Diadenosine tetraphosphate as a potential therapeutic nucleotide to treat glaucoma

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Abstract Glaucoma is a neurodegenerative disease that produces blindness. The main factor associated with this disease is an abnormally elevated intraocular pressure (IOP). To date, some attempts have been made to demonstrate the role of nucleotides modulating IOP, but never in a model of glaucoma. The DBA/2J mouse is an animal that develops the pathology spontaneously, starting from the typical rise in IOP at 9 months of age. Using this animal model, together with a control mouse, C57BL/6J, it has been possible to monitor the elevation in IOP in the glaucomatous mice and to check the ability of the dinucleotide diadenosine tetraphosphate AKA Ap₄A to reduce IOP. The topical application of Ap₄A when IOP is maximal (9–12 months) reduced IOP $30.6 \pm 6.6\%$ in the DBA/2J and $17.9 \pm 4.0\%$ in the C57BL/6J mice. Concentration response curves in both animal strains produced similar pD₂ values; these being 4.9 ± 0.5 and 5.1 ± 0.4 for the normotensive C57BL/6J and the glaucomatous DBA/2J respectively. Antagonist studies showed differences between the control and the glaucomatous animals. In particular, the main receptor reducing IOP in the control animal was the P2Y₁ receptor and in the glaucomatous model the P2Y₆, although the participation of other P2 receptors cannot be ruled out. The long-term effect of Ap₄A applied three times a week for 3 months showed a clear stop in the elevation of IOP in the glaucomatous model, thus indicating the possibility of using Ap₄A as an effective compound for the treatment of glaucoma.

Keywords Ap₄A · Diadenosine tetraphosphate · DBA/2J · Glaucoma · P2 receptors

Introduction

The control of intraocular pressure plays an important role in glaucoma, because it has been accepted that an increase in intraocular pressure (IOP) is a major risk factor for the development of this disease, a pathology that leads to a progressive optic neuropathy characterized by functional and structural impairment of ocular tissues which may result in the loss of vision [1]. Most glaucoma pharmaceutical treatments involve lowering IOP, either by decreasing the production aqueous humour or by improving its outflow, thereby reducing damage of the optic nerve. [2]. Some families of compounds are currently used to induce such IOP reduction, and more are emerging as promising substances to treat this disorder [3].

Among these new potential substances to treat the elevation in IOP associated with glaucoma are nucleotides, emerging as interesting molecules since they are present in aqueous humour [4] and they can induce either an increase or a decrease in IOP, depending on the purinergic receptor that is stimulated [5]. Nucleotides have developed their importance in ocular physiology since they are involved in many crucial processes in most of the ocular structures [6].

One of these nucleotides is diadenosine tetraphosphate, Ap₄A, which is present, both in animal and human aqueous humour [7–9]. Ap₄A can activate P2X and P2Y purinergic receptors as well as its own dinucleotide receptor [10]. In the eye, this dinucleotide activates mainly P2Y₂ receptors [11] modifying several physiological factors such as intraocular pressure [12]. In this way, it has been possible to see that in normotensive New Zealand white rabbits, the topical application of Ap₄A can produce a reduction in IOP [7].

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Nevertheless, when the actions of this dinucleotide are studied individually, either in the trabecular meshwork or in the ciliary body, the results seem to be contradictory [13, 14]. To complicate the complexity of dinucleotides in the regulation of aqueous humour dynamics even more, studies have shown that glaucomatous patients present increased levels of Ap₄A in their aqueous humour [7], which were almost 15 times higher in glaucoma patients than in healthy individuals. This increase in the dinucleotide levels has also been described in the case of ATP [15].

Several animal models have been developed to study new glaucoma treatments, most of them based on elevating IOP in such animals. Artificial IOP rises with biochemical agents like chymotrypsin or surgical procedures such as episcleral veins cauterization or laser trabeculoplasty are efficient protocols for elevating IOP. Currently, however, these treatments damage several tissues that might be the targets to reduce ocular hypertension and glaucoma. Therefore, besides the necessity of an elevation of IOP, a glaucoma animal model needs to be minimally invasive in order to guarantee that most of the ocular structures will be intact and suitable for drug treatment [16].

The DBA/2J mice provide a model of inherited glaucoma. This animal model develops a pigmentary form of glaucoma characterized by a pigment-dispersing iris disease, increased IOP and optic nerve degeneration [17]. It has been demonstrated that pigment dispersion leads to a partial occlusion of the aqueous humour drainage pathways, followed by an increase of IOP and subsequent glaucomatous changes in the retina and the optic nerve [18].

