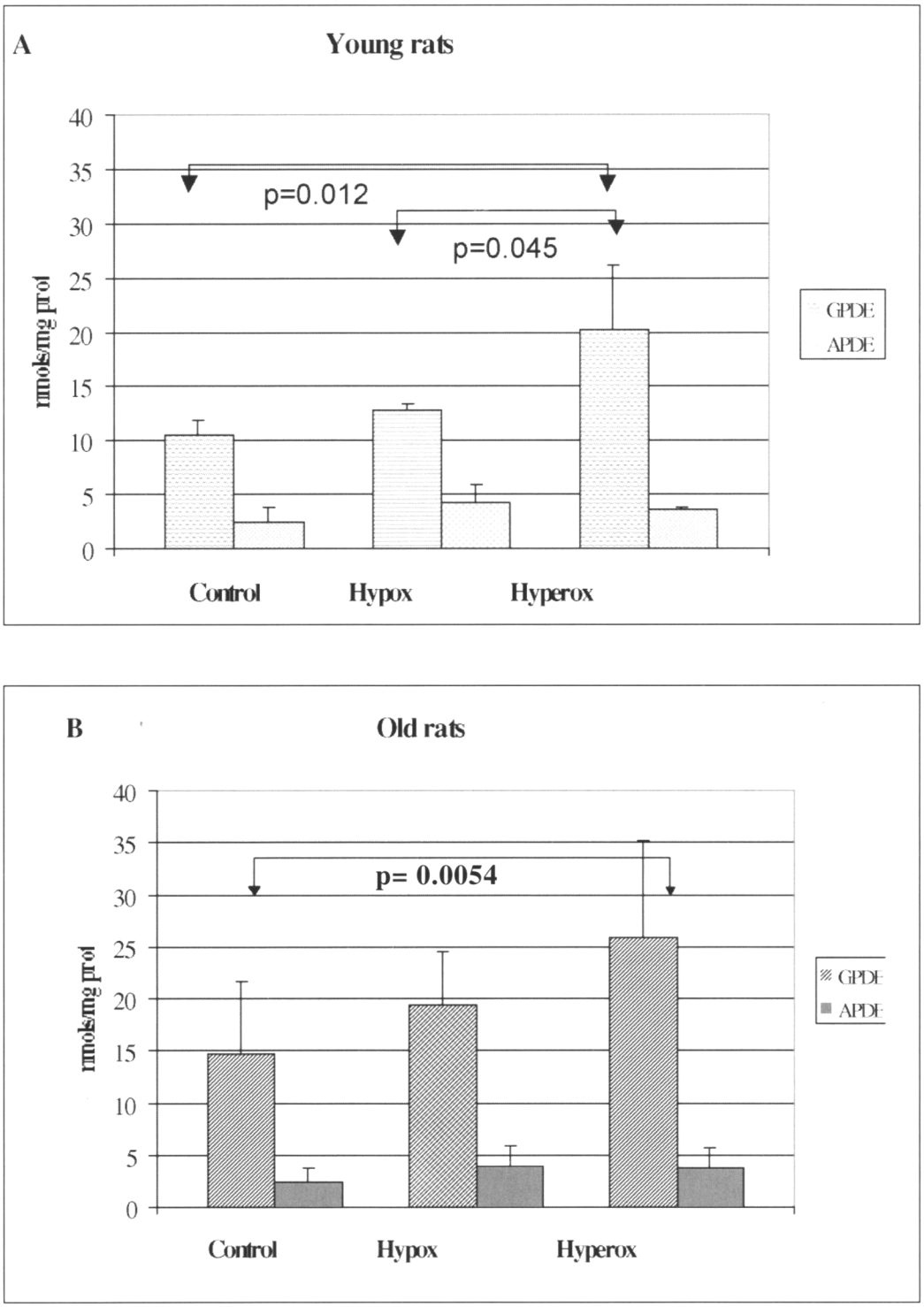

Fig. 1. *Phosphodiesterase activity in eyes of young and old rats.*

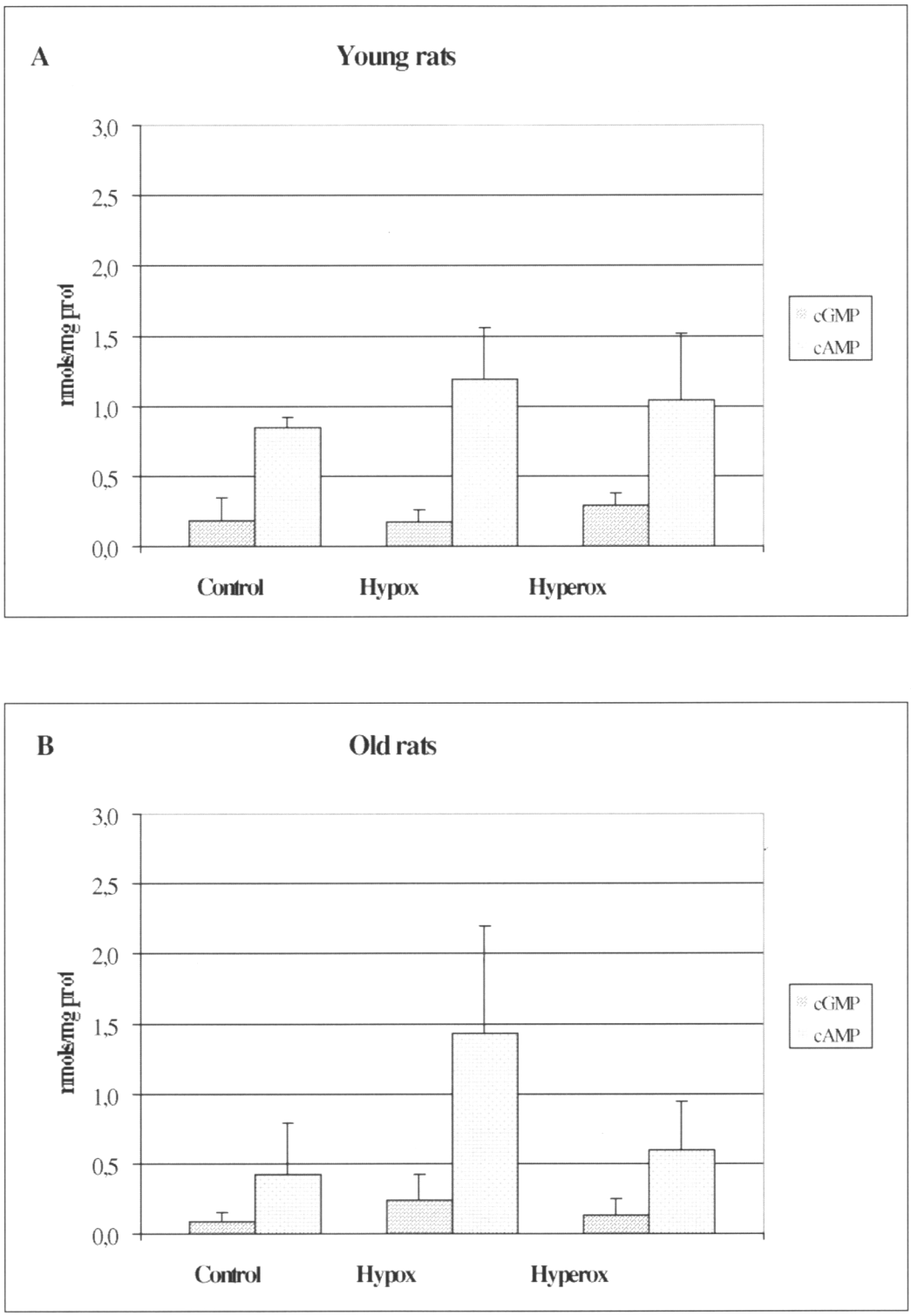

Fig. 2. *Cyclic nucleotides in eyes of young and old rats.*

group , with values below 5% and p= 0.0123 in young and 0.0054 in old rats; statistically significant differences were present also between the hypox group and the hyperox group in the young rats (p value= 0.0458). Figure 1 shows how oxygen supply to the eye affects the expression of phosphodiesterase activity in different ways, according to whether we are looking at old or young rats, and how this influence is different in cGMP PDE and cAMP PDE activity. The cAMP PDE activity increases in old and young rats, passing from normobaric conditions to hyperoxic ones and finally to hypoxia but was no statistically significant differences in APDE values in young and in old rats.

In cGMP-PDE activity, we have behaviour similar to cAMP-PDE one with an increase both in hypoxic and in hyperoxic conditions but mostly in the latter.

