ORIGINAL ARTICLE

Beta2 adrenergic receptor silencing change intraocular pressure in New Zealand rabbits

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KEYWORDS
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Abstract
Purpose/aim: Glaucoma consists of a group of progressive optic neuropathies that are characterized by degeneration of the optic nerve and irreversible visual field loss. Elevated intraocular pressure is the only proven treatable risk factor and commercial products used for glaucoma treatment are focused in lowering intraocular pressure. These drugs can have various undesirable side effects and this invites to look for new strategies. The purpose of this work is to study the use of a siRNA (small interfering RNA) to selectively silence beta2 adrenergic receptors and to see whether it reduces IOP (intraocular pressure).

Material and methods: Topical instillation of beta2 adrenergic receptors small-interfering RNA (siRNA, 25–250 μg) was applied and IOP was measured with a Tonopen XL up to 9 consecutive days. The effect of such siRNA was compared to commercial compounds such as Timoftiol, Trusopt and Xalatan, and it was also analyzed if some anatomical changes occurred by microscopy. Results: siRNA designed for beta2 adrenergic receptor induced a reduction of intraocular pressure (IOP) of 30 ± 5%, compared to a control (scrambled siRNA). The results in terms of IOP decrease were similar to that found with commercial compounds but a long-lasting hypotensive action was shown by beta2 adrenergic receptor siRNA treatment as compared to commercial drugs. No apparent side effects were observed in the ocular structures.

Conclusion: The use of siRNA against the beta2 adrenergic receptors could provide an interesting therapeutic strategy for glaucoma treatment.

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Introducción

El glaucoma es una grupo de enfermedades del ojo que muestran una amplia gama de presentaciones clínicas y etiologías, que conduce al deterioro progresivo del nervio óptico y pérdida visual irreversible debido a daño nervioso. Si se deja sin tratamiento, el glaucoma puede llevar a la ceguera irreversible. La tasa de prevalencia de glaucoma aumenta significativamente con la edad, y puede afectar a cualquier persona a cualquier edad. Los glaucomas se clasifican en dos grandes grupos: glaucoma abierto y glaucoma cerrado. El glaucoma abierto es la causa más común de ceguera en el mundo occidental. El glaucoma cerrado es menos común y se caracteriza por el edema del nervio óptico y la elevación del ángulo palpebral superior o inferior. El glaucoma es una enfermedad crónica y no curable, pero el tratamiento adecuado puede prevenir la progresión de la enfermedad y posponer la ceguera.

El tratamiento del glaucoma debe centrarse en reducir la presión intraocular (IOP) para prevenir la progresión de la enfermedad. La reducción del IOP se puede lograr mediante fármacos topícos, fármacos orales, tratamiento con láser o cirugía. Sin embargo, la eficacia y seguridad de las terapias tradicionales pueden variar ampliamente, lo que puede llevar a la necesidad de buscar alternativas. Recientemente, la terapia de silenciamiento mediante RNA interferente ha mostrado resultados prometedores en el tratamiento del glaucoma.

Cambio de la presión intraocular en conejos de Nueva Zelanda mediante silenciamiento del receptor adrenérgico Beta2

Objetivo: El glaucoma consiste en un grupo de neuropatías ópticas progresivas caracterizadas por degeneración del nervio óptico y pérdida irreversible del campo visual. La elevación de la presión intraocular es el único factor de riesgo tratable probado, centrándose los productos comerciales para el tratamiento del glaucoma en la reducción de la presión intraocular. Estos fármacos pueden tener diversos efectos secundarios indeseados, lo cual invita a buscar nuevas estrategias. El objetivo de este trabajo es estudiar el uso de un ARNip (ARN pequeño de interferencia) para silenciar selectivamente los receptores adrenérgicos beta2, y comprobar si reduce la PIO (presión intraocular).

Material y métodos: Se realizó instilación tópica de ARN pequeño de interferencia para los receptores adrenérgicos beta2 (ARNip, 25-250 μg), midiéndose la PIO con Tonopen XL hasta nueve días consecutivos. Se comparó el efecto de dicho ARNip con componentes comerciales tales como Timoftlol, Trusopt y Xalatan, analizándose asimismo mediante microscopio si se producía cualquier cambio anatómico.

Resultados: ARNip diseñado para el receptor adrenérgico beta2 indujo una reducción de la presión intraocular (PIO) de 30 ± 5%, en comparación al control (ARNip de secuencia aleatoria). Los resultados en términos de reducción de la PIO fueron similares a los encontrados utilizando componentes comerciales, aunque el tratamiento con ARNip para el receptor adrenérgico beta2 reflejó una acción hipotensora de larga duración en comparación con los fármacos comerciales. No se observaron efectos secundarios aparentes en las estructuras oculares.