To date, most of the experiments performed with Ap₄A have been carried out in normotensive animals [12] but not in an animal model that develops the disease spontaneously. Therefore, the present work describes the progression of IOP in glaucomatous mice with age, showing that a topical instillation of Ap₄A into mice's eyes lowers IOP and how this nucleotide can reduce the rise of IOP observed in the non-treated animals.

Materials and Methods

Mice

Experiments were performed on adult female C57BL/6J ($n = 15$) (control animals) and DBA/2J ($n = 15$) (glaucomatous animals) mice obtained from the European distributor of Jackson Laboratories Mice (Charles Rivers Laboratories). All animal maintenance and experimental procedures followed Spanish and European guidelines for animal care in the laboratory and animal research (Guide for the Care and Use of Laboratory Animals) and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Mice

were housed (one to four mice per cage) in temperature and light-controlled rooms maintained according to a 12-h light/dark cycle; all animals were fed ad libitum. The mice were 4 weeks old when they arrived, and they were housed for 2 months until they reached 3 months of age, which was when the study started. DBA/2J and C57BL/6J mice were studied at 3, 6, 9 and 12 months of age.

IOP measurements

Intraocular pressure was measured using a non-invasive rebound tonometer (Tono-lab®; Tiolat, OY, Helsinki, Finland). The tonometer was fixed so that the probe tip was aligned with the optical axis of the eye, at a distance of 1–4 mm. To avoid the putative effect of the circadian rhythm, the IOP was always tested at the same time of the day, in the morning from 8.30 a.m. to 14.30 p.m. Six consecutive measurements were taken for each reading, and three readings were obtained on each eye.

In order to study the effect of Ap₄A, two IOP measurements were taken before Ap₄A was instilled and once every hour for 6 h. For dose-response studies, Ap₄A was applied at concentrations ranging from 10^{-8} to 10^{-3} M, measuring IOP at 3 h, which corresponded to the maximal hypotensive effect of the dinucleotide.

Pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (PPADS), suramin and reactive blue 2 (RB-2, a non-selective P₂ antagonists), 2'-deoxy-*N*⁶-methyladenosine 3',5'-bisphosphate tetrasodium salt (MRS 2179, a P₂Y₁ antagonist) and *N,N'*-1,4-Butanediylbis[*N'*-(3-isothiocyanatophenyl) thiourea (MRS 2578, a P₂Y₆ antagonist) were used as antagonists of purinergic receptors, all tested at 100 μM. To study the effect of these antagonists, they were instilled 2 μl 30 min before Ap₄A at a concentration of 100 μM, measuring IOP in the same fashion as previously described.

Control IOP measurements were performed in DBA/2J and C57BL/6J mice at 3, 6, 9 and 12 months of age to see the variation of IOP. The mentioned compounds were tested when animals were between 10 and 12 months of age, with a minimum of 3 days between experiments.

Another group of 18 animals was selected to test the preventive long-time effect of Ap₄A. To perform the experiment, basal IOP was measured from the fifth to sixth month of age, and then animals were randomized in two groups: control or Ap₄A treated. To test the hypotensive effect of Ap₄A, the compound was applied three times a week, from 6 to 9 months, measuring IOP once a week and comparing IOP with the values in the control group (non-treated). In all experiments, control animals were instilled with 10 μl of saline solution (NaCl 0.9%, pH = 6). Long-term effect of Ap₄A started on Monday and therefore IOP was measured every Monday at 10:00 a.m. during the 3-month period of the experiment.

Anaesthesia

Mice were anaesthetised by inhalation of isoflurane. A Matrx VIP 3000 Calibrated Vaporized (Midmark, OH, USA) was used. This instrument supplies oxygen from an attached tank at 50–55 lb per square inch. Oxygen is mixed with isoflurane (IsoFlo, Abbot, Berkshire, UK) and sent to two outflows at 500 cm³/min, delivering 2.5% of isoflurane in oxygen to the animal. One outflow enters a box where mice were placed into for initial sedation. After about 2 min, the sedated animal was positioned for IOP measurement and clinical examination with a nose cone delivering the isoflurane gas/oxygen mixture. The nose cone permitted access to the eyes.