Such an increase in phosphodiesterase activity could belong to the organism adaptive mechanisms to variations of oxygen levels in the atmosphere. The fact that PDE activity is not significantly higher in young rats, could exclude that there is a decrease in sensitivity, due to aging, for that mechanism in eyes, as is instead described in literature for other tissues (22).

We also tested the endogenous presence of cAMP and cGMP (Figure 2). Considering the middle values of normoxic controls, cAMP and cGMP concentrations are doubled in young rats (0.182 and 0.849, respectively) in comparison with old ones (0.091 and 0.423, respectively). Atmospheric oxygen quantities influence cAMP and cGMP concentrations, too. Particularly, increases in cAMP-PDE activity coincide, both in old and young rats, with increases in cAMP concentrations: it increases passing from normoxic controls to hyperoxic conditions and finally to hypoxia. Instead, cGMP concentrations follow increases in cGMP PDE activity only in young rats, while in old rats, the concentrations always increase in comparison with controls, but with maximum rates in hypoxic conditions.

Data processing with Fisher's PLSD, Scheffe and Bonferroni/Dunn showed no statistically significant differences in cGMP and cAMP concentration values.

DISCUSSION

The PDEs are involved in the intracellular transduction process and in the modulation of the intracellular concentration of cyclic nucleotides. The cGMP PDE enzyme belongs to this protein family, whose members differ in structure, enzymatic activity, and specificity for regulatory factors. It is the key effecter enzyme in vertebrate photoreceptors regulating the visual process. In the retinal photoreceptors, the process leading from light absorption by photopigment molecules to cell membrane hyperpolarization, involves an enzymatic cascade resulting in the activation of cGMP PDE; the cGMP PDE, in turn, hydrolyses the nucleotide guanosine 3'-5' cyclic monophosphate that regulates the permeability of light sensitive channels. This enzyme consists of catalytic a and b subunits and two inhibitory g subunits that block PDE activity in the dark (22).

Several studies have shown that the light-sensitive outer segments of rod photoreceptor cells are particularly rich in cyclic GMP and its metabolic enzymes (3-4); each of these enzyme activities increase in conjunction with photoreceptor cell differentiation and maturation. Either PDE enzyme or oxygen is important in the process of energy supply, and as modulator and mediator of several cellular functions. Acute and chronic hypoxia cause different reactions with several adaptations; the body tends to compensate hypoxia with an increase in ventilation, an increase in heart rate and haemoglobin stimulation with erythropoietin release from the kidney. But while we are more prepared to adapt to hypoxia (people living at high altitude or with congenital heart or pulmonary disease) hyperoxia promptly produces toxic effect. After 92 hrs of exposure to hyperoxia (100%), it is well known that all rats die due to hyperoxia effects on cell metabolism. Oxygen tension affects a series of physiological functions, influencing the expression of several enzymes. PDEs seem to be particularly susceptible to hyperoxia and hypoxia exposure suggesting that cyclic nucleotides are important in the cellular adaptive response to change in oxygen tension. In the present work, we describe phosphodiesterase activity in the eye of young and old rats kept

under hypoxic or hyperoxic and normoxic conditions. We have studied separately enzymatic activity of cAMP PDE and cGMP PDE enzymes.

We observed that in control group of rats (normoxic) the cAMP PDE level is similar between young and old rats, while the cGMP PDE is higher in the elder. Physiologically, cAMP PDE activity decreases with aging, while cGMP PDE increases.

These results may present the error of considering total values of cyclic nucleotides metabolic transformation without attributing a specific value to each single family of phosphodiesterases. Nevertheless, the enzymatic activity values of different cellular lines are significantly different and show different metabolism of cyclic nucleotides, cAMP and cGMP. We evaluated whether hypoxia or hyperoxia caused a significant cAMP and cGMP PDE activity increase, comparing young and old rats. The cAMP PDE activity is higher in hypoxic conditions; contrarily the cGMP PDE increases mostly statistical significant in hyperoxia conditions.

The results of the present investigation clearly indicate that PDE activity is well preserved during aging, that the activity of young and old rat eye PDE is influenced in different ways by hypoxia and hyperoxia. The activation of PDEs is a homeostatic attempt to maintain the oxygen supply at a minimum for basal activity of cell in transduction of signals. The oxygen sensitive mechanism in the eye is less responsive during aging and hyperoxia.

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