Light-induced ATP release from the lens

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Abstract

The recent discovery of the photoreceptor melanopsin in lens epithelial cells has opened the possibility of modulating this protein by light stimulation. Experiments carried out on New Zealand white rabbits have demonstrated that the release of ATP from the lens to the aqueous humor can be reduced either when a yellow filter or a melanopsin antagonist is used. Compared to control ($1.10 \pm 0.15 \mu\text{M}$ ATP), the application of a yellow filter ($\lambda 465\text{--}480$) reduced ATP in the aqueous humor 70%, while the melanopsin antagonist AA92593 reduced the presence of ATP 63% ($n = 5$), an effect which was also obtained with the PLC inhibitor U73122. These results indicate that when melanopsin is blocked either by the lack of light, a filter, or an antagonist, the extracellular presence of ATP is significantly reduced. This discovery may be relevant, on the one hand, because many ocular physiological processes are controlled by ATP and, on the other hand, because it is possible to stimulate ATP release with just light and without using any added substance.

Keywords AA92593 · ATP · Eye · Lens · Light · Melanopsin

Introduction

Light has been illuminating the earth before any living being existed [1]. Its influence on life from the very beginning was rapidly exploited by organisms which created primitive structures to detect light. These primitive eyes developed over millions of years until becoming the structures we recognize today in many living beings.

Eyes permit images to form by means of the photoreceptors present in the retina, but this structure at the back of the eye has a group of specialized cells that can absorb light without being involved in the image-forming process. The termed *intrinsically photosensitive retinal ganglion cells* present a photoreceptor named melanopsin [2] which can detect the blue component ($\lambda 460\text{--}485$ nm) present in sunlight (or any artificial white light) indicating to the body whether it is day or night and concomitantly initiating or stopping the synthesis of melatonin in the pineal gland and regulating what are called circadian rhythms [3].

Recently, it has been possible to demonstrate the presence of the melanopsin photoreceptor in human lens epithelial cells [4]. Melanopsin, when illuminated by light, inhibits the local synthesis of melatonin but under darkness conditions, it is produced normally. A question that arises is if it is possible that light, as in the case of melatonin, can also control the synthesis or release of other substances in the lens [5].

In this sense, it is interesting to point out that the lens is the richest tissue containing ATP in the whole body. This ATP is used to keep all the active transport systems working in the lens but it also presents a physical effect by absorbing the noxious UV light and preserving the retina from its damage [6].

Since melanopsin has been recently discovered in the lens and the lens is the richest tissue containing ATP, in this current study, it is described the release of ATP triggered by light as a consequence of melanopsin stimulation.

Material and methods

Animals

Male New Zealand white rabbits were kept in individual cages with free access to food and water. The control group ($n = 5$) and filter group ($n = 5$ for each filter, yellow filter, $\lambda 465\text{--}480$ and magenta filter $\lambda 505\text{--}595$) were kept under controlled 12-h day-night cycles. For all the experiments involving light and

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filters, a constant illumination of 400 lx was used. This study followed the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the European Communities Council Directive (86/609/EEC).

Aqueous humor collection and HPLC measurements

Aqueous humor collection was carried out after anesthetizing the animals with a mixture of ketamine (7.5 mg/kg, Imalgene 1000, Merial, Barcelona, Spain) and Domtor (0.25 mg/mg, DOMTOR®, Orion Pharma, Espoo, Finland). Samples of the aqueous humor (100 µl) extracted with a syringe connected with a 30-gauge needle were stored in -20°C until HPLC analysis.

Studies carried out with ex vivo lenses were performed as follows: animals were euthanized with an overdose of sodium pentobarbital. After enucleation the eyes were immediately taken and the lenses were removed and kept in multiwells with culture media to be used for the experiments indicated later. In each particular experiment, supernatants were taken and measured by HPLC as indicated in the following paragraphs.