Compounds

Ap₄A was purchased from Sigma, St. Louis, USA. MR2578, MR2179, PPADS, suramin, and reactive blue 2 were purchased from Tocris, Minneapolis, MN, USA. All compounds were diluted in saline solution 0.9% pH 6 and prepared at 100 µM concentration and applied in drops to the cornea at a fixed volume of 2 µL.

Data analysis

All data are presented as the mean ± S.E.M. Statistical differences between treatments were calculated using the ANOVA test. Plotting and fitting were carried out by GraphPad Prism 6 computer program (GraphPad Software).

Results

IOP changes related to age and glaucomatous pathology development

IOP was compared between the control C57BL/6J and the glaucomatous DBA/2J mice, from 3 to 12 months of age, every 3 months. In DBA/2J, IOP changed from 16.3 ± 0.8 mmHg at 3 months to a maximal of 26.2 ± 0.7 mmHg at 12 months of age. As it can be seen, it is already significantly higher from 9-month-old (22.5 ± 0.4 mmHg) compared to the 3-month-old mice ($n = 32$; *** $p < 0.001$; two-way ANOVA with Tukey post-test), reaching 155% of basal IOP at 12 months old, as shown in Fig. 1. IOP did not significantly differ throughout in C57BL/6J mice.

Effect of Ap₄A in IOP in DBA/2J and C57/BL mice

In order to study the effect of Ap₄A on intraocular pressure, a single dose of 100 µM (2 µL) was tested and the time-course was followed until IOP returned to the initial values (6 h).

Fig. 1 Evolution of IOP in C57BL/6J and DBA/2J from 3 to 12 months of age. Changes in IOP measured in both control C57BL/6J and DBA/2J mice, expressed as % of control for both animals at 3 months of age (IOP was 11.7 ± 0.4 and 10.6 ± 1.0 mmHg for C57BL/6J and DBA/2J, respectively, at 3 months of age). IOP peaked at 12 months of age in glaucomatous mice but not in the control one (IOP at 12 months was 11.8 ± 0.4 in the C57BL/6J and 16.3 ± 0.8 mmHg in the DBA/2J) ($n = 32$; *** $p < 0.001$ vs. 3 months value; two-way ANOVA with Tukey post-test)

Ap₄A treatments were applied in DBA/2J and C57BL/6J mice when the animals were between 10 and 12 months of age, since as described before, this was the period when IOP was stably higher.

As can be seen in Fig. 2, Ap₄A produced a reduction in IOP of 30.6 ± 6.6% in DBA/2J and of 17.9 ± 4.0% in C57BL/6J, with a maximum effect at 3 h in both animals, when the animals were studied at 12 months of age compared to the corresponding animals which received the same volume of vehicle ($n = 14$, * $p < 0.05$ and ** $p < 0.01$ vs. control; two-way ANOVA with Tukey post-test).

Concentration–response behaviour for Ap₄A on IOP

Ap₄A was tested in a broad range of concentrations starting from 10⁻⁸ to 10⁻³ M in order to see its effect on IOP in a concentration-dependent manner. As shown in Fig. 3, Ap₄A depicted a sigmoidal behaviour presenting a pD₂ of 4.9 ± 0.5 and 5.1 ± 0.4 for the normotense C57BL/6J and the glaucomatous DBA/2J respectively, which were equivalent to EC₅₀ of 9.0 and 8.9 µM. Interestingly, concentrations above 10⁻⁴ M did not produce any further decrease in IOP (Fig. 3, $n = 4$, * $p < 0.05$ and ** $p < 0.01$ vs. control. Two-way ANOVA test with Tukey post-tests).

Antagonist studies

The effect of Ap₄A was tested in the presence of P2 purinergic antagonists. Ap₄A has been described as P2Y₁, P2Y₂ and P2Y₆ selective agonist and P2Y₁₃ selective antagonist [19–22].