Conclusión: El uso de ARNip contra los receptores adrenérgicos beta2 podría aportar una estrategia terapéutica interesante para el tratamiento del glaucoma.

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reduces the amount of compound needed diminishing potential side effects outside the eye. Moreover, the immune system has a limited access to the eye; therefore immune responses to the compound are less likely to occur.13 As the localized delivery of siRNA to the eye is less challenging than for other tissues, there has been significant progress made toward its use as a therapeutic procedure for eye diseases.14 In fact, several siRNA-based therapeutic agents for ocular disorders such as wet age-related macular degeneration and diabetic retinopathy have already reached clinical trials.15-17

In the present work, we analyze the use of siRNA, designed to silence β2-adrenoceptors, in order to reduce IOP in rabbits, studying the characteristics of different concentrations of siRNA on the animal responses and comparing this novel strategy with classical glaucoma treatments.

Materials and methods

Animals

24 male normotensive New Zealand white rabbits, with 6–8 weeks of age, were kept in individual cages with food and water ad libitum and maintained under a controlled 12 h/12 h light/dark cycles. All the procedures complied with the ARVO Statement for the Use of Animals in Ophthalmology and Vision Research. Animal studies were approved by the Ethical Committee of Complutense University (UCM-CEA) which follows the tenets of the European Communities Council Directive (86/609/EEC).

siRNA and commercial drug application

All the siRNA experiments were performed and measured at the same time. A siRNA targeting the beta2 adrenergic receptor (sc-39866) and siRNA scramble (sc-37007) were purchased from Santa Cruz (Hercules, CA, USA).

The siRNAs were topically applied in one eye and the contralateral eye received the same volume of saline solution (0.9% NaCl). The single eye treated with the siRNAs received an amount of 250 μg of the siRNA dissolved in 40 μL of 0.9% NaCl, daily in a single dose on four consecutive days. For dose–response studies, concentrations of 125 μg and 25 μg were dissolved in 40 μL of 0.9% NaCl. Experiments were performed using a single blind design: no visible indication was given to the experimenter as to the applied solution (agent or vehicle). IOP was measured for more than 8 days and, afterwards, animals were killed and both eyes were collected.

In the experiments performed with commercially available drugs 40 μL of the commercial drugs: Trusopt (dorzolamide) (Merck Sharp & Dohme de España S.A., Madrid, Spain), Xalatan (latanaprost) (Pfizer, Alcobendas, Madrid, Spain) and Timolol (timolol) (Santen Oy, Tampere, Finlandia) were instilled daily over a period of four consecutive days. IOP levels of rabbits treated with commercial drugs or with siRNAs were measured every hour for a total of 8 h, starting 1 h after the last administration. In addition, to estimate the mean time effect of these compounds, IOP was also measured for 8 days as described above.

Intraocular pressure (IOP) measurements in rabbits

IOP was measured by means of a Tono-Pen XL contact tonometer (Mentor Massachusetts Inc., Norwell, MA). Topical anesthesia (Colircusi, Laboratorios Cusí, Spain: 0.1 mg/ml tetracaine plus 0.4 mg/ml oxybuprocaine in 0.9% saline, diluted 1:3 in 0.9% saline) was applied (10 μL) to the cornea before each measurement to avoid animal discomfort.

Side effects

The assessment of the ocular tolerance of the RNAi sequences, were followed several aspects and parameters from the tear film, ocular anexa and ocular surface. The analysis were performed and graded before and after the topical application of siRNA with the Efron grading scales. A Topcon SL-8Z slit lamp and a Topcon SP-2000P specular microscope (Topcon Spain, Madrid, Spain), as well as a digital camera and a ‘‘de visu” exam were used for every experiment. Slit-lamp images were captured under 10× augmentations, a diffuser filter and a medium degree of illumination. The images were recorded and analyzed by IMAGEnet 2000 system software (Topcon).

Data analysis

All data are presented as the mean ± S.E.M. Significant differences were determined by two-tailed Student’s t-tests. The plotting and fitting of all the data was carried out with the computer program Prism GraphPad v.4.0 (Prism, USA).

Results

Effect and specificity of beta2 adrenergic siRNA on IOP

The effect of a siRNA designed for silencing the beta2 adrenergic receptor was tested together with a scramble siRNA in order to confirm the specificity of the tested siRNA. Measurement of intraocular pressure (IOP) was monitored during more than 8 days applying both siRNA under the conditions described in methods (250 μg of the siRNA in 40 μL).