The HPLC consisted of a Waters (Milford, MA) 1515 isocratic HPLC pump, a 2487 dual absorbance detector and a Rheodyne injector, all managed by the Breeze software from Waters. Analysis was carried out with the following mobile phase: 0.1 mM KH_2PO_4 , 2 mM tetrabutylammonium hydrogen sulfate, and 15% acetonitrile, pH 7.5. The column was a NovaPak C-18 (15-cm length, 0.4-cm diameter; Waters). Detection was studied at 260-nm wavelength. To confirm that the released nucleotide measured by HPLC was ATP, a sample taken from the rabbit aqueous humor under white light conditions was incubated with alkaline phosphatase (EC 3.1.3.1) 0.3 U/ml (Sigma, St. Louis, Mo, USA) and the digested products were analyzed by HPLC.

Experimental procedures

To confirm that ATP was released mainly from the lens, lenses were incubated under light or darkness conditions for up to 4 weeks. After this period of time, supernatant samples (100 µL) were taken and analyzed to measure extracellular ATP by HPLC as previously described. It is important to bear in mind that incubation volumes were 250 µL, since this is the estimated volume of the aqueous humor within the rabbit eye.

To block melanopsin, 20 µL of AA92593 (Melanopsin antagonist, Sigma, St. Louis, Mo, USA) was used at a final concentration of 100 µM, once every 8 h [4]. Also, and since melanopsin is coupled to phospholipase C (PLC), lenses were incubated with the PLC inhibitor U73122, applied once every 8 h [4] (Sigma, St. Louis, Mo, USA) and the inactive compound U73433 (negative control) both at 3 µM, the experiments being performed under illumination with white light. At

the indicated time, (4 weeks), supernatants were collected as previously indicated.

Data analysis

All data are presented as the mean $\pm$ S.D. Statistical differences between treatments were calculated using the Student *t* test. Plotting was carried out by GraphPad Prism 6 computer program (GraphPad Software).

Results

Light-induced ATP release

A group of five rabbits were submitted to white light (control), and another five were submitted to the same light but their cages had a yellow filter to cut the blue component of white light ($\lambda 460\text{--}485\text{ nm}$). When the levels of ATP were studied in their aqueous humor by HPLC, the transparent liquid that bathes the lens and the inner anterior part of the eye, it was possible to see remarkable changes. While the animals submitted to white light have a concentration of ATP $1.10 \pm 0.15\text{ }\mu\text{M}$ (control), in the animals under the yellow filter for a period of time of 4 weeks, ATP presented levels of $0.34 \pm 0.08\text{ }\mu\text{M}$ (Fig. 1a, b). When the animals were returned back to white light, in 2 weeks, they had recovered the normal ATP values of around $1\text{ }\mu\text{M}$, almost identical to that already described in the literature [7]. Moreover, if we inhibit melanopsin with the inhibitor AA92593 under white light conditions, or the animals were submitted to constant darkness, or even the antagonist was tested in the darkness, the concentrations of ATP in the aqueous humor obtained were $0.41 \pm 0.11\text{ }\mu\text{M}$, $0.32 \pm 0.15\text{ }\mu\text{M}$, and $0.38 \pm 0.09\text{ }\mu\text{M}$ respectively, almost the same as the value obtained with the yellow filter ($n = 5$, Fig. 1a, b). To fully confirm that the measured concentrations of ATP were due to the effect of white light, yellow filter, or darkness, another set of five animals were kept for 4 weeks under a magenta filter ($\lambda 505\text{--}595$, which permits blue and red light to pass through). As observed in Fig. 1a, b, magenta filter provided ATP concentrations similar to those obtained with white light ($0.96 \pm 0.21\text{ }\mu\text{M}$, $n = 5$), suggesting that the yellow filter, blocking the blue component of white light, can reduce the ATP that is released from the lens.