Fig. 2 Time-course of the effect of Ap_4A along 6 h in control and glaucomatous mice. Effect of Ap_4A (100 μM , 2 μL) in the glaucomatous model (upper traces) and on the non-glaucomatous model (lower traces). In each model, DBA (white squares) and C57 (black circles) represent the time course in the presence of vehicle ($n = 14$, $*p < 0.05$ and $**p < 0.01$ vs. control; two-way ANOVA with Tukey post-test)

Single doses of 100 μM of MRS2179 (P2Y_1 antagonist); MRS2578 (P2Y_6 antagonist) and a cocktail of suramin, PPADS and RB-2 (all at 2 μL instillation volume) were tested. Ap_4A decreased IOP $30.6 \pm 6.6\%$ in DBA/2J mice and $17.9 \pm 4.0\%$ in C57BL/6J, as shown before. In the presence of the mentioned antagonists, the effect of Ap_4A in lowering IOP was antagonized completely by MRS2179 and by the cocktail of suramin, PPADS and RB-2 in C57BL/6J mice. In DBA/2J, Ap_4A was completely inhibited by MRS2578 and the cocktail of suramin, PPADS and RB-2 (Fig. 4, $n = 4$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ vs. A_4A ; one-way ANOVA with Dunnett's multiple comparisons test).

Long-time effect of Ap_4A

Ap_4A is able to produce a reduction in IOP measurable during a few hours (see Fig. 2). To fully understand the effect of Ap_4A during the development of the glaucomatous process, this compound was applied three times a week during three consecutive months (from the sixth to the ninth month of age of both control and glaucomatous animals). As shown in Fig. 5, there was a clear trend in those animals treated with 100 μM Ap_4A , reducing the IOP rise observed in those glaucomatous animals treated with saline. Although the trend was visible from the second month of treatment (eighth week), it was only possible to see differences statistically speaking

Fig. 3 Concentration-response curves for Ap_4A . Ap_4A concentration-response curves in the glaucomatous model (DBA/2J, upper traces) and control (C57BL/6J, lower traces) testing the ability of Ap_4A to modify IOP from 10^{-8} to 10^{-3} M ($n = 4$, $*p < 0.05$ and $**p < 0.01$ vs. control; two-way ANOVA with Tukey post-test)

Fig. 4 Effect of P2 purinergic receptor antagonists on Ap₄A on C57BL/6J and DBA/2J mice models. Ap₄A effect was antagonized by MRS2578 and the cocktail of suramin, PPADS and RB-2 in C57BL/6J mice (left

panel) and by MRS2179 and the cocktail of suramin, PPADS and RB-2 in DBA/2J mice (right panel) ($n = 4$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ vs. Ap₄A, one-way ANOVA with Dunnett's multiple comparisons test)

during the last 2 weeks of measurements (Fig. 5, $**p < 0.01$, $***p < 0.001$ vs. C57). At this point, the differences in IOP were about 19% of reduction but with a clear stop in the IOP increase compared to the non-treated animals.

Discussion

Rodents share similar anatomical and developmental characteristics in the anterior segment, especially in the aqueous outflow pathway, with those of the human [23]. Advances in IOP measurements in small eyes have also accelerated the use of rodents in glaucoma research [24, 25]. Therefore, the research in ocular hypertension and glaucoma using mice instead of other animal models is providing more valuable information when trying to translate it to the treatment of human beings. An interesting mouse model in this regard is the DBA/2J. This model has a significant increase of its IOP as they

grow up, in a similar fashion to humans when developing ocular hypertension and glaucoma [26].

This model is especially attractive since it permits us to test compounds with ocular hypotensive properties, not only by measuring the IOP reduction during a few hours but also by studying the effect through several months. In this way, the present article has been focused on the effect of Ap₄A, which was tested previously as an IOP reducer, but never tested in a real glaucoma model. Our results clearly show that in the glaucoma model, the hypotensive effect is more robust than in the normotensive mouse duplicating the effect. This effect was concentration-dependent and mediated by P2 receptors according to the antagonists tested.

Concerning the effect of the dinucleotide on the glaucomatous model, it was possible to observe a reduction in IOP of 30%, which was significantly better than the one obtained in the normotensive animal. In the DBA/2J, the reduction was equivalent to the one observed for other compounds in the same model. For instance, melatonin or its