As observed in Fig. 1, whereas beta2 adrenergic receptor siRNA application clearly induced a hypotensive effect reducing IOP 74.0 ± 2.0% (n = 8), after the treatment with the scramble siRNA, rabbit IOP did not significantly change during the time of the experiment, confirming both the effect and the specific action of beta2 adrenergic receptor siRNA. Concerning the mean time effect (duration of the effect), the application of the aforementioned siRNA produced a maximal effect that lasted for about 120 h (Table 1).

Effect of different doses of beta2 adrenergic receptor siRNAs on IOP

To see whether the silencing of beta2 adrenergic receptors produced changes in the IOP when different doses were applied, the rabbits were treated with the siRNAs against the beta2 adrenergic receptor and monitored in their IOP
for more than 8 days. Three different concentrations of the beta2 adrenergic receptor siRNA were tested: 250 µg, 125 µg and 25 µg, dissolved in 40 µL of 0.9% NaCl (Fig. 2). As it shown in Fig. 2A the lowest dose of siRNA tested only induced a slight modification in IOP with a reduction of 8% 102 h after application. The dose of 125 µg evoked an IOP decrease of around 10% at 24 h, with the highest effect (14% of reduction) observable 60 h after application and sustained in the next 24 h.

The highest dose of beta2 adrenergic receptor siRNA (250 µg), induced a robust reduction in IOP which started to be measurable 24 h after the instillation of the siRNA and which was statistically significant for 5 days. The maximal reduction obtained after silencing the beta2 receptor was 30 ± 5% compared to control. This and similar values were maintained for 1–2 days, with the IOP remaining low for three more days (with a mean of 30% of reduction) and then returning slowly toward control values (Fig. 2A). The maximal reduction in IOP obtained for each given dose is presented in Fig. 2B, where it can be seen that the reduction of IOP is dependent of the doses assayed. Interestingly, it was possible to observe that the higher the dose of beta2 adrenergic receptor siRNA was applied the longer mean-time effect was obtained (Table 1).

**Comparative studies with commercial drugs**

The effect of beta2 adrenergic receptor siRNA on IOP of New Zealand White rabbits was compared with that induced by ophthalmic commercial drugs: Trusopt (dorzolamide), Xalatan (latanoprost) and Timofoyl (timolol). As observed in Fig. 3, any of those compounds produced a decrease in IOP of between 20 and 35%, this reduction in IOP was similar to that found with beta2 adrenergic receptor siRNA treatment. In addition, the effect of the beta2 adrenergic receptor siRNA in terms of time effect was also compared with conventional pharmacological agents and the mean time effect of each compound was calculated as the difference between the half maximal time-point of recovery and the time at which the half maximal effect on IOP was achieved (Table 1). Commercial drugs showed mean time effect values between 5 and 7 h, whereas the effect of the beta2 adrenergic receptor siRNA was significantly longer exhibiting values between 78 and 120 h for the higher doses assayed.
Beta2 adrenergic receptor silencing change intraocular pressure in rabbits

Figure 3  Maximal IOP reduction elicited by siRNA against the beta2 adrenergic receptor and anti-glaucomatous drugs. Maximal IOP reduction (% of control) for beta2 adrenergic receptor siRNA tested (250 μg) was compared with the hypotensive action induced by Xalatan, Trusopt and Timoptol. Control (100%) represents the intraocular pressure before the application of any drug (15.2 ± 1.8 mm Hg). Values represent the mean ± S.E.M. (n = 8).

Discussion

Glucoma is one of the leading causes of blindness in the world. Anti-glaucomatous drugs currently prescribed can have various undesirable effects and their efficacy is also subject to a reduction after prolonged use. Moreover, to maintain adequate levels of IOP, frequent instillation is necessary, which can lead to poor compliance of the patient. Therefore, the development of new therapeutic strategies to circumvent the drawbacks associated to the presently available pharmacologic classes is required. In this context, we analyzed siRNA-based therapy for glaucoma.