Finally, to demonstrate that the peak measured by HPLC was ATP, a sample obtained under light condition was collected and treated with alkaline phosphatase. As observed in Fig. 1c, after 10 min of treatment, ATP peak decreased and it was possible to observe the presence of both ADP and AMP as a consequence of the enzyme action, thus confirming that the original peak was ATP.

Fig. 1 Levels of ATP in the aqueous humor of New Zealand white rabbits under light/darkness and filters. **a** HPLC elution profiles showing the release of ATP under white light (WL), yellow filter (YF), darkness with the melanopsin antagonist AA92593 (D + Antag), white light plus the melanopsin antagonist AA92593 (WL + Antag), and darkness and magenta filter (MF). **b** Quantification of the experiment presented in **a** ($***p < 0.001$ vs. white light, $n = 5$, the Student t test). **c** Alkaline phosphatase digestion of the putative ATP under the conditions defined in methods. As a consequence of the enzyme action, ATP peak gets reduced and a concomitant presence of ADP and AMP occurs

ATP is released mainly from the lens

The presence of ATP in the aqueous humor could be due to the lens contribution but also from the ciliary body [8]. To see if the light-induced ATP release was due to the lens, lenses were isolated and submitted to light and darkness conditions and the supernatant levels of ATP were measured following the protocols described in the methods. As presented in Fig. 2a, the cultured lenses under light condition presented an ATP concentration that was higher than that obtained when the lenses were in the dark ($0.79 \pm 0.18 \mu\text{M}$ and $0.27 \pm 0.13 \mu\text{M}$, respectively, $n = 4$).

To confirm that the effect triggered by light was due to melanopsin stimulation, lenses in culture were treated with some antagonists and inhibitors. In this sense, lenses under white light conditions but in the presence of the selective melanopsin antagonist AA92593 did abolish the light-induced ATP release, indicating that this effect is due to this photoreceptor stimulation

(Fig. 2b, c). ATP concentrations changed from $0.82 \pm 0.14 \mu\text{M}$ under light conditions to $0.31 \pm 0.11 \mu\text{M}$, in the presence of AA92593 ($n = 4$). To fully ratify the involvement of melanopsin, the PLC inhibitor U73122, which has been currently used to confirm the involvement of melanopsin, was used [4]. In Fig. 2b, c, it can be seen that the active PLC inhibitor reduced the light-triggered ATP release to $0.33 \pm 0.15 \mu\text{M}$ ([ATP] under light conditions $0.81 \pm 0.10 \mu\text{M}$, $n = 4$), while the inactive compound U73433 did not affect the light-induced ATP release (its concentration being $0.77 \pm 0.16 \mu\text{M}$, $n = 4$).

Discussion

In this current study, it has been possible to see that ATP is released from the lens as a consequence of light stimulation and in particular by the blue component of white light. The

Fig. 2 Effect of light/darkness and agents on the light-induced ATP release. **a** Experiment performed with isolated rabbit lenses submitted to light or darkness. Under light conditions, ATP peak in the HPLC chromatogram is higher than that obtained when the lens is in the darkness. **b** HPLC elution profiles obtained from the supernatants of isolated lenses submitted to white light (WL), white light plus the melanopsin antagonist AA92593 (WL + Antag), white light and the PLC inhibitor U73122 (WL + U73122), or the inactive form U73433 (WL + U73433). **c** Quantification of the experiment presented in **b** (***p* < 0.001 vs. white light, *n* = 4, the Student *t* test)

activation of the photoreceptor melanopsin present in lens epithelial cells by light induces the release of ATP. This release can be abolished if the selective antagonists of melanopsin, the compound AA92593 or the PLC inhibitor U73122, are used [4].