Fig. 5 Effect of Ap₄A long-term application in the normal and glaucomatous model. Time course showing the evolution of IOP from the fifth to the ninth month in both C57/BL6J (control) and DBA/2J (glaucomatous) mice. In the glaucomatous mice, it is observed from the

eighth week of treatment, a reduction in IOP which is statistically significant at the end of the treatment ($n = 18$, $**p < 0.01$, $***p < 0.001$ vs. C57, two-way ANOVA with Tukey post-test)

analogue 5-MCA-NAT reduced IOP between 29 and 32% in the DBA/2J and, as happened in the present paper, the effect on the normotensive animal was not so marked [27]. Other authors who used classical compounds such as the carbonic anhydrase inhibitor dorzolamide have demonstrated a similar effect [28], reducing IOP. These authors observed reductions of approximately 60%, which are far from the ones here described. The reasons for such differences are the targets of Ap_4A and dorzolamide. While Ap_4A can act on different purinergic receptors, some producing ocular hypertension (P2Y_2 [14]) and other hypotension (P2Y_1 [13] and P2Y_6 [29]), dorzolamide acts straight on carbonic anhydrases, reducing aqueous humour production [30]. So, it is clear that the dinucleotide final effect on IOP is the consequence of acting on several receptors (some reducing, some increasing IOP) while dorzolamide acts only on carbonic anhydrases. It has been also demonstrated that other commercial antiglaucomatous drugs apart from dorzolamide, such as brimonidine tartrate and travoprost, also produced a reduction in IOP in DBA/2J and C57BL/6J. Interestingly, the classical beta-blocker timolol maleate did not lower IOP in these mice [31]. The differences between the percentage of action of the commercial drugs and Ap_4A can be due to the same reason mentioned before; the selectivity of action of each compound.

This paradigm, one dinucleotide several receptors, is a matter of discussion. According to the antagonist study carried out in this work, it seems that the hypotensive effect is due mostly to a P2Y_1 (due to the MRS2179 effect) in the C57 and by a P2Y_6 in the DBA/2J, although other receptors such as P2X and the P2X_2 cannot be excluded [32] (due to suramin, RB-2 and PPADS).

The effect reducing IOP through P2Y_1 receptors triggered by Ap_4A has been quoted previously [13]. Moreover, this effect has been linked to the presence of P2Y_1 receptors in the place when the aqueous humour is drained, the trabecular meshwork [33]. On the other hand, the effect on P2Y_6 has also been described and moreover, it has been suggested that it is connected to a change in the ciliary body blood vessels [29].

The other side of the coin is the existence of a P2 receptor increasing IOP. In this sense, a P2Y_2 receptor located in the ciliary body, the area where the aqueous humour is synthesized, has been demonstrated that causes a rise in IOP [14]. This means that when Ap_4A is topically applied to the mouse eye, the observed effect on IOP is the final balance among hypotensive receptors P2Y_1 , P2Y_6 and P2X_2 purinoceptors and a hypertensive P2Y_2 .

Another interesting aspect is the existing differences between Ap_4A actions in the normotensive mice (C57BL/6J) and the glaucomatous DBA/2J. According to the antagonist study, in the case of the normotensive, the effect seems to be mostly due to the P2Y_1 , while in the case of the glaucomatous, one was mainly in the P2Y_6 . Interestingly, the expression of P2 purinergic receptors change during the animal's age and

also with the development of the pathology. In the case of the DBA/2J, there was a difference between young and old glaucomatous mice, and the expression of both P2Y_2 and P2Y_6 were increased [34]. There were also other differences between both animals and topical application of Ap_4A . Long-term effect of Ap_4A was able to stop the rise of IOP in DBA/2J from the eighth week, while in C57BL/6J, there were no changes. This means that more experiments are necessary to fully understand the differences between normotensive and hypertensive mice concerning the possible treatment of glaucoma by means of nucleotides and dinucleotides.

In summary, Ap_4A is an active dinucleotide reducing IOP in a glaucomatous model. The possible activation of P2Y_6 receptors in the ciliary processes that may reduce IOP shows strong support for the use of this nucleotide for the treatment of those pathologies in which intraocular pressure is abnormally elevated.

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Compliance with ethical standards The animal treating procedures were approved by the Committee for the Welfare of Laboratory Animals of the University Complutense de Madrid and the Comunidad de Madrid laws and experiments conducted in the accordance with the guidelines of the European Community Council (Directive 86/609/EEC) and the ARVO Statment for the use of Animals in Ophthalmic and Vision Research. All efforts were made to minimize the number of animals used and their suffering.

Conflict of interest Begoña Fonseca declares that she has no conflict of interest.

Alejandro Martínez-Águila declares that he has no conflict of interest.

María J. Pérez de Lara declares that she has no conflict of interest.

Jesús Pintor declares that he has no conflict of interest.

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- astrocytes, Muller cells, lens, trabecular meshwork, cornea and lacrimal gland. *Exp Eye Res* 127:270–279
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