In particular, we focused our attention on beta2 adrenergic receptors, since their commercial inhibitors are effective in lowering IOP, the primary indication of glaucoma. The hypotensive action of a commercial sequence of beta2 adrenergic receptor siRNA was tested in rabbits. The results confirmed the reduction of the IOP in rabbits, in a marked and sustained way. The frequency of application is a factor to be considered to achieve this IOP reduction in a reproducible mode. In this case, beta2 adrenergic receptor siRNA was administered daily in a single dose on four consecutive days in rabbits. This approach, the use of siRNA to reduce IOP has been also observed by other authors, with similar results to the ones here presented. In a clear difference with those works, the reduction in IOP observed at the highest siRNA concentration showed a decreased in IOP around 30% while in the work by Moreno-Montañes and co-workers it only reduced around 20%. Also, the concentrations used by these authors, up to 900 μg/day are far from the doses here studied, which were 250 μg/day. The present study was performed in rabbits while the one of 900 μg/day was carried out in humans. Although the aqueous humors in rabbits and humans are not so different, it would be interesting to carry out experiments in humans to confirm the potential higher efficacy of the siRNA used in our work.

The value of IOP reduction obtained using siRNA was similar to that found with drugs currently prescribed for glaucoma treatment such as Xalatan, Trusopt or Timoptol. Although the recommended dosage for Trusopt or Timoptol in some patients is one drop in the affected eye twice a day, we administrated these drugs following similar experimental conditions than those used for siRNA application (a daily single dose on four consecutive days). Clearly, differences in application frequency of therapies can have influence in the results. In order to compare the hypotensive effect induced by siRNA treatment with the effect produced by drugs we used the same dosage regimen.

The effect of beta2 adrenergic siRNA treatment was significantly longer in terms of time-effect as compared to conventional pharmacological agents. While the commercial compounds reduced IOP for a few hours, the siRNA effect lasted for several days. In a previous study, a single dose of a siRNA for the beta receptor, provided a mean time effect of 91 h, while in our case it was about 120 h. Therefore, it seems that comparing commercial compounds as well as other siRNA treatments already published, it seems that the characteristics of the sequence we use is also better producing a longer lasting effect.

Comparing to the commercial drugs, it is a clear that siRNA effect, which uses a different molecular mechanism to those used by commercial medicines, provide a more profound and sustained reduction in IOP. When RNAI therapy is used, upon cessation of treatment the silenced protein has to be re-synthesized in order to recover its activity and consequently it prolong the duration of the treatment as compared to conventional treatments. Thus, siRNA against beta2 adrenergic receptor enables to obtain a significant reduction in IOP with a long-term effect, whereas continuous application of commercial drugs would be necessary to induce a sustained effect on IOP.

This long-lasting reduction of IOP may be one of the most attractive features of this new approach for the treatment of ocular hypertension and glaucoma since it could allow reducing the frequency of instillation and improving patient compliance. Moreover, taking into account that the frequent application of some commercial drugs currently used at high concentrations to achieve the desired therapeutic effects, can lead to adverse side effects due to systemic absorption (e.g., timolol can generate deleterious effect in the heart) the use of siRNA therapy could minimize these side effects. Likewise, it has been demonstrated, that on the contrary to an isolated compartment as the eye, siRNA stability in serum is decreased as a consequence of RNases activity. Thus, a reduced siRNA half-life in serum also would contribute to reduce the likelihood of systemic side effects. A potential limitation of our study is that we have not evaluated those possible systemic side effects. However, previous reports suggest the safety of siRNA treatment. In animal biodistribution studies only trace amounts of siRNA were detected in systemic organs (lung, liver and kidney) and the absence of 5’-phosphorylated antisense strand of siRNA (a marker of intracellular delivery of the siRNA) indicated that the siRNAs were not internalized by the cells and accordingly they are not biologically active. In addition, the analysis of several parameters (clinical signs, electrocardiogram, hematology) in toxicity studies performed with animals that received ocular administration of siRNA did
not reveal any alterations, indicating the safety of siRNA treatment.\textsuperscript{18} Supporting these data, in a study with humans using a siRNA targeting the β2 adrenergic receptor,\textsuperscript{19} no significant changes were observed during the comprehensive clinical evaluations, monitoring of vital signs or electrocardiograms.

On the other hand, although the eye content of RNases is lower than in other tissues and, indeed, a long-lasting effect was achieved by simple topical administration of a naked siRNA, perhaps the use of a non-viral vector carrier, preventing siRNA degradation, could even prolong the hypotensive action of the siRNA. Finally, the siRNA application did not alter the integrity of ocular structures and neither other ocular side effects were observed.

In summary, our results support a new generation of drugs to treat ocular hypertension and glaucoma based on RNAi therapy, which allows targeting of specific sequences of single genes and local delivery into the eye. The long-term effect on IOP reduction induced by siRNA could make possible to reduce the dose of siRNA minimizing side effects in other tissues as well as simplifying patient compliance.

**Conflicts of interest**

The authors report no conflicts of interest.

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