Light has been considered as an important factor for evolution due to the ability of many living organisms to have eyes and therefore to receive information from the surrounding environment. Nonetheless, in transparent structures such as those existing in the eye, light can perform a different task, acting as an active agonist by stimulating melanopsin [2]. The existence of such a protein in the lens and the modulation of the synthesis and release of some substances have been quoted. In this sense, it has been possible to demonstrate that light can modify the synthesis and release of the substance melatonin [4]. Interestingly, and in clear contrast to what is described in this current study, where light triggers the release of ATP, the effect of light on melatonin release is the opposite; therefore, under light conditions, melatonin is not released [4, 5]. A possible explanation for such differences may rely on the fact that melatonin inhibition by light in the darkness is related to changes in the expression of the melatonin synthesis enzyme

AANAT [4]. In the case of ATP, and considering that this ocular structure contains huge amounts of this nucleotide [6], light effect could be a consequence of an increase in its release rather than a change in its synthesis. The mechanism why melanopsin activation can produce the release of ATP is not yet known; nonetheless, some ideas can be suggested. Melanopsin activation by light is coupled to PLC and this signaling cascade may modify intracellular Ca^{2+} concentrations mobilizing this ion from intracellular stores [9]. Such a rise in the concentrations of intracellular Ca^{2+} may change the activity of lens connexin and pannexin hemichannels that open, thereby facilitating the release of ATP from the lens (Fig. 3) [10, 11]. Further experiments are necessary to fully confirm this hypothesis.

The release of ATP from the lens induced by light has been proved in vivo and ex vivo by means of filters and antagonists/inhibitors. Nevertheless, one needs to be aware that the lens should not be the only source of ATP within the eye. The ciliary body is a source of ATP that contributes to this nucleotide concentration in the aqueous humor [8]. Unfortunately, it was not possible, in the in vivo experiments, to evaluate the

Fig. 3 Suggested mechanism of action of melanopsin activation and the corresponding light-induced ATP release. The light entering the eye reaches the lens and stimulates the photopigment melanopsin. This protein is coupled to PLC which can rise Ca^{2+} levels and this cation can stimulate lens connexin and pannexin (PX) hemichannels that open facilitating the release of ATP

contribution of the lens and the ciliary body. Moreover, it could be the case that the ciliary body also presents melanopsin, but to date, there is no information available regarding this.

An interesting, unanswered question is why, after the light stimulation for several weeks, ATP concentrations remain elevated when compared to those obtained under darkness conditions. Melanopsin is a photoreceptor that does not suffer from inactivation when light is present even after long exposure times [12]. This may explain why, under the conditions of permanent light described here, the light-induced ATP release still occurs. The release of ATP when light is present is important to regulate some ocular physiological processes such as the regulation of intraocular pressure (IOP). In this sense, P2X receptors present in the ciliary body are responsible for reducing IOP, hence controlling this physiological parameter. Interestingly, the IC_{50} value for ATP, α, β -meATP, and β, γ -meATP on these P2X receptors regulating IOP was around $1.0 \mu\text{M}$ [13]. This indicates that under light conditions, P2X receptors located in the ciliary body will be stimulated and will maintain IOP under normal physiological values. Thus, $1.0 \mu\text{M}$ ATP under light conditions produces a reduction in IOP of roughly 50% but when darkness occurs, ATP concentration is reduced to $0.30 \mu\text{M}$, this concentration scarcely reducing IOP more than 16% [13]. This suggests that ATP becomes a natural regulator of IOP during the day, leaving the same role of keeping IOP under physiological values to melatonin at night, when the concentration of this molecule is more elevated [4].

In summary, these findings open a new perspective for ocular pharmacology: it is possible to regulate the release of ATP without using any drug, just by modulating white light, for example, by suppressing the blue component by using filters. This is of interest since the role of the purinergic system in the eye has already been demonstrated [11]. Therefore, and since ATP and other nucleotides have been involved in the physiopathology of relevant diseases such as glaucoma or cataracts [13, 14], it is possible that in the future, some of these diseases may be treated or at least ameliorated by wearing filtered glasses or contact lenses.

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Compliance with ethical standards

Conflicts of interest Jesús Pintor declares that he has no conflict of interest.

Ethical approval This study followed the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the European Communities Council Directive (86/609/EEC).

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